THE EFFECT OF FLUID SHEAR STRESS ON GROWTH PLATE CHONDROCYTES

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THE EFFECT OF FLUID SHEAR STRESS ON GROWTH PLATE CHONDROCYTES

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LIST OF SYMBOLS AND ABBREVIATIONS

1,25	$1\alpha25OH_2D_3$ or 1,25 dihydroxy vitamin D
24,25	$24R,25(OH)_2D_3$ or 24,25 dihydroxy vitamin D
α	angle of cone to plate
$\alpha5\beta1$	integrin alpha 5 beta 1
A	adenine
ADAM	A Disintegrin and Metalloproteinase (a peptidase protein)
ADAMTS5	a member of the ADAM protein family
AGG	aggrecan
ALPHA 2	integrin alpha 2
ALPHA 5	integrin alpha 5
ANOVA	analysis of variance
ATDC5	ATDC5 Cell line
$\beta1$	integrin beta 1
BCA	bicinchoninic acid
BETA 1	integrin beta 1
BETA 3	integrin beta 3

BMP	bone morphogenetic protein
C	cytosine
COL I	collagen type I
COL2	collagen type II
COLX	collagen type X
COMP	cartilage oligomeric matrix protein
ddH2O	double-distilled water
DEPC-H20	diethylpyrocarbonate treated water
DEVD-pNA	aspartic acid-glutamic acid-valine-aspartic acid-(p-nitroanilide)
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	media of half DMEM and half Ham's F12
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
dyn	dynes (or for some figures dynes/cm^2)
ECM	extracellular matrix
ERK	extracellular signal-regulated kinases
F12	Hams nutrient mixture F-12 medium
FBS	fetal bovine serum

G	guanine
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glvr-1	type III sodium dependent phosphate transporter
$[^3H]$	tritium
HA	hyaluronic acid
ITGB1	integrin beta 1
μ	viscosity
MGP	matrix Gla Protein
MMP	matrix metalloproteinases 13
MMT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
Na_2HPO_3	dibasic sodium phosphate
NaH_2PO_3	monobasic sodium phosphate
NC	negative control

NO	nitric oxide
PBS	phosphate buffered saline
PC	positive control
PCR	polymerase chain reaction
PFA	phosphonoformic acid
Pi	inorganic phosphate
r	radius or radial distance from center of cone
R	analogous defined ratio to Reynolds number for cone-plate
RC	resting zone chondrocyte
Re	Reynolds Number
RGD	arginine - glycine - aspartic acid (peptide sequence)
RNA	ribonucleic acid
RT	reverse transcriptase
$[^{35}S]$-SO_4	sulfate (SO_4) with radioactive sulfur isotope ($[^{35}S]$)
SEM	standard error of mean
shβ1	small hairpin loop integrin beta 1 silenced ATDC5 cells
SOX9	SRY (sex determining region Y)-box 9

T	thymidine
τ	shear stress
Tω	shear stress
TCA	trichloroacetic acid
TE	Tris ethylenediaminetetraacetic acid
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloric acid
Veh	vehicle or diluent for hormone treatment
WT	wild type
υ	kinematic viscosity

SUMMARY

Cartilage tissue provides compressive resistance in diarthrodial joints, and has been shown to be regulated by mechanical signals, in particular with regard to production of extracellular matrix proteins. However, less is understood about how chondrocytes in regions not solely purposed to provide compressive resistance may also be affected by mechanical forces. The growth plate is a small layer of cartilage that functions to facilitate longitudinal growth of the long bones from *in utero* through post-adolescent development. The growth plate maintains distinct regions of chondrocytes at carefully regulated stages of endochondral ossification that are in part characterized by their morphology and differential responsiveness to vitamin D metabolites. Understanding if mechanical cues could be harnessed to accelerate or delay the process of endochondral ossification might be beneficial for optimizing tissue engineering of cartilage or osteochondral interfaces. This study focused on three aims to provide a basis for future work in this area: 1) Develop a cell line culture model useful for studying growth plate chondrocytes, 2) Determine the response of primary growth plate chondrocytes and the cell line model to fluid shear stress, and 3) determine if expression of integrin beta 1 is important for the observed responses to shear stress. The findings of this study suggest that inorganic phosphate can promote differentiation in coordination with the $24,25(OH)_2D_3$ metabolite of vitamin D, and that fluid shear stress generally inhibits differentiation and proliferation of growth plate chondrocytes in part through an integrin beta 1 mediated pathway.

CHAPTER 1. SPECIFIC AIMS

Cells are known to be responsive to mechanical stimuli, and for some types of cells mechanical environment may be an important parameter for homeostatic function. Numerous studies have considered the effect of various mechanical stimuli on chondrocytes involved in the joints, such as articular cartilage or meniscal fibro-cartilage, both in normal or arthritic conditions. Less is known about the effect of mechanical stresses on chondrocytes found in the growth plate. While various types of mechanical stimuli modulate chondrocyte function, cartilage is a highly hydrated tissue and a certain amount of fluid shear stress should be experienced by cells as water is expelled during loading, and osmotically drawn back into the tissue during unloading. A similar process is likely to occur in the cartilage of the growth plate.

The growth plate is a remaining center of endochondral ossification that persists after in utero development of the long bones to be closed later during post-adolescence or adulthood. Some evidence suggests that mechanical loading may even begin to play a role in joint development in the fetal temporal-mandibular joint [1, 2] and hip [3]. How growth plate chondrocytes experience shear is not well understood. Moreover, it may change markedly with the state of chondrocyte maturation within the growth plate. The chondrocyte phenotype is uniquely staged through endochondral ossification in both a temporal and spatial manner. Not only do the cells show changes in shape and size, but they also vary in their extracellular matrix composition and in their responses to local and systemic factors. One characteristic of the resting zone chondrocytes found in the growth plate is the responsiveness of these particular cells to the specific Vitamin D metabolite

$24R,25(OH)_2D_3$, whereas less differentiated mesenchymal cells lack responsiveness to this hormone and more differentiated hypertrophic growth plate chondrocytes lose responsiveness, becoming sensitive to $1,25(OH)_2D_3$ [4-6]. We will take advantage of this marker of endochondral development to establish our experimental model for the studies proposed below.

The **overall goal** of this project is to contribute to the understanding of how fluid shear stress effects the differentiation of chondrocytes in an endochondral ossification pathway. A better understanding in this area will contribute to the basic understanding of the growth plate and developmental biology, as well as potentially progress tissue engineering strategies that involve the growth plate, bone fracture callus healing [7], osteochondral plugs [8], or even developmental biology of joints [2]. This project will focus on the **general hypothesis** that fluid shear stress modulates the differentiation of resting zone chondrocytes.

Specific Aim 1: To develop a cell line culture model that can be induced and controlled to a chondrocytic phenotype with sensitivity to $24R,25(OH)_2D_3$, indicating comparability to resting zone growth plate chondrocytes.

The ***objective*** of this study was to establish a relevant chondrocytic cell culture model that can be shown to be phenotypically comparable to resting zone chondrocytes in their responsiveness to $24R,25(OH)_2D_3$. This also provided an additional cell source for later experiments, allowing for some experimental possibilities that were more suited for a cell line. The **hypothesis** was that the ATDC5 cell line, which has been shown extensively in the literature to be chondrogenic, can be induced to a differentiated state in

which it is responsive to $24R,25(OH)_2D_3$. We used biochemical and radioactive assays to determine if the cells are responding significantly to $24R,25(OH)_2D_3$.

Specific Aim 2: To determine the effect of fluid shear stress on rat costochondral resting zone chondrocytes as well as on ATDC5 cells differentiated to a resting zone-like phenotype.

The ***objective*** of this study was to determine if fluid shear stress will alter the differentiation or phenotypic expression of resting zone chondrocytes in either a time or dose dependent manner. Both primary rat costochondral resting zone cells and ATDC5 cells differentiated to an RC-like phenotype were exposed to shear stress at differing lengths of time or amount of shear stress and then measured for changes in markers of chondrocytic phenotype. The working **hypothesis** was that shear stress would affect some or all of the differentiation markers in a dose dependent manner.

Specific Aim 3: To determine if expression of integrin β1 is important for the effects of shear stress on resting zone chondrocyte differentiation.

The ***objective*** of this study was to determine if integrin β1 expression is important for translating the effects of fluid shear stress on chondrocyte differentiation. The ATDC5 cell line was transduced with lentiviral particles delivering a plasmid inducing permanent expression of a small hairpin loop RNA that targeted the degradation of integrin β1 mRNA. The effectiveness of silencing integrin β1 was validated at both the mRNA and protein expression level. The most effective shear stress treatment from Aim 2 that altered differentiation of the chondrocytes was used to determine if expression

level of integrin β1 was critical to the shear-induced effects on differentiation. The working **hypothesis** was that a reduction in integrin β1 expression will result in a reduced effect of the shear stress.

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CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

ENDOCHONDRAL OSSIFICATION

Mammalian skeletal development occurs mostly through a complex and carefully regulated process known as endochondral ossification. In this process, primary cartilaginous members formed in the embryo begin to mineralize at multiple initiation sites to lay the first calcified matrix that will be remodeled to proper bone tissue. However, the primary cartilaginous members are not completely calcified into bone at birth or even well into adulthood. Cartilage tissue remains at the end of long bones to form the protective cushion at diarthrodial joints. This articular cartilage will be needed to protect the underlying bone tissue from increased compressive loading that will stress the skeletal frame at joint interfaces as the individual increases ambulatory motion and weight bearing with longitudinal growth. The cartilage at joints is intended to remain well into adulthood to perform its needed function. However, this tissue often suffers degeneration from osteoarthritis in many individuals as they age beyond adulthood. Endochondral ossification is mostly relevant to skeletogenesis, however, the regulation of this process or related processes may be crucial for postnatal functions such as maintenance of articular cartilage, proper function of growth plates, and the ossification of a bone fracture cartilage callus. If this process could be regulated in a controlled ex vivo setting, it would open great potential for orthopedic tissue engineering [1].

Growth Plate

Along with articular surfaces of diarthrodial joints, another post-natal cartilage tissue is preserved at least until adult height is achieved. This important cartilage tissue is called the growth plate and consists of a corrugated, thin layer of cartilage at the metaphyseal region of long bones. The function of the growth plate is to enable much slower processes of endochondral ossification to occur over the development of the individual until full adult skeletal size is achieved. At this point the growth plate may fuse and remodel to strictly bone tissue, depending on anatomical location of the growth plate and species.

Despite the relatively thin width of the growth plate in comparison to the rest of the bone, the growth plate has a very distinct layered morphology (Figure 2.1). Near the epiphyseal border marks the resting zone of the growth plate, where the chondrocytes exhibit the least mature phenotype. In this region the cells are relatively small and dispersed randomly throughout significant amounts of matrix. Moving towards the diaphyseal center of the bone, the growth plate becomes more populated with larger cells that have assembled in columnar fashion and are surrounded by reduced amounts of matrix. This area is known as the growth or proliferative zone. Below this region, the chondrocytes enter a hypertrophic zone where the cells have undergone significant hypertrophy, displaced much of the extracellular matrix, and also have begun to mineralize their matrix at the lower end of this region. This mineral matrix interfaces with the underlying mineral of the bone tissue where leading edge angiogenesis occurs allowing for chondroclast and osteoclast cells to approach and migrate to mineral surfaces. These cells resorb mineral surfaces and allow for osteoblasts to follow

afterwards for deposition of newly layered mineralized matrix following the pattern of bone, including the deposition of collagen type I.

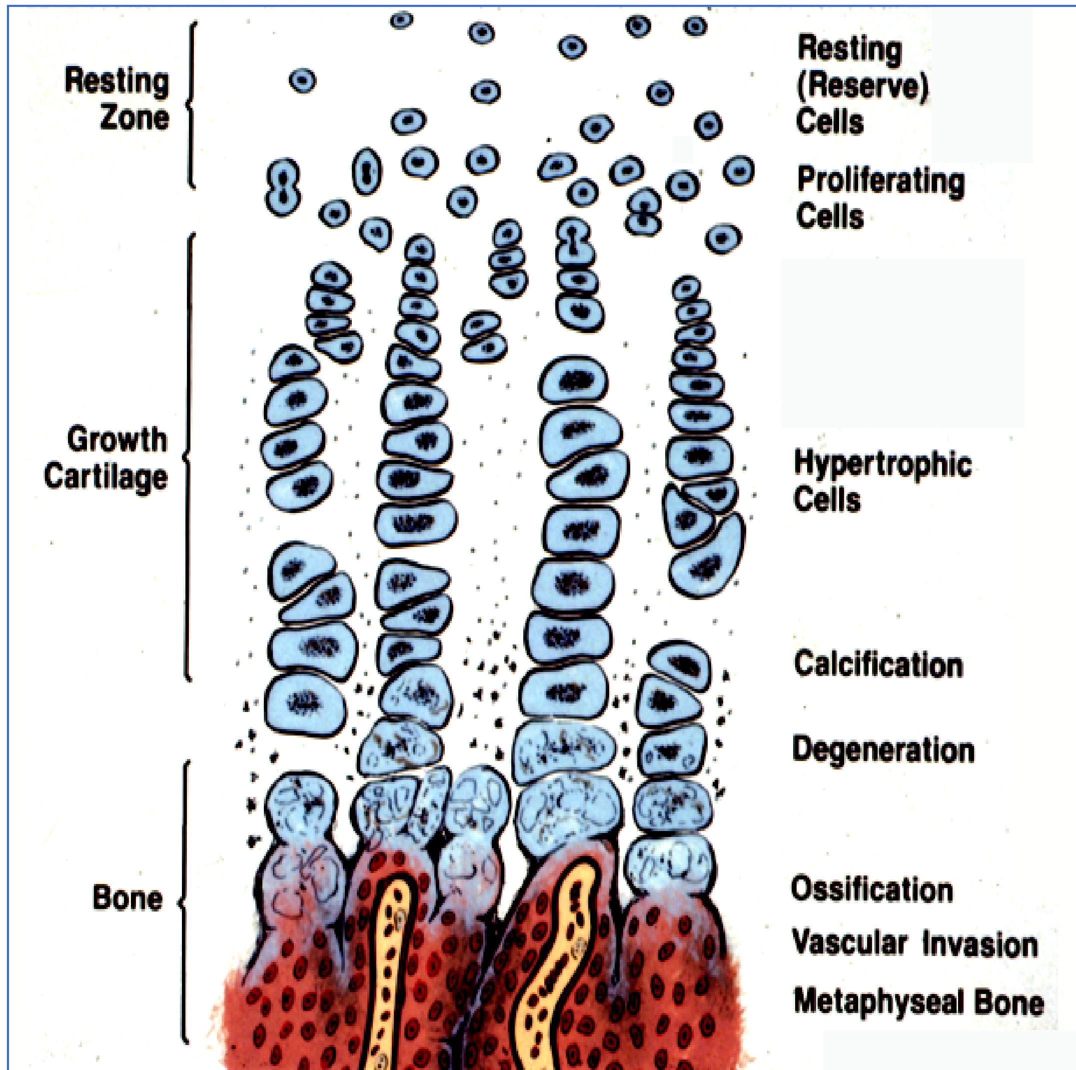

Figure 2.1. Schematic of the growth plate. Resting zone cells are the least mature chondrocytes near the epiphyseal region of the bone. Descending through the regions of the growth plate the chondrocytes exhibit a distinctive morphology as they progress in differentiation and the stages of endochondral ossification. Cells in the proliferating zone organize in columnar arrangement, followed by hypertrophic growth of the cells and ultimately calcification of the matrix surrounding the cells to interface with the mineral of the underlying and remodeling bone.

Vitamin D Metabolite Regulation of Growth Plate Zones

Furthermore, the thin-layered anatomy of the growth plate is not only morphologically distinct, but also has been shown to be regulated by tightly controlled chemical inducers of differentiation and signaling. In particular, vitamin D metabolites have shown to act very specifically at unique stages within the differentiation spectrum of the growth plate. The metabolites $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ have been shown to have specific targeted actions in the growth zone and resting zone, respectively [2]. Growth zone chondrocytes exhibit nuclear receptor mediated genomic responses to $1,25(OH)_2D_3$ as well as evidence of membrane-associated rapid response regulation by this hormone as well. Additionally, the resting zone chondrocytes have exhibited rapid responses to the $24,25(OH)_2D_3$ metabolite strongly suggesting evidence of membrane receptor regulation of this hormone also [3]. Moreoever, the membrane actions of these hormones is significant when considering that they are shown to regulate matrix vesicles that contain no genomic material, yet are uniquely regulated by specific metabolites when derived from resting or growth zones. These signals allow for the cells to carefully regulate the deposition of mineral that can be initiated from matrix vesicles deposited into the matrix after the cells have achieved a preferred distance apart. These data suggest that growth zone chondrocytes are capable of metabolizing their own specific autocrine and paracrine hormone signals to direct mineralization and differentiation carefully.

Inorganic Phosphate Regulation of Endochondral Ossification

Other factors have been shown to regulate progression of endochondral ossification. Growth plate chondrocytes become competent for mineralization of their surrounding matrix as they enter the hypertrophic stage of differentiation, which inherently requires the regulation of high amounts of phosphate ions as the cells prepare to use this ion as a source of mineral formation. It has also become apparent that this ion itself can act as a signal to the chondrocytes to increase their markers of differentiation [4] and eventually increase apoptosis [4, 5].

Apoptosis in the hypertrophic region is important to allow the remaining mineralized matrix to be turned over into bone tissue, thus preventing the problematic extension of the growth plate. This process appears to be regulated in part by Bcl-2 family proteins regulate Pi-induced apoptosis of the growth plate, which is crucial to proper lengthening and mineralization of the hypertrophic region of the growth plate [6]. Apoptosis may not be absolutely crucial to calcification occurrence, as suggested by one avian model [7], but generally both apoptosis and calcification are nearly concurrent activities in the growth plate.

Cell Line Model for Endochondral Ossification

Well characterized cell lines offer a useful tool for studying cellular physiology. The ATDC5 cell line has been a useful for studying the process of chondrogenesis and differentiation. This cell line was clonally derived from a murine embryonic limb bud, and was shown to differentiate through a chondrogenic pathway when cultured with insulin after confluency [8]. Studies with additional factors besides insulin have also

shown insightful findings about other growth and differentiation factors. Palmer et al. present a study that indicates that TGFβ-1 upregulates expression of inorganic phosphate (Pi) transporter Glvr-1 [9]. Magne et al. have presented multiple studies indicating that Pi itself can act as a signal to ATDC5 cells [4, 10], which corroborates with other studies in other cell types [11-14]. Other studies have indicated that ATDC5 cells produce factors that help regulate mineralization in their matrix [10, 15-17]. Shukunami et al. also showed that BMP-2 can regulate phases of differentiation in this cell line [18]. The ATDC5 cell line has become a well accepted cell line in the literature for studying chondrogenesis and the chondrocytic stages of endochondral ossification that occur in the growth plate. No reported work has tried to define this cell line with progressive differentiation as defined by responsiveness to multiple vitamin D metabolites.

MECHANICAL STIMULATION AND HOMEOSTASIS IN CARTILAGE

Many tissues perform a function that requires the ability to withstand certain levels of mechanical loads and stresses, and often the tissues are innately tuned to respond to the variations in these loads with an adaptive response. This can be in a short or long time scale as may be suitable for the optimal function and capability of the tissue. For example, blood vessels are able to respond to higher blood pressures and flows by releasing NO to induce an expansion in the arterial wall allowing for a reduction in pressure [19]. On a much longer time spectrum, bone tissue has long been observed to alter its density over several days and weeks in adaptive response to increased or decreased loading. This is observed in astronauts subjected to extended microgravity

who lose significant bone mineral density during time in space [20]. Alternatively, patients with osteoporosis are often encouraged to do weight-bearing exercise because it is well known to promote bone density (or at least delay bone loss in osteoporosis patients) [21]. This phenomenon of bones modifying their architecture in response to mechanical loading was first observed by Julius Wolff in the 19th century and has been coined as Wolff's Law [22]. In the case of Wolff's Law, there is an apparent balancing act for the body to provide the necessary density and strength to the skeletal bones so they can withstand the load bearing forces applied to them, with the alternate need of the body to not store unnecessary calcium or phosphate mineral in the bone tissue.

Cartilage is also responsive to mechanical loading. In an analogous sense to bone, cartilage also responds to mechanical stimuli by trying to provide the essential extracellular matrix components that are necessary to withstand the experienced mechanical loads and to offset any damage that may be accrued from the loading as well. It has been shown that chondrocytes will alter their rates of matrix synthesis and catabolism in response to different types and durations of mechanical loading.

Cartilage Matrix Regulation by Mechanical Signals

Cartilage is a highly hydrated tissue with a matrix that is composed mostly of collagen type II and proteoglycan molecules. The proteoglycan molecules contain glycosaminoglycans that are highly sulfated and because of their charged nature are very hydrophilic. This allows for the compressive resistance of cartilage because water is not easily removed from the matrix by compression. The collagen molecules offer tensile strength to cartilage. These trimeric molecules exhibit a random orientation and allow for

tensile strength in multiple directions, although may tend to align in regions of the cartilage where tensile forces or mostly unidirectional such as the superficial zone of articular cartilage [23]. One other important though much less abundant protein in cartilage tissue is cartilage oligomeric matrix protein (COMP). This pentameric glycoprotein facilitates proper three-dimensional arrangement of other matrix components, particularly collagen molecules [24, 25]. The absence or mutation of COMP can result in significant pathological phenotypes such pseudoachondroplasia [26].

Chondrocytes can experience both compressive and tensile forces in vivo and also fluid shear stress when water is relocated during compression [27]. It is not surprising given the necessity of the cartilage matrix to serve its biomechanical function, that chondrocytes would be responsive to mechanical stresses in attempt to properly maintain the composition of the matrix. However, studies have yielded varied results as to the response of chondrocytes in their matrix maintenance in response to diverse mechanical stimuli. Furthermore, as cartilage is by necessity an avascular tissue, it also has limited self-repair capability following injury since migration of stem cells cannot be easily achieved into the area. When cartilage is severely damaged from traumatic injury or excessive loading, it may result in surviving chondrocytes experiencing abnormal physical stresses not occurring within normal tissue. These abnormal stresses can exacerbate the cartilage degradation as the cells are unable to respond with proper matrix synthesis under the altered mechanical conditions [28].

In Vitro Application of Shear Stress

Various devices have been crafted and adapted for the application of various types of mechanical forces on cultures of cells. For this project, the application of fluid shear stress was implemented with a cone-plate viscometer device. This apparatus has been adapted for application of shear stress on monolayer cell culture in a Petrie dish. The design is based on a rotational cone and a stationary plate (the Petrie dish) with liquid media filling the space between the two parts. When the cone rotates at a low velocity then flow is usually characterized be a small Reynolds number ($Re < 1$), which means that the flow will be essentially azimuthal (or predominantly only in the rotational direction) and laminar. In this case, shear stress can be approximated by the equation $\tau = \mu\omega/\alpha$ where τ is shear stress, μ is fluid viscosity, ω is angular velocity, and α is angle of the cone from the surface of the plate (Figure 2.2). The device used in these experiments allowed for 8 individual culture samples to be sheared simultaneously in the same experiment (Figure 2.3). This device has been characterized generally in previous literature [29-31] and related models have been reported for studies involving chondrocytes [32, 33] and endothelial cells [34-37].

Mechanoregulation of Proteoglycan

Aggrecan as a major component of the proteoglycan complex, has been shown to be altered in expression in response to mechanical stress. Le Maitre et al showed that aggrecan was highly reduced in oscillatory compression in chondrocytes from the nucleus pulposa from the intervertebral disc of the human spine [38] and that this result may be mediated by integrin $\alpha 5\beta 1$ in healthy, but not in degenerate discs, suggesting the

possibility of multiple mechanotransduction pathways, that may be altered in certain pathological conditions. This assertion was strengthened by evidence published by Holledge et al that showed that mechanical stimulation in normal chondrocytes resulted in increased GAG production, but not in osteoarthritic chondrocytes [39]. Salter et al showed that osteoarthritic and normal chondrocytes have different responses to mechanical stress in their membrane polarization [40].

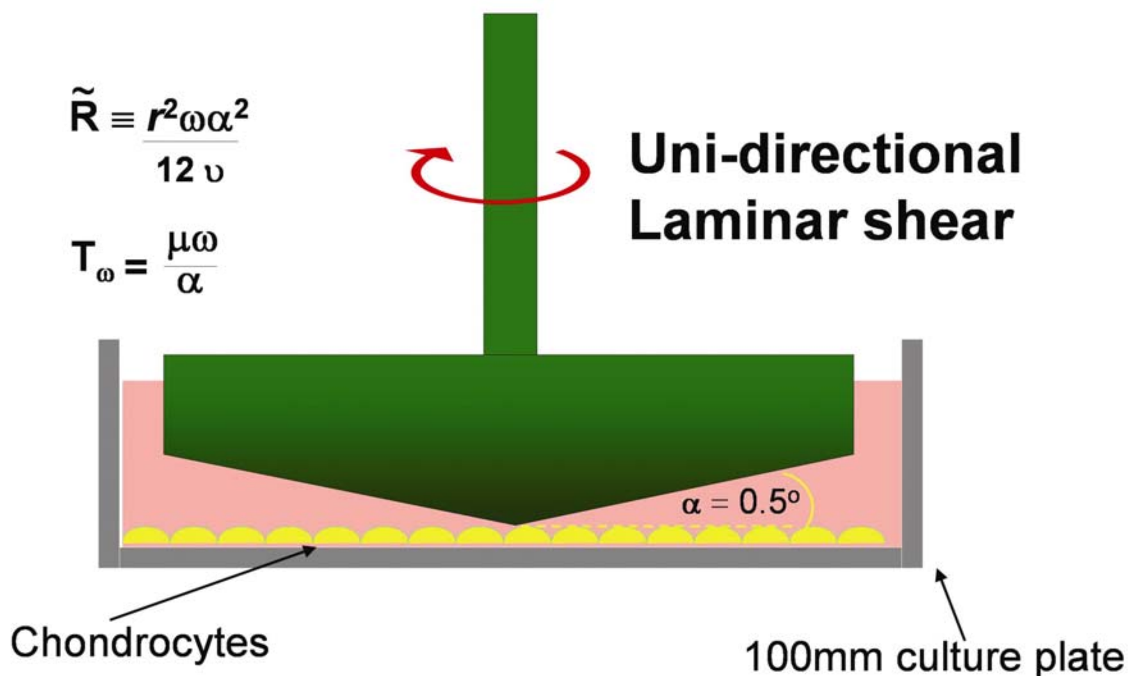

Figure 2.2. Schematic of Cone-Plate Shear Apparatus. The base of a cone is submerged into the culture media above the cell monolayer. Laminar, azimuthal (following direction of rotation) flow occurs when a dimensionless ratio (analogous to Reynold's Number) R < 1 where R is defined to be R = $r^2\omega\alpha/12\upsilon$ where r = radial distance from center, ω is the angular velocity, α is the angle of the cone surface from the plate, and υ is the kinematic viscosity. Under these parameters the shear stress (T_ω) across the plate can be estimated by $T_\omega = \mu\omega/\alpha$ where μ is viscosity of the media. Image provided courtesy of Dr. Hanjoong Jo and adapted slightly from [37].

Figure 2.3. Photograph of Cone-Plate Shear Apparatus. This particular model can hold 4 separate cone-plate devices driven by a magnetic stir plate (cone contains a magnetic stir bar). Petrie dishes are securely suctioned onto a base plate via a vacuum pump to limit movement and curvature of the base of the Petrie dishes. For this study, two similar devices as shown above were available allowing for 8 concurrently sheared samples in a single experiment. Static control samples were cultured in the same incubator during shear.

Mechanoregulation of Collagen Type II

Aggrecan and Collagen type II mRNA was increased with hydrostatic pressure [41]. Collagen type II was upregulated in a parallel plate designed bioreactor for tissue engineering, suggesting steady, laminar shear flow can in some cases also upregulate desired matrix proteins and that this shear type may be useful for bioreactor design

potentially [42]. Other reports suggest that fluid shear stress reduces expression of aggrecan and collagen type II [43].

<u>Mechanoregulation of Cartilage Oligomeric Matrix Protein</u>

Cartilage oligomeric matrix protein has also been shown to be responsive to shear. It is known that COMP serum levels are elevated in marathon runners [44] and in osteoarthritic patients [45, 46]. COMP appears to be important in the binding of glycosaminoglycans, integrins, and collagen [47], and is thought to be important to organizing the arrangement of these molecules. Wong et al showed that unconfined cyclic compression of chondrocytes resulted in a large increase in COMP expression [48]. Members of this group also went on to show that blocking integrin Beta 1 could inhibit this upregulation of COMP by mechanical stimulation [49]. Oscillatory motion was shown to upregulate COMP expression in chondrocytes in a 3D-scaffold [50].

Apoptosis and Proliferation of Chondrocytes Under Shear Stress

Under some conditions, shear stress has been shown to induce signals that promote apoptosis in chondrocytes. Abulencia et al demonstrated that fluid shear stress can induce apoptosis in monolayer chondrocytes at higher levels of shear (10-20dyn/cm^2) via a COX-2 mediated pathway. However, apoptosis was less apparent in these cells at lower shear (4 dynes/cm^2) even when exposed for up to 24 hours [51]. Shear stress increased release of nitric oxide (NO) and nucleosomal degradation in osteoarthritic chondrocytes as well as decreased the level of anti-apoptotic factor bcl-2 [33]. Another study from that group showed that NO release during shear affected matrix molecule mRNA production. Shear stress up to 24 hours and then incubated at static conditions for

24 hours showed a decrease in collagen type II mRNA and aggrecan mRNA. Inclusion of NO antagonists reduced this effect of the shear, suggesting these effects occur through NO induction [52]. Martin and Buckwalter showed that human articular cartilage under high shear stress can increase apoptotic activity due to shear-induced oxidative stress [53].

Shear stress and fluid flow can regulate cell proliferation as well. Lin et al showed that chondrocytes perfused in a bioreactor with constant fluid flow could increase the proliferation of cells dependent on level of shear over long exposure time [54]. Other reports indicate that chondrocytes could increase proliferation after 96 hours of fluid shear likely via a TGF-Beta1 pathway [55].

Shear stress has been shown to alter proliferation differently in other cell types. Endothelial cells have been shown to increase proliferation measurements under shear stress, especially under oscillatory flow [56], but were also shown to be inhibited in laminar fluid flow via a cyclin-dependent kinase inhibitor p21 [57]. Osteoblasts have also been shown to exhibit shear-induced proliferation via multiple pathways [58], including through Erk1 and Erk 2 [59]. The variety of reports of fluid shear effects on cells of different phenotypes indicate much is still not understood about common effects of mechanical stresses on general cellular behavior, and which responses are specific to cell type.

Mechanical Loading during Endochondral Ossification

Mechanical Influence During Embryonic Development

It has also been observed through creative experimental methods that mechanical stresses may play a role in endochondral ossification as early in development as in utero. Habib et al. showed that by suturing the jaw of mice embryos so that fetal jaw movement was restricted resulted in disorganized osteochondral boundary formation of the temporal-mandibular joint, indicating that motion and mechanical stresses may help develop proper boundaries of this joint [60, 61]. Other studies have shown related results in the rat hip [62] and chick skeleton [63].

Fracture Callus Loading

When a bone fracture occurs of significant size or severity, it generally will heal through a process of endochondral ossification. This occurs via the formation of a callus that forms within the fracture, converts to cartilage, and ultimately remodels back to bony tissue in favorable circumstances. The time scale of this process however is much quicker than the process occurring in the growth plate and has been shown to be responsive to appropriate mechanical loading [64]. A combination of hypoxia and compressive stress is thought to guide the cells to differentiate into chondrocytes at the center of the callus, with slightly altered responses at other regions of the callus [65]. Goodship et al showed that even low-magnitude loading of fracture callus at high frequency can increase the stiffness and mineral deposition around the fracture site compared to rigidly fixed fractures [66].

Growth Plate Loading

Compressive loading of the growth plate in vivo has shown that mineralization rate can be inhibited and hypertrophic chondrocytes can increase in number [67]. Another study showed that sustained in vivo compression inhibited longitudinal growth more than only half-time loading periods [68]. However, ex vivo bending of mouse bone anlagen suggest that bending increased bone collar formation but did not alter chondrocyte differentiation on bone elongation [69]. In vivo studies provide relevant information for the greater physiological perspective, but are also subject to significant variability like in vitro studies, and sometimes the underlying source of mechanisms involved can be hard to decouple such as which cells are effected most by the mechanical signals and are secreting the signals that are most influential to the overall response of the entire tissue or organ. No studies have shown specifically how mechanical loading can affect uniquely chondrocytes from the least mature stage in endochondral ossification, such as those found in the resting zone of the growth plate.

INTEGRIN MEDIATED MECHANOTRANSDUCTION

Mechanotransduction refers to the process whereby mechanical signals are converted to chemical mediators that ultimately realize the cellular response to physical stimuli. There has been evidence that many possible pathways may be involved in cellular responses to mechanical stimuli, including cell-cell interactions such as cadherins, stretch activated ion channels, and streaming potentials induced by convection of extracellular ion concentrations. Additionally, more and more evidence is continuing

to implicate a significant role for integrin receptors in mechanotransduction, including in chondrocytes.

Structures both inside and outside the cell contribute to the mechanical properties of a tissue, but integrins provide the primary attachment site between a cell and its extracellular matrix. Thus, if a mechanical stimulus is experienced in the ECM or across the surface of an adherent cell, integrin-ligand interactions will be a significant anchor point where that stimulus can be relayed from outside to inside the cell. Integrins consist of a family of heterodimeric, transmembrane receptors that bind to specific ligands in the ECM and result in cellular adhesion sites. Each integrin has an α and β subunit that interact noncovalently and over 20 combinations of these dimer pairs have been found to occur [70]. Both subunits have a large amino-terminal head of over 700 residues that is expressed outside the membrane. Each subunit has a single domain that crosses through the membrane and a smaller cytoplasmic tail domain that ranges between about 13-70 residues [71].

Integrins are a logical candidate to serve as a mechanoreceptor due to their significant role in cell adhesion and spreading. They influence cell shape and are tension points where stresses acting on the whole cell body would be transmitted to the portions of matrix adherent to the cell. Integrins are also involved in transmitting cellular signals. When integrins bind, they can recruit other integrins to their proximity and also participate in the assembly of what is known a focal adhesion complex. These complex assemblies form when the transmembrane integrin receptor is bound to an extracellular protein and involve the intracellular recruitment of focal adhesion kinase, talin, paxillin, and actin assemblies among others.

Integrin Mediated Mechanotransduction in Chondrocytes

Many integrin subunits have been found to be expressed in chondrocytes, including alpha 1,2, 5, v and beta 1 and 3. The β1 integrin subunit has been shown to be prominently expressed in chondrocytes [72] including the growth plate [73] and to play a role in chondrocyte adhesion to cartilage under shear stress [74] and cyclical pressure-induced strain [75]. Little to no work has focused on the role of integrin β1 in mechanotransduction in the growth plate, particularly with respect to differentiation along an endochondral pathway. It has been shown in a conditional knock out mouse that integrin beta 1 is important for the proper development and orientation of chondrocytes in the growth plate [76], suggesting this receptor subunit could potentially be important for mechanically-induced differentiation within the context of ligand signaling from the extracellular matrix.

Integrin beta 1 has especially been shown to be involved in mechanotransduction in chondrocytes, especially with its dimeric partner alpha 5 [77]. This integrin receptor binds primarily to the protein fibronectin, but also can bind to some collagen molecules and vitronectin. The receptor α5β1 recognizes the peptide sequence RGD (arginine, glycine, aspartic acid). It has been shown that mechanically upregulated expression of COMP can be inhibited by inclusion of integrin beta 1 antibodies in the medium [49]. Also, the use of RGD peptides prevented a mechanically induced decrease in aggrecan mRNA expression in non-degenerative nucleus pulposa cells (from cartilaginous intervertebral discs). Interestingly, RGD containing peptides had not effect on cells from degenerative nucleus pulposa cells, suggesting that this pathological condition may include an alteration in mechanotranduction pathways [38]. Similar evidence was seen

for osteoarthritic chondrocytes in their modification of membrane polarity in shear when compared to normal cells [77]. TGF-Beta3 induced human chondrocytes to be responsive to dynamic compression by upregulating sulfate incorporation and thymidine incorporation, and these effects of compression were blocked by RGD containing peptides, but not by nonsense peptides [78]. This suggests some effects of mechanical stimulation require coordination or preparation by growth factors or other chemical modifiers.

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CHAPTER 3. INORGANIC PHOSPHATE MODULATES RESPONSIVENESS TO 24,25$(OH)_2D_3$ IN CHONDROGENIC ATDC5 CELLS

INTRODUCTION

The formation of mammalian long bones occurs through the process of endochondral development, which begins with mesenchymal condensation in the embryo to form cartilaginous limb buds. Primary and secondary centers of ossification develop within the cartilage, ultimately becoming bone. The ends of the bones, the epiphyses, are separated from the metaphyses and diaphysis by a region of cartilage called a growth plate, which is spatially organized into zones defined by the differentiation state of chondrocytes resident in that region of the tissue. Nearest to the epiphysis is the reserve or resting zone. Chondrocytes in this region produce an extracellular matrix enriched in type II collagen and proteoglycan aggregates containing sulfated glycosaminoglycans. In embryonic bone, this region is relatively small as cells are rapidly progressing along the endochondral developmental pathway. In contrast, in post-natal growth plates, the resting zone serves as a chondrocyte reservoir and represents a larger component of the tissue. At the base of the resting zone, chondrocytes appear to align in columns to form the proliferative zone, in which they undergo rapid division, providing the major contribution of the growth plate to longitudinal bone growth [1]. Following proliferation, the cells undergo a prehypertrophic phase, transitioning into hypertrophy, a period in which the cells remodel their extracellular matrix to accommodate their increase in size and to prepare the matrix for calcification [2]. During this phase, many of the hypertrophic chondrocytes also undergo apoptosis, which causes the growth plate to

retain a consistent length despite continued growth of the bone. This process depends upon coordinated mineralization of the matrix. In conditions like vitamin D and phosphate deficient rickets, where the growth plate fails to become calcified, the hypertrophic zone continues to increase in length [3].

Previous work examining mouse and rat growth plates has shown that two metabolites of vitamin D, 24,25-dihydroxy vitamin D3 [24,25$(OH)_2D_3$] and 1,25-dihydroxy vitamin D3 [1,25$(OH)_2D_3$], each play a role in regulating the process of endochondral development [4-6]. Chondrocytes from the resting zone exhibit specific sensitivity to 24,25$(OH)_2D_3$, whereas cells in the growth zone no longer exhibit the same responses to 24,25$(OH)_2D_3$ but have acquired specific sensitivity to 1,25$(OH)_2D_3$. 24R,25$(OH)_2D_3$ stimulates extracellular matrix production by resting zone cells, increasing production of sulfated glycosaminoglycans [7]. In addition, it causes resting zone chondrocytes to produce extracellular matrix vesicles containing neutral metalloproteinases [8] and reduces total matrix vesicle metalloproteinase activity in vitro [9] and in vivo [10]. In contrast, 1α,25$(OH)_2D_3$ inhibits DNA synthesis in prehypertrophic and hypertrophic chondrocytes and reduces synthesis of sulfated proteoglycans [7], while increasing production of alkaline phosphatase-enriched matrix vesicles that contain increased metalloproteinase activity [10, 11]. Moreover, 1α,25$(OH)_2D_3$ acts directly on matrix vesicles produced by these cells, activating resident phospholipases, causing loss of membrane integrity and release of matrix processing enzymes [12]. These observations suggest that 24R,25$(OH)_2D_3$ enhances matrix production and maintenance of resting zone cartilage, whereas 1α,25$(OH)_2D_3$ modulates the rate and extent of matrix degradation during chondrocyte hypertrophy. Interestingly,

1α,25(OH)$_2$D$_3$ induces production of 24R,25(OH)$_2$D$_3$ by growth zone chondrocytes [13], suggesting cross-talk among cells at different maturation states in endochondral development.

Inorganic phosphate (Pi) has also been implicated in the differentiation of the growth plate by acting as a signal affecting the differentiation of mineralization-competent cells [14-16]. The extracellular concentration of Pi is relatively high in the extracellular matrix produced by hypertrophic chondrocytes, in part due to the increased activity of matrix vesicle alkaline phosphatase [17]. Studies examining the effects of exogenous Pi on chondrocyte phenotype in post-fetal growth plates show that Pi can induce apoptosis [3, 18]. This suggests a feed-back loop in which 1α,25(OH)$_2$D$_3$ activates matrix vesicle alkaline phosphatase, releasing Pi into the matrix and Pi then acts back on the chondrocytes to induce apoptosis.

It is less clear how Pi might interact with 24R,25(OH)$_2$D$_3$. To address this question, we took advantage of the embryonic ATDC5 cell model. This prechondrocyte cell line offers a useful culture system for studying the progression of endochondral development. When confluent cultures of ATDC5 cells are grown in high insulin media, they form cartilage nodules that exhibit the differentiation sequence typical of long bone growth plates [19, 20]. 1α,25(OH)$_2$D$_3$ has been shown to inhibit proliferation and differentiation of ATDC5 cells [21], but it is not known if these cells are regulated by 24,25(OH)$_2$D$_3$. Interestingly, Pi has been shown to be a regulator of chondrogenic differentiation and apoptosis in these cells, including upregulation of collagen type X, a marker of maturation in the hypertrophic zone of the growth plate [22, 23]. Pi was also shown to regulate expression of matrix Gla protein (MGP) via ERK1/2 in both ATDC5

cells and primary growth plate organ cultures [24]. MGP is an inhibitor of matrix calcification [25], suggesting that Pi may induce production of factors that retard endochondral ossification like 24R,25$(OH)_2D_3$, as well as production of factors that stimulate chondrocyte maturation and apoptosis.

The purpose of the present study was to determine if Pi treatment causes ATDC5 cells to become responsive to 1α,25$(OH)_2D_3$ or 24R,25$(OH)_2D_3$ and if so, what are the consequences to endochondral maturation of the cells. The physiological importance of Pi is supported by the observation that active ion transport through the membrane is required [26].

METHODS AND MATERIALS

Cell Culture

ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 ratio of DMEM/F12 media (Cellgro, Manassas, VA) with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 10 μg/ml human transferrin (Sigma Chemical Company, St. Louis, MO), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 3 x 10^{-8} M sodium selenite (Sigma). After reaching confluence cells were cultured with differentiation media, which is identical to maintenance media with the addition of 10 μg/ml bovine insulin (Sigma) and 50 μg/ml ascorbic acid (Sigma) [19, 27]. At 10 days post-confluence, cells were cultured for 24 hours in differentiation media supplemented with Pi (0 to 20 mM beyond media basal level) and 10% FBS (10% FBS was used to ensure sufficient serum proteins such as fetuin that help regulate pathologic precipitation of calcium phosphate crystals [28, 29]). To make Pi-supplemented media, a more

concentrated volume of Pi was dissolved in warm DMEM/F12 (37˚C) using molar ratios of 4 moles of dibasic sodium phosphate Na_2HPO_3 (Sigma) to one mole of monobasic sodium phosphate NaH_2PO_3(Sigma) [16]. When the phosphate salts were completely dissolved, the pH was adjusted to 7.4, and the solution was filter sterilized. An appropriate aliquot from the concentrated Pi solution was added to the media preparation to result in the desired final concentration. Control cultures were also treated on day 10 with differentiation media with 10% FBS. Some experiments also included concurrent treatment with the Pi transporter inhibitor phosphonoformic acid (PFA) (sodium phosphonoformate tribasic hexahydrate) (Sigma) to test the effect of phosphate transport inhibition. Cells were returned to differentiation media with 5% FBS on day 11 for treatment with 24R,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ or ethanol vehicle (Sigma).

Cell Number

Effects of Pi and 24R,25$(OH)_2D_3$ on proliferation were determined by measuring cell number at harvest and also as a function of DNA synthesis (described below). To measure cell number, ATDC5 cells were treated with Pi for 24 hours followed by treatment with 24R,25$(OH)_2D_3$ for 24 hours. At harvest, cells were washed twice with DMEM and trypsinized (Invitrogen) for 10 minutes. Cells were resuspended in saline, and counted on a Beckman Coulter Z1 particle counter.

Alkaline Phosphatase Activity

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline]-specific activity was used as an indication of chondrocyte differentiation. Harvested cells were suspended in 0.05% Triton-X. After 3 freeze-thaw cycles to lyse the cells, alkaline

phosphatase activity in the cell lysates was determined [30] and normalized to protein content using the Macro BCA Protein Assay Kit (Pierce, Rockford, IL).

[^{35}S]-Sulfate Incorporation

To determine the role of inorganic phosphate in mediating the effects of 24R,25$(OH)_2D_3$ on extra-cellular matrix production, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation as described previously [31, 32]. Four hours prior to harvest, [^{35}S]-sulfate (Perkin Elmer) was added to the cultures. Cell layers were collected and dialyzed to remove any unbound [^{35}S]-sulfate. Radiolabeled incorporated into the cell layer was expressed as disintegration per minute normalized to protein levels for each sample.

Histology

To verify that the ATDC5 cells produced a cartilage extracellular matrix, cultures were examined for collagen II protein by immunohistochemistry. Cells were seeded into four well chamber slides. Once the cells reached confluence they were cultured with differentiation media and were treated with 20 mM Pi on Day 10 and 10^{-7} M 24,25$(OH)_2D_3$ on Day 11, respectively, or with the appropriate vehicle. The cells were fixed for 30 min in 4% formalin in PBS, after which the cells were rinsed three times in PBS and stored in 75% absolute ethanol. At time of staining, the fixed cultures were etched with 0.25% pepsin to expose the antigen, followed by PBS washes. Cell monolayers were blocked with 2% horse serum in PBS. Cells were then treated with the mouse anti-collagen II antibody (Hybridoma Bank, University of Iowa). After more PBS washes a biotinylated horse anti-mouse IgG antibody was applied. An alkaline

phosphatase ABC kit was used to visualize the biotin. Samples were counterstained with haematoxylin, and visualized with light microscopy.

RNA Extraction and RT-PCR

Cellular RNA was harvested using the Trizol® reagent kit (Invitrogen). RNA samples were converted to cDNA using the Omniscript RT kit (Qiagen, Valencia, CA) and then PCR was performed using HotStar Taq Master Mix Kit (Qiagen). PCR product was visualized using gel electrophoresis in 5% TBE Ready Gels (Biorad, Hercules, CA) and visualized on a Versadoc Model 1000 (Biorad). To better visualize qualitative differences between groups, densities of the visualized bands were measured using Quantity One 4.4.1 Software (Biorad). The gene specific primers (MWG Biotech, Huntsville, AL) used to amplify mRNA were as follows: aggrecan - 5'ATC ACA GCC ACC ACT TCC 3' (sense) and 5' CTC CAC TCA CAG ATG TTA TAC C 3' (anti-sense), collagen type I - 5' GGC TCC TGC TCC TCT TAG 3' (sense) and 5' TCT TCT GAG TTT GGT GAT ACG 3' (anti-sense), collagen type II - 5' GCG GTC CTA ACG GTG TCA G 3' (sense) and 5' ACC AGC CTT CTC GTC ATA CC 3' (anti-sense), collagen type X - 5' GCA CCT ACT GCT GGG TAA GC 3' (sense) and 5' GCC AGG TCT CAA TGG TCC TA 3' (anti-sense), cartilage oligomeric matrix protein (COMP) - 5' CCA CTG ATG ATG ACT ATG C 3' (sense) and 5' GAT GTA GCC AAC TTG AGG 3' (anti-sense), SOX9 - 5' GAA CGA GAG CGA GAA GAG ACC 3' (sense) and 5' GGC GGA CCC TGA GAT TGC 3' (anti-sense), and glyceraldehyde phosphate dehydrogenase (GAPDH) - 5' TTC AAC GGC ACA GTC AAG G 3' (sense) and 5' TCT CGC TCC TGG AAG ATG G 3' (anti-sense). The negative control was RNA from

mouse liver tissue and the positive control was RNA from mouse cartilage (Zyagen, San Diego, CA).

DNA Synthesis

The effects of inorganic phosphate and 24R,25$(OH)_2D_3$ on DNA synthesis were determined by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA, Sigma) insoluble cell precipitates as previously described [33]. ATDC5 cells were treated for twenty-four hours with Pi followed by treatment with 24R,25$(OH)_2D_3$. Two hours prior to harvest, [^{3}H]-thymidine (Perkin Elmer, Waltham, MA) was added. Radioactivity in TCA-precipitable material was measured by liquid scintillation spectroscopy.

Assays for Apoptosis

DNA Fragmentation: Cells were pre-labeled with [^{3}H]-thymidine (Perkin Elmer) for 4 hours and then treated with Pi for 24 hours followed by 24R,25$(OH)_2D_3$ for 24 hours, or with Pi followed by vehicle. Cell monolayers were washed with DMEM three times to remove any residual unincorporated [^{3}H]-thymidine and cells were lysed with TE buffer (10mM Tris-HCl, 1mM EDTA, 0.2% Triton X-100) for 30 minutes. Cell lysates were centrifuged at 13,000g for 15 minutes to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of [^{3}H]-DNA.

Caspase-3 Activity: Caspase-3 activity was assessed using the colorimetric CaspACE™ Assay System (Promega, Madison, WI). Cells were harvested 24h post treatment with 200µl cell lysis buffer followed by two 10 second periods of sonication. After harvest, 2µl of the caspase-3 selective substrate DEVD-pNA were added to each

well containing 100µl of cell lysate and incubated at 37^0C for 4h. DEVD-pNA cleavage into the colorimetric product pNA was measured at 405nm. Caspase-3 activity was normalized to protein content as determined by the Pierce Macro BCA Protein Assay Kit.

Statistical Analysis

Data are presented as means $\pm$ standard error of the mean (SEM) for six independent cultures for each variable. The results for individual experiments are shown. To ensure validity of the results, all quantitative experiments were repeated at least two or more times. Data were analyzed with ANOVA followed by Bonferroni's modification of Student's T-test. Differences in means were considered to be statistically significant if the P value was less than 0.05.

RESULTS

Pi treatment alone did not affect cell number except at the highest concentration (20 mM) tested (Figure 3.1A). $24R,25(OH)_2D_3$ caused a small but significant decrease in the control cultures and further decreased the effects of 20mM Pi. The expanded dose response (Figure 3.1B) confirmed that the effects of Pi on response to $24R,25(OH)_2D_3$. Pi reduced ATDC5 cell number at 20mM. Effects of $24R,25(OH)_2D_3$ depended on Pi concentration and were dose-dependent from 19.5 to 20.25 mM with the greatest effect at 20mM.

Pi had a biphasic effect on alkaline phosphatase activity in the ATDC5 cell lysates, with an increase over control levels at 20mM Pi (Figure 3.1C). Effects of $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ on alkaline phosphatase were also sensitive to Pi pretreatment. $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ reduced enzyme activity in control

cultures. At 5 mM Pi, only 10^{-8}M $1\alpha,25(OH)_2D_3$ reduced enzyme activity over that seen in Pi treated cells. At 15 mM Pi, both $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ stimulated activity but at 20mM Pi, $24R,25(OH)_2D_3$ caused a 30% increase in alkaline phosphatase. The stimulatory effect of 20mM Pi on $24R,25(OH)_2D_3$-dependent alkaline phosphatase activity was confirmed in the expanded dose-response study (Figure 3.1D).

The response of ATDC5 cells to $24R,25(OH)_2D_3$ was dose-dependent following pretreatment with 20 mM Pi. The reduction in cell number was greatest at 10^{-7} M (Figure 3.2A) and the stimulatory effect of alkaline phosphatase was greatest at 10^{-7} M $24R,25(OH)_2D_3$ (Figure 3.2B). The effects of 20 mM Pi were specific based on their inhibition by phosphonoformic acid (PFA), which is a specific competitive inhibitor of the type III sodium dependent phosphate transporter Glvr-1. PFA caused a dose-dependent decrease in the Pi-induced reduction in cell number (Figure 3.2C) and a dose-dependent decrease in Pi-activated alkaline phosphatase (Figure 3.2D).

Treatment with 20mM Pi on Day 10 reduced [^{35}S]-sulfate incorporation at the end of Day 11, but this was restored to control levels when Pi was followed by treatment with 10^{-7} M $24R,25(OH)_2D_3$ (Figure 3.3A). ATDC5 cells produced an extracellular matrix containing type II collagen, regardless of the treatment regimen. Cell layers stained positively with anti-type II collagen antibody whether they were untreated or treated with Pi followed by $24R,25(OH)_2D_3$ (Figure 3.3B). Semiquantitative analysis by RT-PCR showed that mRNA expression of chondrogenic markers was affected (Figure 3.4). Pi treatment reduced collagen II mRNA, but dramatically increased collagen X mRNA. During direct exposure to Pi there was an increase in aggrecan and decrease in COMP mRNAs. By itself, $24R,25(OH)_2D_3$ had minimal effect on any markers, but the

steroid rescued collagen II mRNA and enhanced collagen X mRNA after pretreatment with Pi.

Pi caused an increase in ATDC5 apoptosis. Pi increased DNA fragmentation (Figure 3.5A), increased caspase-3 activity (Figure 3.5B), and reduced DNA synthesis (Figure 3.5C) by the end of Pi treatment. The stimulatory effect of Pi on apoptosis was reversed by subsequent treatment with $24R,25(OH)_2D_3$. $24R,25(OH)_2D_3$ blocked DNA fragmentation in Pi-treated cells (Figure 3.5D), decreased caspase-3 activity (Figure 3.5E), and increased DNA synthesis (Figure 3.5F).

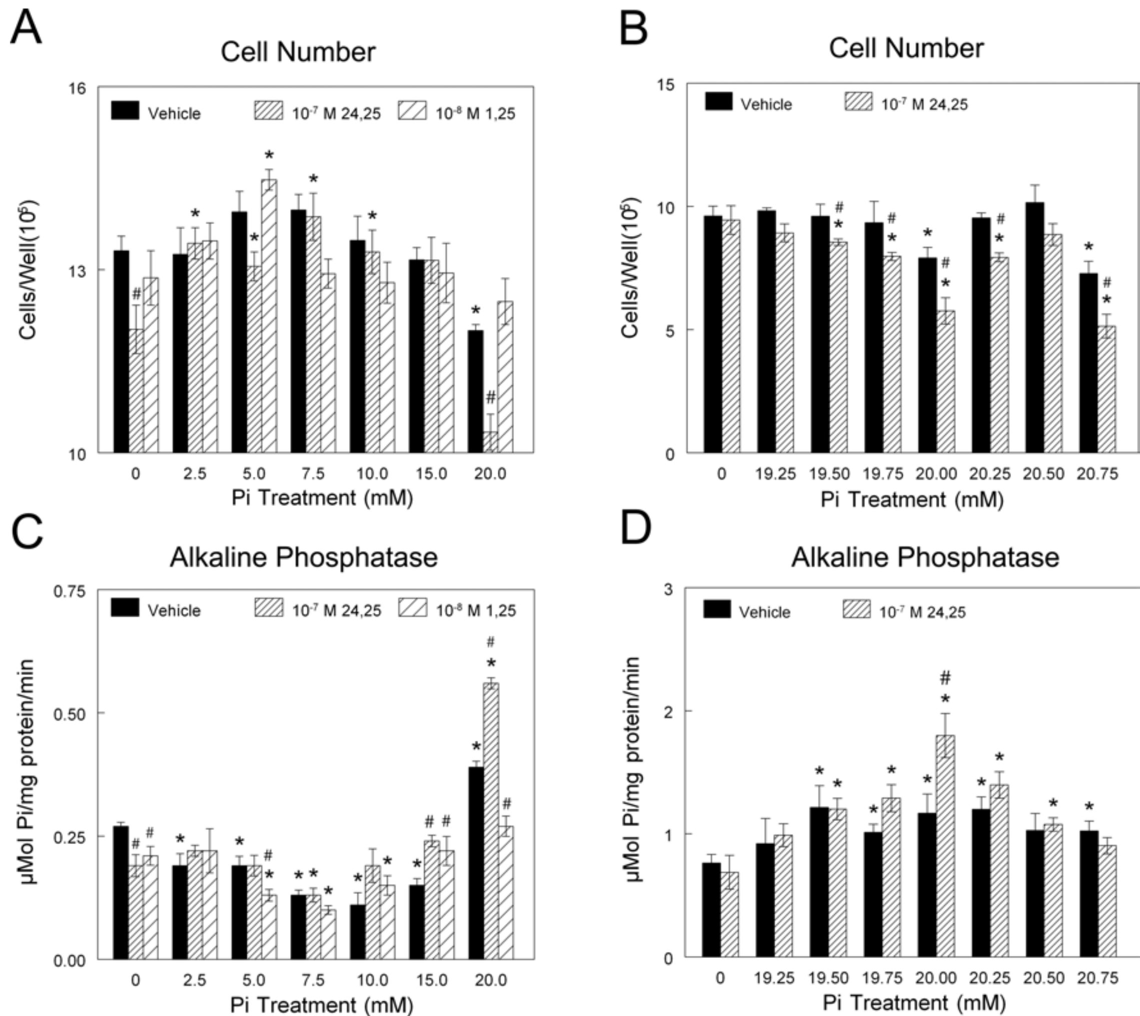


Figure 3.1. Pi dose-dependently induces $24R,25(OH)_2D_3$ sensitivity in ATDC5 cells. Cells were pretreated with control media or media with additional Pi (2.5 mM to 20.75 mM) on Day 10 followed by vehicle, 10^{-8} M 1,25, or 10^{-7} M 24,25 on Day 11. Cells were harvested at end of Day 11 for cell number (A,C) and alkaline phosphatase activity(B,D). # $P < 0.05$ v. vehicle within same Pi-treated group, * $P < 0.05$ v. 0mM Pi. (B,D).

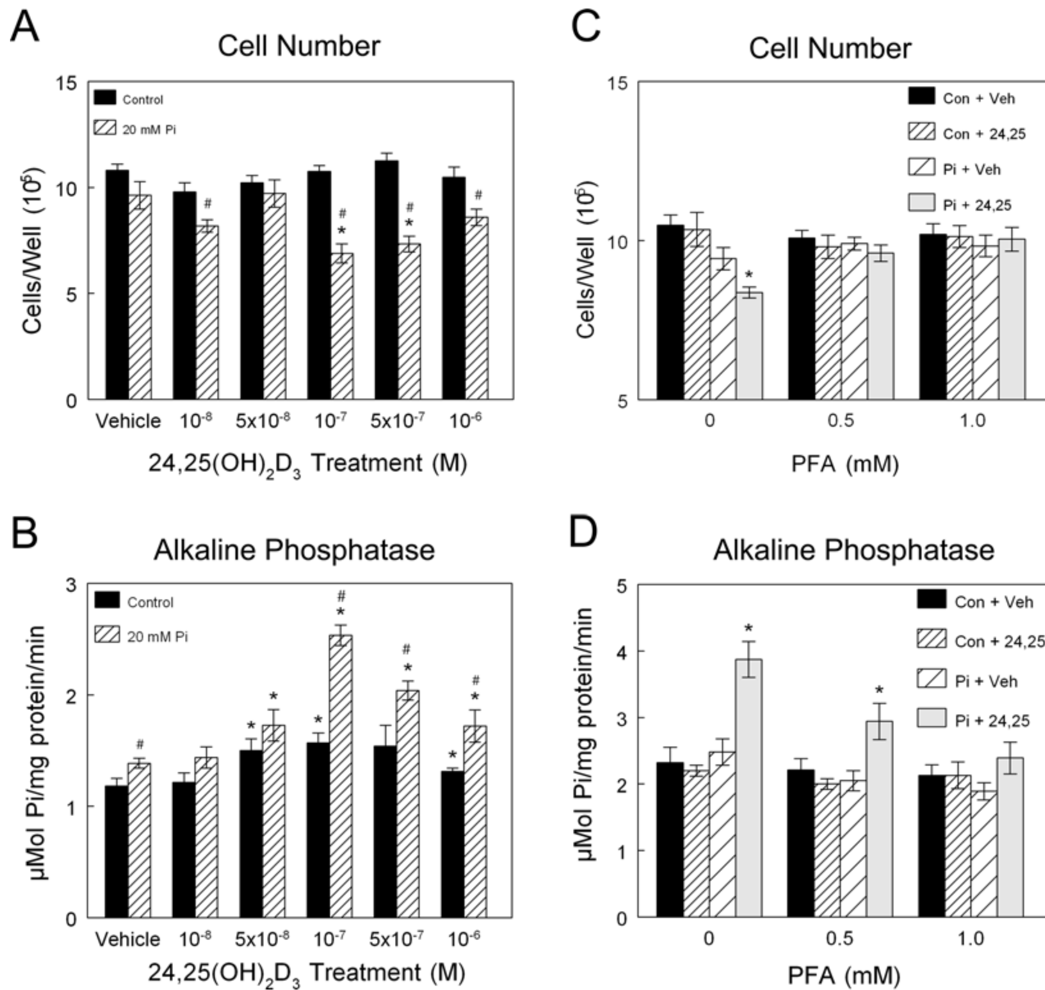
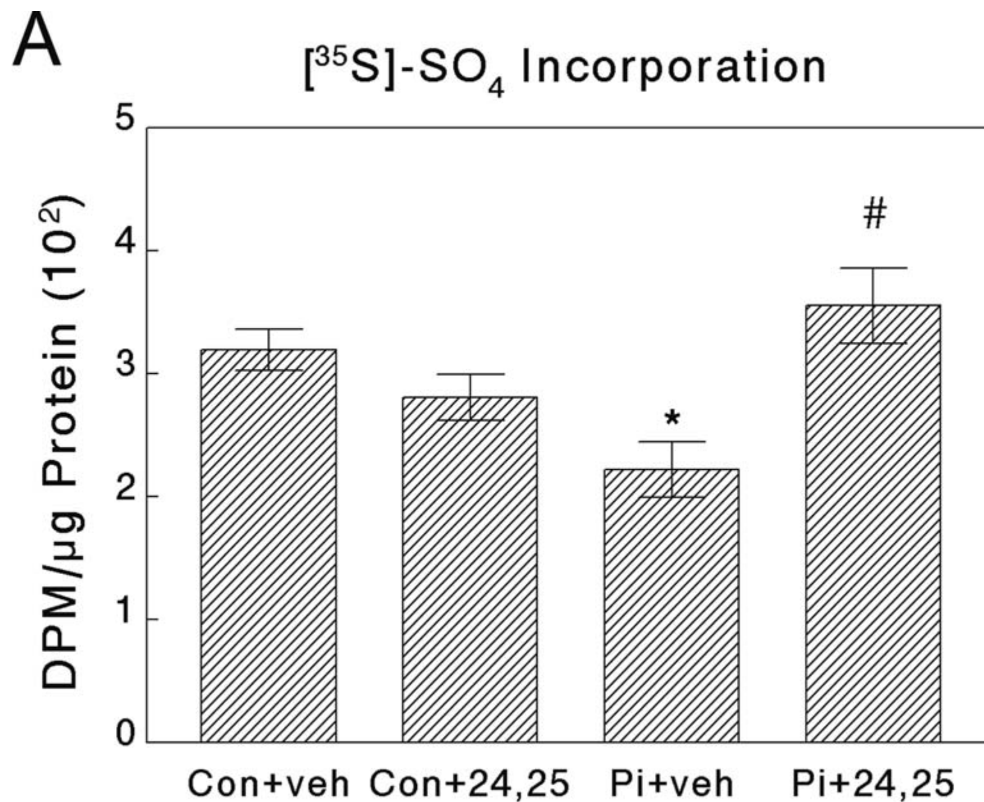


Figure 3.2. Response to $24R,25(OH)_2D_3$ is dose-dependent on Pi pretreatment, and Pi transport is required for Pi-induced $24R,25(OH)_2D_3$ sensitivity. (A-B) Cells were treated with vehicle or 10^{-8} to 10^{-6} M $24R,25(OH)_2D_3$ after pretreatment with 20 mM Pi. Cell number (A) and alkaline phosphatase activity (B) were measured. # $P < 0.05$ v. Control, * $P < 0.05$ v. no $24,25(OH)_2D_3$. (C-D) Cells were treated with control or 20mM Pi on Day 10 with 0-1mM of the Pi transport inhibitor PFA, and then treated on Day 11 with vehicle or 10^{-7} M $24R,25(OH)_2D_3$. Cell number (C) and alkaline phosphatase (D) were then measured. * $P < 0.05$ treatment v. control for each PFA concentration.



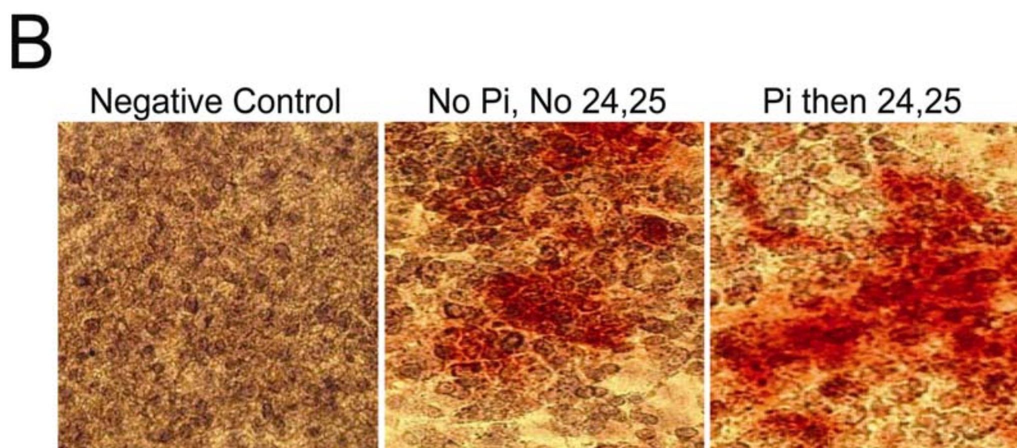


Figure 3.3. 24R,25$(OH)_2D_3$ recovers Pi-induced reduction of sulfate incorporation, but neither treatment necessary for collagen type 2 protein expression. (A) ATDC5 cells were treated with 20 mM Pi or control media on Day 10 and with vehicle or 10^{-7} 24R,25$(OH)_2D_3$ on Day 11. Pi on day 10 resulted in lower sulfate incorporation by the end of day 11, but this effect was not seen for cells that received subsequent 24R,25$(OH)_2D_3$ on day 11. * $P < 0.05$ vs. control, # $P < 0.05$ vs. vehicle. (B) ATDC5 cells treated with 20 mM Pi on Day 10 and then 10^{-7} M 24R,25$(OH)_2D_3$ on Day 11 or with neither of these treatments were tested with immunohistochemical staining for collagen type II expression at the end of day 11. Negative control was performed without primary antibody to test for nonspecific staining.

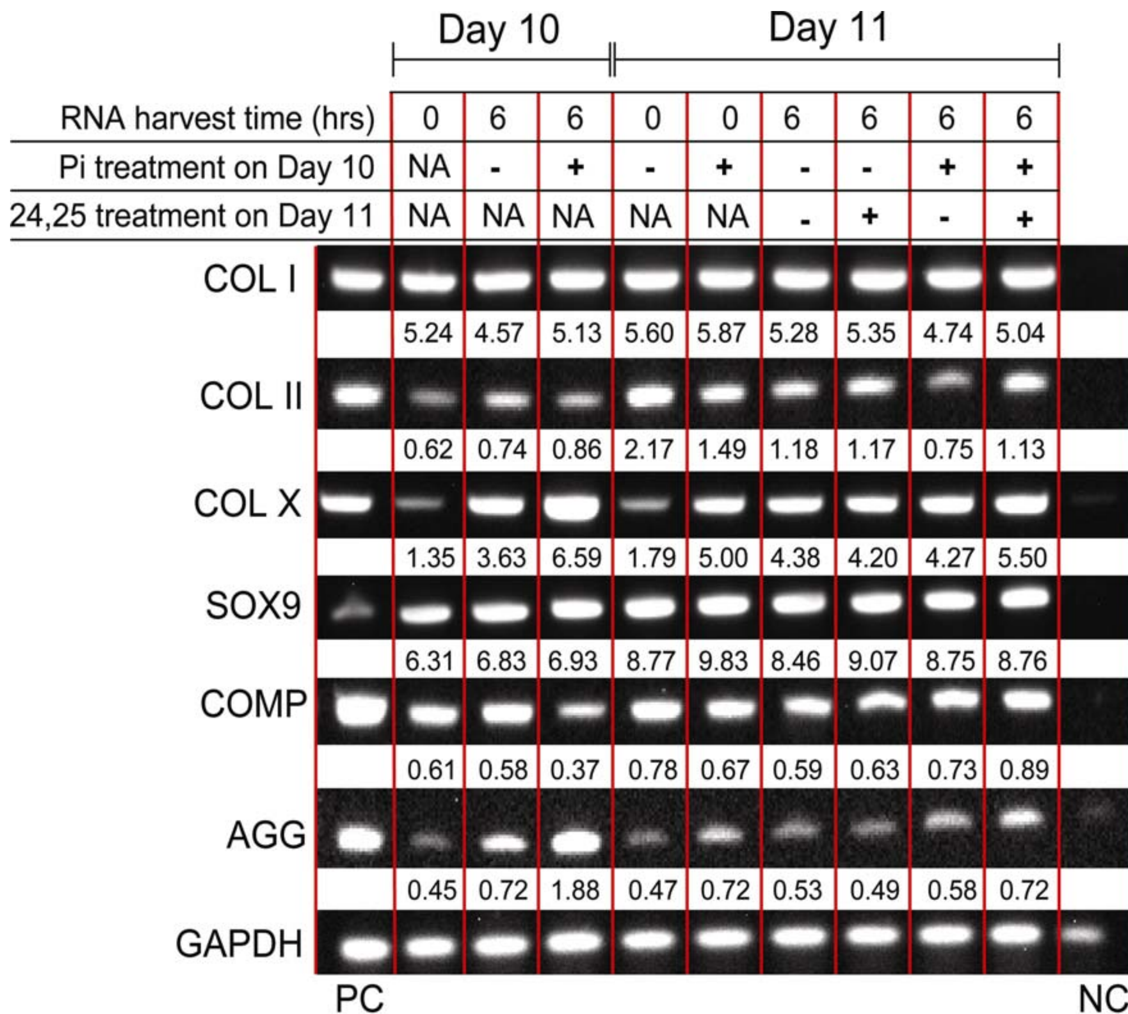


Figure 3.4. Chondrocyte marker mRNA expression in ATDC5 cells during Pi and 24R,25$(OH)_2D_3$ treatment. ATDC5 cells were treated +/- 20 mM Pi on Day 10 and +/- 10^{-7} M 24R,25$(OH)_2D_3$ on Day 11. RNA was extracted at 0 and 6 hrs on Day 10 and Day 11 and RT-PCR was performed to assess mRNA expression of collagen I, II, and X and also SOX9, COMP, and aggrecan (AGG). GAPDH was also assessed to confirm consistent mRNA levels between samples. The density of each band compared to the density of the respective GAPDH band is shown numerically underneath each sample. Positive control (PC) is mRNA from mouse cartilage tissue and negative control (NC) is mRNA from mouse liver. GAPDH band for negative control is shown to demonstrate RNA present in negative control sample.

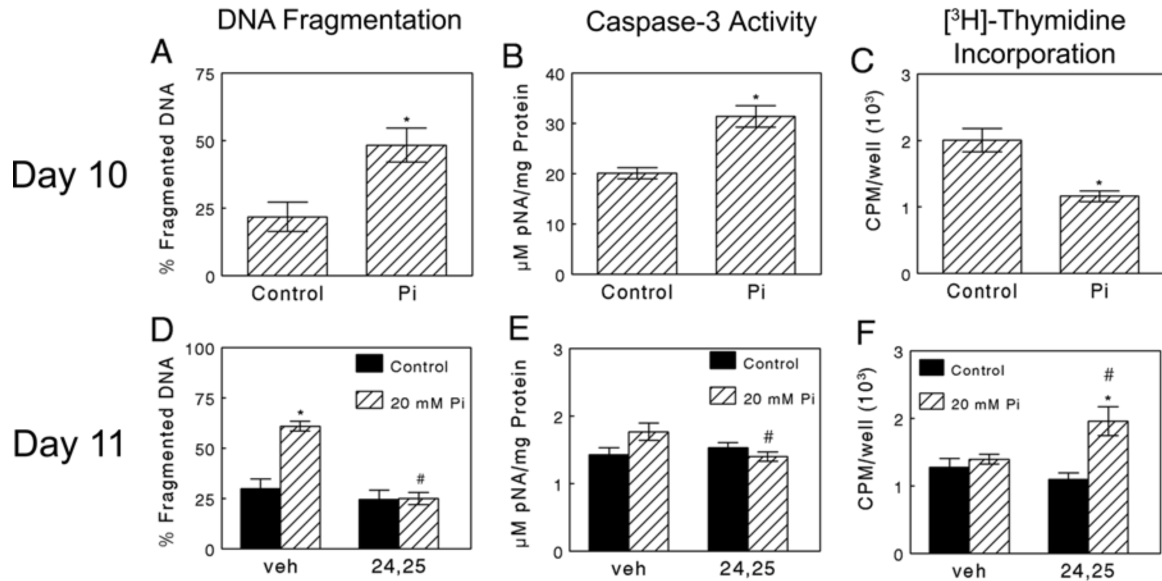


Figure 3.5. Time course effects of Pi and 24R,25$(OH)_2D_3$ on apoptosis and [3H]-thymidine incorporation. ATDC5 cells were treated with 20 mM Pi or control on Day 10 and then tested after 24 hours for DNA fragmentation (A), caspase-3 activity (B), or [3H]-thymidine incorporation(C). At the start of Day 11 ATDC5 cells then received vehicle or 10^{-7} M 24R,25$(OH)_2D_3$ and were tested for the same assays after 24 hours (D-F). 24R,25$(OH)_2D_3$ reduced apoptosis and increased proliferation on day 11 when treated with Pi on day 10. * P<0.05 vs. control. # P<0.05 vs. vehicle.

DISCUSSION

The results presented here demonstrate that exogenous Pi is a potent inducer of endochondral development, not only for hypertrophic cells as has been reported previously[2], but also for prechondrocytes. In response to relatively high levels of Pi, ATDC5 cells exhibited increased levels of mRNA for type II collagen and aggrecan. These cells also exhibited markers of endochondral development, including reduced expression of the early differentiation marker Sox 9, reduced cell numbers and increased alkaline phosphatase specific activity as well as elevated expression of the later-stage marker of hypertrophic chondrocytes, collagen type X. Others have reported a dose dependent increase in collagen X in ATDC5 cells treated with Pi in the range of 3-30um

[23], supporting our finding. Moreover, our results confirm that the effects of Pi on endochondral development were specific and were dependent on active transport of the ion because treatment of the cells with the phosphate transporter inhibitor phosphonoformic acid blocked the Pi-induced responses.

Interestingly, the Pi-induced chondrocytes were sensitive to both $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ with respect to reduced cell number and increased alkaline phosphatase at Pi concentrations below 20 mM, but in cultures treated with 20 mM Pi, there was a very specific enhancement of response to the $24R,25(OH)_2D_3$ metabolite of vitamin D3. This was unanticipated since studies using rat [32, 34] and mouse [6] costochondral growth plate chondrocytes have shown that resting zone cells are the primary target for $24R,25(OH)_2D_3$, whereas prehypertrophic and hypertrophic chondrocytes are primary targets for $1\alpha,25(OH)_2D_3$. Moreover, 20 mM Pi induced sensitivity of the ATDC5 cells to 10^{-7} M $24R,25(OH)_2D_3$, which is the concentration at which costochondral resting zone cells exhibit maximal responses to the seco-steroid [6] and similar to the level of endogenous $24R,25(OH)_2D_3$ produced by these cells when stimulated in culture [35].

These observations suggest that $24R,25(OH)_2D_3$ may serve to protect the early endochondral chondrocytes from premature terminal differentiation due to high levels of exogenous Pi. Our results support this hypothesis. $24R,25(OH)_2D_3$ blocked the inhibitory effect of Pi on $[^{35}S]$-sulfate incorporation. Moreover, it blocked the stimulatory effects of Pi on apoptosis, based on two different indicators of cell death. $24R,25(OH)_2D_3$ increased DNA synthesis, reduced DNA fragmentation, and reduced caspase-3 activity in Pi-treated ATDC5 cells.

The fact that the effects of Pi treatment on sensitivity to $24R,25(OH)_2D_3$ were so narrowly focused in terms of dose may indicate that one or more critical conditions must be met with precision to invoke the need for response to the steroid during endochondral ossification in the embryo. The spatial and temporal sequence of events in embryonic bone formation differs from post-fetal bone growth. Thus, Pi and $24R,25(OH)_2D_3$ may act together to reduce proliferation and begin the process of hypertrophy, but as alkaline phosphatase increases generating higher levels of exogenous Pi, $24R,25(OH)_2D_3$ acts as a brake on the apoptotic process induced by the active uptake of Pi.

In summary, our study demonstrates the value of the ATDC5 prechondrocyte model for studying factors that modulate endochondral ossification, as noted by others [19, 24, 36]. Our results confirm the importance of exogenous Pi in regulating the differentiation and maturation of chondrocytes in endochondral development. Most importantly, they show that Pi treatment induces sensitivity to vitamin D metabolites $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ in a dose-dependent manner and at the higher concentrations of Pi, the cells become specifically responsive to $24R,25(OH)_2D_3$. This metabolite acts with Pi to reduce cell number and increase endochondral differentiation, but at the same time it blocks the activation of apoptosis, suggesting a role for modulating the rate of terminal differentiation in embryonic bone formation.

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CHAPTER 4. FLUID SHEAR STRESS INHIBITS DIFFERENTIATION OF GROWTH PLATE RESTING ZONE CHONDROCYTES AND CHONDROGENIC ATDC5 CELLS

INTRODUCTION

Many cell types have been shown to be responsive to mechanical stimuli, and for some types of cells including chondrocytes, mechanical environment may be an important parameter for homeostatic function [1]. Numerous studies have considered the effect of shear stress on mature normal [2] or arthritic [3, 4] chondrocytes located in tissues around the joints, such as articular cartilage [5, 6] or meniscal fibro-cartilage [7, 8]. Cartilage is a highly hydrated tissue that experiences the movement of fluid when compression is applied to the tissue, resulting in potential fluid shear stress at or near the cellular membrane [9]. Studies of tissue engineered cartilage show that changes in fluid shear stress in a bioreactor can modulate the growth and differentiation of the cells and that effects change as the cells produce extracellular matrix [10-12]. Articular chondrocytes in arthritic tissues experience increased shear stress due to loss of the protective integrity of the surrounding matrix. The cells respond to increased shear by escalating activity that can advance arthritis such as decreased matrix production [2] and cell proliferation [13] and increased apoptosis [3, 14].

Some evidence suggests that mechanical loading may begin to play a role in joint formation as early as during fetal development, such as in the temporal-mandibular joint [15, 16] and hip [17]. Less is known about the effects of fluid shear on chondrocytes in the growth plate. The mammalian growth plate is a remaining center of endochondral

ossification that persists after embryonic development of the long bones. How growth plate chondrocytes experience shear throughout the course of endochondral ossification is not well understood. Moreover, it may change markedly with the state of chondrocyte maturation within the growth plate.

The purpose of the present study was to determine how changes in fluid shear force modulate the behavior of chondrocytes in the growth plate. The chondrocyte phenotype is staged through endochondral ossification in both a temporal and spatial manner. Not only do the cells show changes in shape and size, but they also vary in their extracellular matrix composition and in their responses to local and systemic factors [18-20]. To limit the potential variables in the present study, we focused on chondrocytes in the resting zone of the growth plate, which is characterized by sulfated proteoglycan-rich, type II collagen extracellular matrix. We used two models: primary rat resting zone chondrocytes and also the chondrogenic embryonic murine ATDC5 cell line. Numerous studies have shown that the rat resting zone cells retain phenotypic properties of resting zone cells in vivo, including synthesis of type II collagen but not type X, zone specific responses to vitamin D metabolites $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, and low matrix metalloproteinase activity [18, 21]. The ATDC5 cells were cultured using a method we have previously developed to induce similar phenotypic characteristics to resting zone chondrocytes [22].

METHODS AND MATERIALS

Cell Culture

Primary resting zone (RC) chondrocytes were obtained following a procedure previously shown to retain phenotypic properties of the cells during in vitro expansion up to fourth passage [23]. Briefly, chondrocytes from the resting zone (reserve zone) of the costochondral junction of 125-g male Sprague Dawley rats were cultured in DMEM containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 50 μg/ml ascorbic acid in an atmosphere of 5% CO_2 and 100% humidity at 37 ˚C. At fourth passage, RC cells were plated at a seeding density of 10,000 cells/cm^2 and received shear stress treatment at confluence (typically after 5-7 days of culture).

ATDC5 cells were also plated at 10,000 cells/cm^2 and cultured in maintenance medium consisting of a 1:1 ratio of DMEM/F12 media (Cellgro, Manassas, VA) containing 5% FBS, and 10 μg/ml human transferrin (Sigma Chemical Company, St. Louis, MO), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 3 x 10^{-8} M sodium selenite (Sigma). After reaching confluence (typically after 5 days) cells were cultured with differentiation media, which is identical to maintenance media with the addition of 10 μg/ml bovine insulin (Sigma) and 50 μg/ml ascorbic acid [24, 25]. At 10 days post-confluence, cells were cultured for 24 hours in differentiation media supplemented with 20 mM phosphate (Pi) beyond media basal level and 10% FBS to induce a resting-zone-like phenotype in the ATDC5 cells. This method was previously shown to induce a comparable phenotype to resting chondrocytes based on chondrocytic differentiation and sensitivity to $24,25(OH)_2D_3$ [22]. ATDC5 cells were returned to normal differentiation media and received shear stress treatment on Day 11.

Application of Shear Stress

Shear stress was applied to the cells via a unidirectional cone-plate viscometer device modeled after the design used by Dr. Hanjoong Jo (Emory University, Atlanta, GA) [26-28] and is similar to other previously reported designs [29-31]. The device was operated at a low Reynolds number ($Re < 1$), which allows for shear stress τ to be estimated by the equation $\tau = \mu\omega/\alpha$, where μ is viscosity of the media, ω is the angular velocity of the cone, and α is the angle of the cone to the culture surface. Cultures were exposed to estimated shear forces ranging from 2 - 6.5 dynes/cm^2. A static control set of cultures was included in each experiment. All cells were cultured in 100 mm x 20 mm Petri dishes (BD Falcon, Franklin Lakes, NJ) to accommodate the size of the cone-plate device, and were cultured in a volume of 10 mls of media, including during shear exposure. Eight cone-plate devices were available thus allowing 8 simultaneously sheared samples in a single experiment. Shear exposure was for 24 hours for all data shown. Following shear stress application, cells were either harvested for assay immediately or were given fresh media and allowed up to 24 hours before harvesting.

Cell Number and Viability

At harvest, cells were washed twice with DMEM and trypsinized (Invitrogen) for 10 minutes. Cells were resuspended in saline, and counted on a Beckman Coulter Z1 Particle Counter (Beckman Coulter, Fullerton, CA). To determine if a decrease in cell number was due to cell detachment caused by shear, cell number was also measured immediately following shear in both static control and sheared samples. Both the adherent cell monolayer and cells suspended in the media at the end of shearing were collected and analyzed separately for cell number. To determine if shearing had caused

cell death in the treated cultures of primary RC cells a two-color fluorescent stain was performed using the Molecular Probes LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen). The ATDC5 cultures as used when grown past confluence were too dense to be very appropriate for the use of this kit.

Alkaline Phosphatase Activity

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline]-specific activity was used as an indication of chondrocyte differentiation. Cells were harvested at 24 hours after shear and suspended in 0.05% Triton X-100. After three rapid freeze-thaw cycles to lyse the cells, enzyme activity was measured in cell lysates and normalized by protein content determined by using the Macro BCA Protein Assay Kit (Pierce, Rockford, IL).

DNA Synthesis

The effect of shear stress on DNA synthesis in resting zone chondrocytes was determined by measuring [^{3}H]-thymidine (Perkin Elmer, Waltham, MA) incorporation into trichloroacetic acid (TCA, Sigma) insoluble cell precipitates as previously described [32]. When resting zone chondrocyte cultures were approximately 70% confluent, they received starvation media with only 1% FBS for 48 hours to synchronize the cells, after which they received 24 hours of shear stress. Samples were then assayed for [^{3}H]-thymidine incorporation either immediately following shear or at 24 hours later. Cell cultures received a 2-hour pulse of [^{3}H]-thymidine either immediately after shear or at 22 hours after shear, respectively. Radioactivity in TCA-precipitable material was measured by liquid scintillation spectroscopy. [^{3}H]-Thymidine incorporation was not measured in

ATDC5 cells, because the culture method for them was not compatible with the pre-confluent starvation method used in this assay.

[^{35}S]-Sulfate Incorporation

To determine the role of shear stress in modulating extracellular matrix composition, [^{35}S]-sulfate incorporation was measured as an indicator of sulfated glycosaminoglycan production as described previously [33, 34]. Four hours prior to harvest, [^{35}S]-sulfate (Perkin Elmer, Waltham, MA) was added to the cultures. For samples cultured under static conditions for 24 hours after shear, [^{35}S]-sulfate was pulsed into the media at hour 20 after shear. To determine [^{35}S]-sulfate incorporation directly following shear, the [^{35}S]-sulfate pulse was applied to the cells immediately following the end of shear for 4 hours. Cell layers were collected and dialyzed to remove any unbound [^{35}S]-sulfate. Radiolabeled [^{35}S]-sulfate incorporated into the cell layer was expressed as disintegrations per minute normalized to protein levels for each sample.

mRNA Analysis with Quantitative Real-Time PCR

Extraction of mRNA was performed using a TRIzol reagent kit (Invitrogen) and was quantified using the Nanodrop-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcriptase was performed using the RT Omniscript Kit (Qiagen, Valencia, CA) and random primers (Promega, Madison, WI) to generate a cDNA library of each sample.

Real-time PCR was performed to quantify the effects of shear on expression of aggrecan, collagen type II, and cartilage oligomeric matrix protein (COMP). mRNA was isolated from both RC cells and ATDC5 cells at the end of 24 hours of shear (6.5 dynes/cm^2) and also 12 hours following the end of shear after returning to static culture.

The primer sequences used for the murine ATDC5 cells were as follows: aggrecan, 5'-GGT CTG TGC CAT CTG TGA GG-3' (sense) and 5'-CCC AGT CCA GCC GAG AAA TG-3' (anti-sense); collagen type II, 5'-TGG AGC AGC AAG AGC AAG G-3' (sense) and 5'-GTG GAC AGT AGA CGG AGG AAA G-3' (anti-sense); COMP, 5'-AAT ACG GTC ATG GAA TGT GAT G-3' (sense) and 5'-TCT CGG AGC AGA CTA CGC-3' (anti-sense); and GAPDH, 5'-TTC AAC GGC ACA GTC AAG G-3' (sense) and 5'-TCT CGC TCC TGG AAG ATG G-3' (anti-sense). The primer sequences used for rat RC cells were as follows: GAPDH, 5'-AAG TTC AAC GGC ACA GTC AAG G-3' (sense) and 5'-CAT ACT CAG CAC CAG CAT CAC C-3' (anti-sense); aggrecan, 5'-AGG TGT CAC TTC CCA ACT ATC C-3' (sense) and 5'-GCT TCG CTG TCC TCA ATG C-3' (anti-sense); and sequences for the collagen type II and COMP primers (Qiagen, Valencia, CA) remain undisclosed by the company.

Statistical Analysis

The cone-plate device accommodates 8 simultaneously sheared samples in a single experiment and thus experiments generally had sample size of n = 8 per shear group and also n = 8 for static control. Single independent experiments were analyzed with Student's T-test between shear and static control. Treatment-to-control ratios (fold change) were calculated for individual experiments and multiple fold changes from replicate experiments using equivalent conditions were tested with a one sample t-test to compare to a hypothetical mean of 1. All comparison of fold change across multiple experiments at different shear levels were analyzed with ANOVA followed by Bonferroni's modification of Student's T-test. Differences were considered to be statistically significant if the P value was less than 0.05.

RESULTS

Resting zone cells exhibited a decrease in cell number 24 hours following termination of shear compared to cells cultured under constant static conditions, and this decrease was comparable at all levels of shear stress tested (Figure 4.1A). ATDC5 cell number was also reduced in cultures exposed to shear stress, but this was significant only in cultures exposed to 6.5 dynes/cm^2 (Figure 4.1B),

Alkaline phosphatase specific activity in the cell lysates was decreased in response to increasing shear. Statistically significant decreases were observed in RC cells at 6.5 dynes/cm^2 compared to static control cultures as well as to cultures only receiving 2 dynes/cm^2 (Figure 4.1C). The effects of shear on alkaline phosphatase activity in ATDC5 cells were dose-dependent, with decreased activity in cultures exposed to shear stress greater than 3.5 dynes/cm^2 compared to static control cultures and activity in cultures treated with 5 or more dynes/cm^2 compared to cultures exposed to 2 dynes/cm^2 (Figure 4.1D).

Reductions in cell number following shear were not due to cell detachment. There were no differences in the number of cells in the sheared monolayer compared to the static monolayer for either cell type (Figure 4.2A). Furthermore, the number of cells suspended in the media was less than 1% of the number of cells in the monolayer for both cell types. Primary RC cells exposed to 24 hours of shear stress were also stained for a live/dead assessment at the very end of shear treatment and showed overwhelming positive staining as viable (Figure 4.2B).

The reduction in cell number was due in part to reduced DNA synthesis. At cessation of shear stress, there was a reduction in [^{3}H]-thymidine incorporation in all RC

cell cultures exposed to shear (Figure 4.3). These effects were not dose-dependent. However, following the 24 hour recovery period, DNA synthesis was restored to control levels in nearly all shear-treated groups, except for 3.5 dynes/cm^2.

Exposure to shear stress caused a dose-dependent decrease in [^{35}S]-sulfate incorporation in RC cell cultures (Figure 4.4). In cultures exposed to 5 and 6.5 dynes/cm^2, there was a 50% decrease in incorporation. However, by 24 hours after shear stress, [^{35}S]-sulfate incorporation was restored to static control levels in cultures exposed to 2 dynes/cm^2 and the decrease noted in cultures exposed to 5 dynes/cm^2 was reduced. Only at the highest level of shear stress was incorporation of radiolabel still decreased by 50%. ATDC5 cells were affected in a similar manner, but the effects of shear stress at termination of shear were greater (Figure 4.5).

Shear stress differentially affected mRNA levels for cartilage extracellular matrix proteins (Figure 4.6). Aggrecan mRNAs were reduced during shear in both RC and ATDC5 cells. In contrast, levels of collagen type II mRNA and COMP mRNA were not altered at the cessation of shear in either cell type. However, collagen type II mRNAs were reduced at 12 hours of recovery in both RC and ATDC5 cells whereas only the ATDC5 cells exhibited a decrease in mRNAs for COMP.

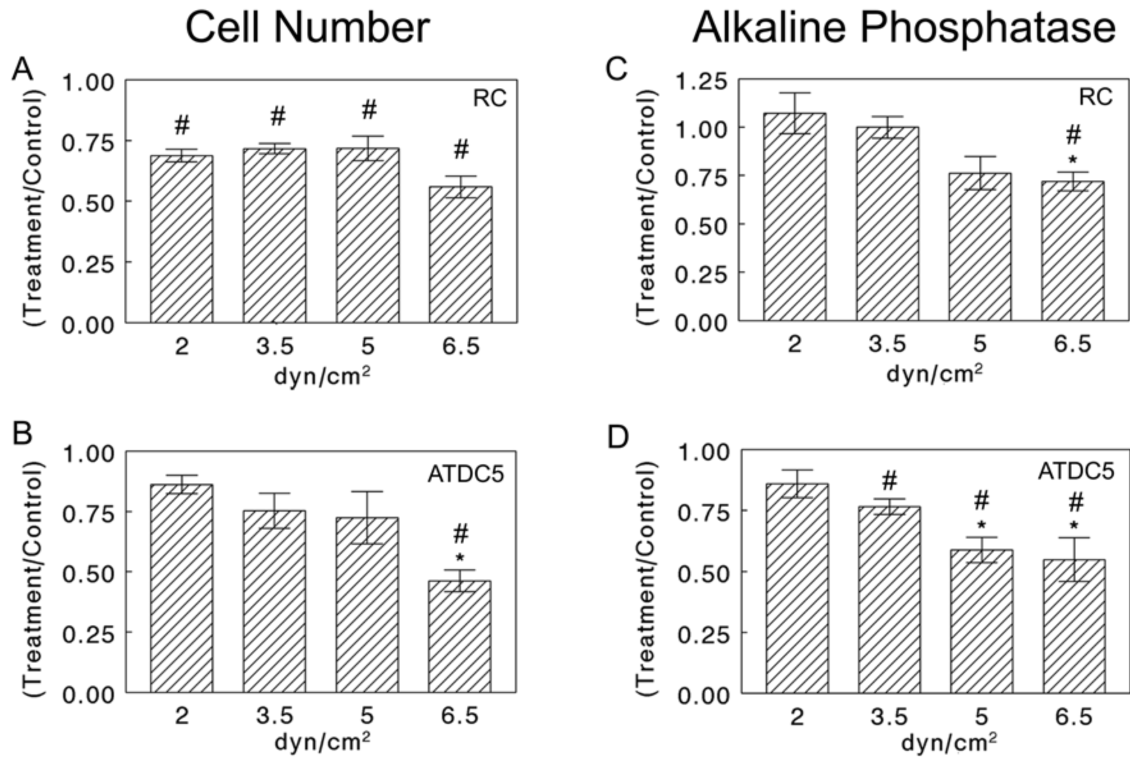

Figure 4.1. Shear stress dose-dependently reduces cell number and alkaline phosphatase activity in resting zone chondrocytes and ATDC5 cells. All bar graphs represent average treatment/control ratios of three independent experiments of cells receiving shear stress treatment (2 - 6.5 dynes/cm^2) for 24 hours followed by another 24 hours of static culture before harvest. (A) Resting zone chondrocyte (RC) cell number. (B) ATDC5 cell number. (C) RC alkaline phosphatase specific activity. (D) ATDC5 alkaline phosphatase specific activity. # $P < 0.05$ vs. hypothetical mean of 1 (or no change from static control within each level of shear treatment), * $P < 0.05$ vs. 2 dynes/cm^2.

Shear + 0 hr

A

RC	Cell Number (10^6)	
	AVG	SE
Static Monolayer	2.48	0.12
Static Media	0.04	0.01
Sheared Monolayer	2.38	0.20
Sheared Media	0.02	0.01

ATDC5	Cell Number (10^6)	
	AVG	SE
Static Monolayer	22.32	0.58
Static Media	0.08	0.01
Sheared Monolayer	21.75	1.46
Sheared Media	0.18	0.04

B

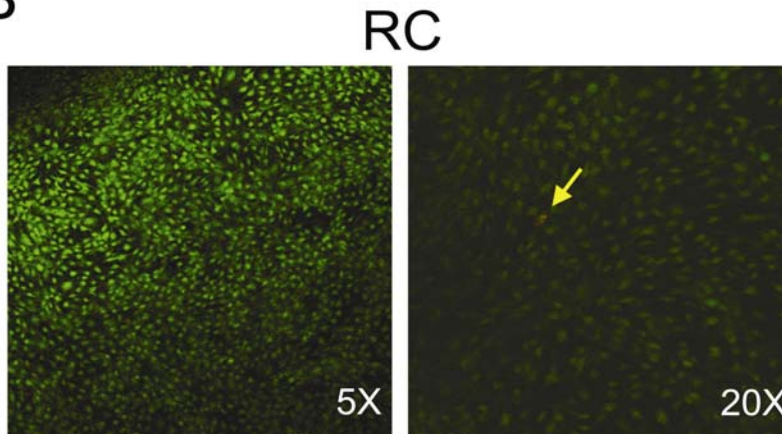

Figure 4.2. Cells remain adherent and viable during highest shear treatment. (A) RC and ATDC5 cells were sheared for 24 hours at 6.5 dynes/cm^2 and then harvested immediately for cell number, with results shown in table. Adherent cells (monolayer) were measured separately from detached cells (media) suspended in the culture media for both sheared samples and static controls. Mean (AVG) and standard error (SE) are shown. No statistically significant differences were observed between groups, and number of cells detached in media was negligible compared to adherent cells. (B) Primary RC cells were sheared for 24 hours at 6.5 dynes/cm^2 and then harvested immediately for live (green) or dead (red) staining. The adherent cells were overwhelmingly shown as viable with very few dead cells observed (example of dead cell shown with arrow at higher magnification).

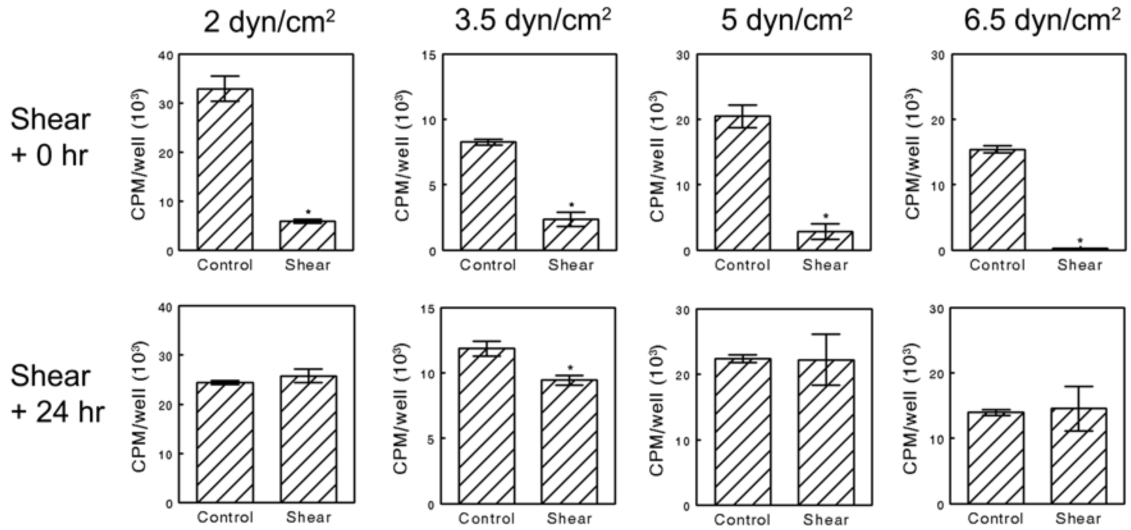

Figure 4.3. [^{3}H]-Thymidine incorporation of resting zone chondrocytes is inhibited during fluid shear stress and recovers after shear. [^{3}H]-Thymidine incorporation by RC chondrocyte cell layers treated for 24 hours with 2 - 6.5 dynes/cm^2 shear stress or under static control conditions. Incorporated label was measured immediately following shear stress (top row) or 24 hours after shear stress (bottom row). Data presented are from a single representative experiment, all with comparable results. Values shown are means $\pm$ SEM for N = 8 static cultures and N = 8 treated cultures for each shear condition. * $P < 0.05$ vs. Static Control.

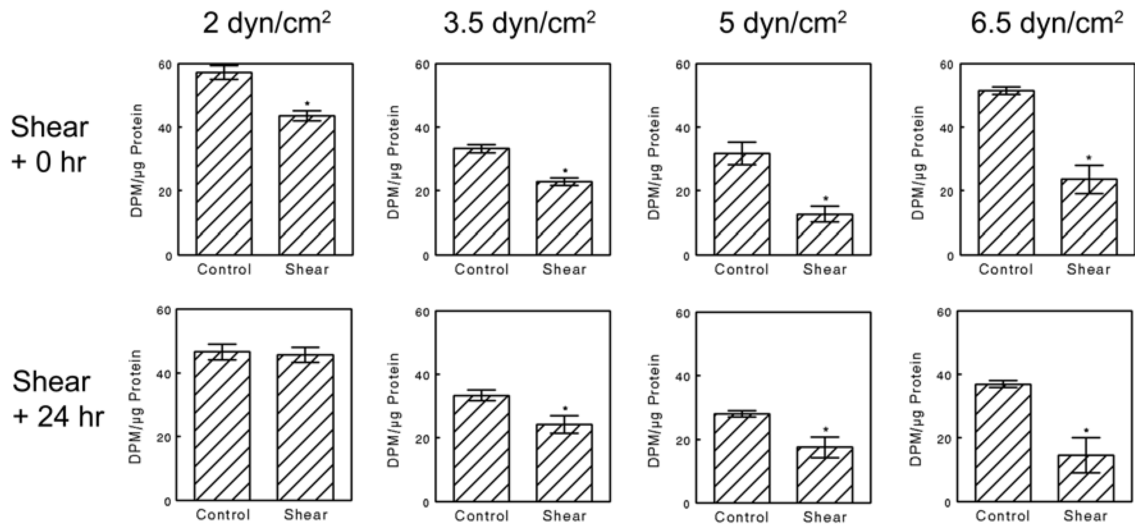

Figure 4.4. Fluid shear stress reduces [^{35}S]-sulfate incorporation of resting zone chondrocytes (RC) and dose-dependently affects post-shear recovery rate. [^{35}S]-Sulfate incorporation into RC chondrocyte cell layers treated for 24 hours with 2 - 6.5 dynes/cm^2 shear stress or under static control conditions. Incorporated label was measured immediately following shear stress (top row) or 24 hours after shear stress (bottom row). Data presented are from a single representative experiment, all with comparable results. Values shown are means ± SEM for N = 8 static cultures and N = 8 treated cultures for each shear condition. * $P < 0.05$ vs. Static Control.

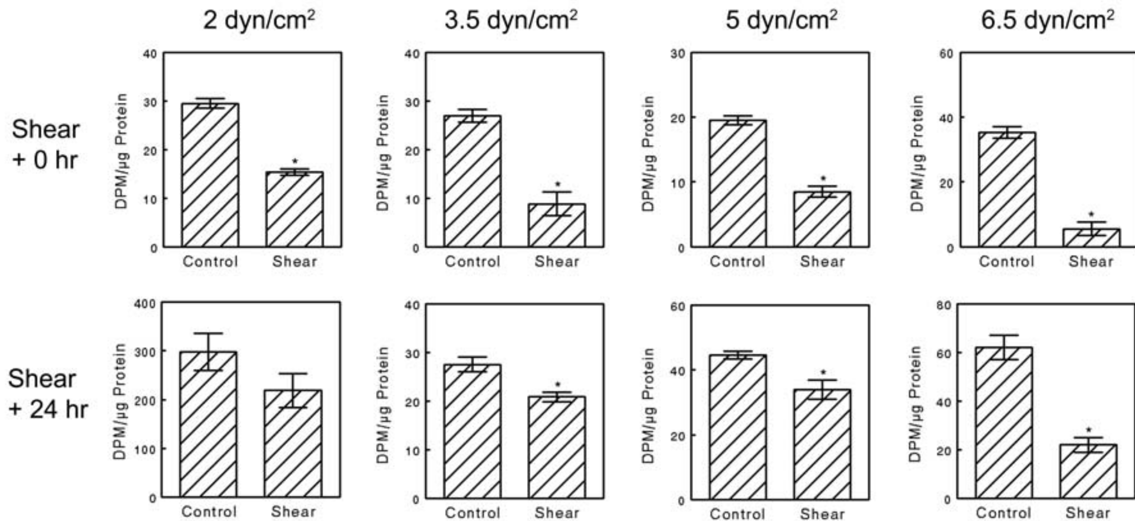

Figure 4.5. Fluid shear stress reduces [^{35}S]-sulfate incorporation of ATDC5 cells and dose-dependently affects post-shear recovery rate. [^{35}S]-Sulfate incorporation into RC chondrocyte cell layers treated for 24 hours with 2 - 6.5 dynes/cm^2 shear stress or under static control conditions. Incorporated label was measured immediately following shear stress (top row) or 24 hours after shear stress (bottom row). Data presented are from a single representative experiment, all with comparable results. Values shown are means $\pm$ SEM for N = 8 static cultures and N = 8 treated cultures for each shear condition. * $P < 0.05$ vs. Static Control.

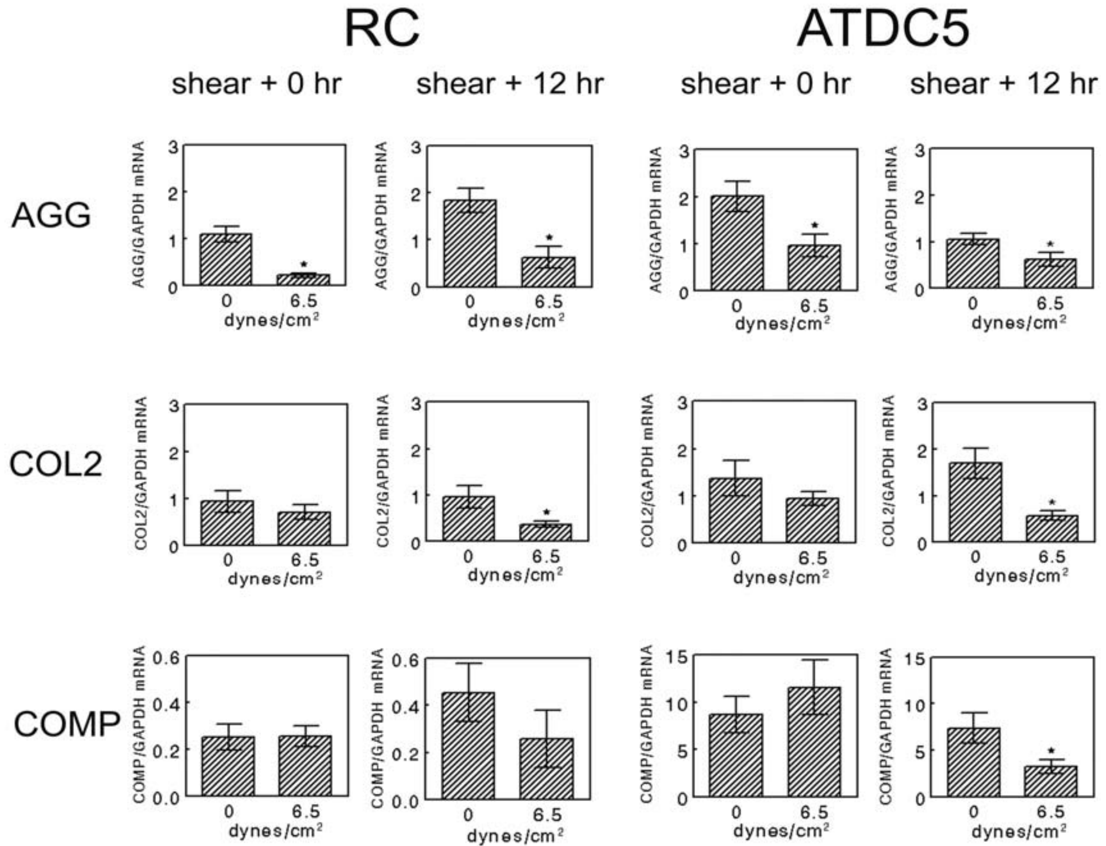

Figure 4.6. Fluid shear stress regulates mRNA of chondrocytic markers. RC cells (columns 1 and 2) or ATDC5 cells (columns 3 and 4) were sheared for 24 hours at 0 (static control) or 6.5 dynes/cm^2 and then either harvested immediately (shear + 0 hr, columns 1 and 3) for RNA extraction or allowed 12 additional hours of static culture before harvest (shear + 12 hr, columns 2 and 4). Real-time PCR was used to analyze quantitative expression of mRNA for aggrecan (AGG, top row), collagen type II (COL2, middle row), and cartilage oligomeric matrix protein (COMP, bottom row). * $P < 0.05$ vs. static control.

DISCUSSION

Our results demonstrate that growth plate chondrocytes are sensitive to fluid shear stress. They respond to stress with reduced proliferation and differentiation and changes in extracellular matrix synthesis. Moreover, the concurrence of observations using primary rat resting zone cells and murine ATDC5 cells supports the conclusion that the murine model accurately reflects the behavior of RC cells in culture and supports the hypothesis that our observations indicate a general property of growth plate chondrocytes in vivo.

The decrease in cell number noted in cultures exposed to 6.5 dynes/cm^2 shear stress was not due to cell detachment by mechanical force. All cultures were observed for significant cell detachment following shear. On the occasion that a culture showed obvious loss of cell layer due to an abnormal disturbance during shear, then that culture was discarded. Furthermore, the dramatic decrease in [^{3}H]-thymidine incorporation seen at the end of 24 hours of shear stress in resting zone chondrocytes indicates that fluid shear almost completely ablates proliferation of the cells. While, most cultures returned to baseline [^{3}H]-thymidine incorporation by 24 hours in static conditions after shear, it is possible that proliferation rates were not restored until almost the end of that recovery time. This may explain why in one experiment, DNA synthesis was still reduced at 24 hours following shear. Cessation of proliferation at some point after the start of shear exposure along with a slow recovery to normal levels may contribute substantially to the drop in cell number observed at 24 hours of post-shear static culture.

It is not known from these experiments whether apoptosis was induced by shear in the cell cultures. Studies have reported that shear can induce apoptosis in

chondrocytes via the production of nitric oxide (NO) [3], so it is possible that apoptosis also contributed to the shear-induced reduction in cell number. The higher levels of primary cells staining positive for viability following shear suggests that cells have not undergone significant apoptosis at the end of shear; however, the ultimate onset of apoptosis may occur during the static period following treatment in sheared groups still contributing to decreased cell number by 24 static hours after shear. This may contribute to the decrease in collagen type II and COMP mRNA at 12 hours after shear. Future work should more fully characterize the timing and severity of the onset of any apoptosis induced by the shear.

Our results show that fluid shear stress reduced differentiation of the cells with respect to alkaline phosphatase specific activity. Alkaline phosphatase is indicative of differentiation state for both chondrocytes [20, 35] and osteoblasts [36]. Alkaline phosphatase activity has not previously been reported as sensitive to fluid shear stress in resting zone chondrocytes, although it was shown to be decreased by cyclical strain in hypertrophic growth plate chondrocytes [37]. Shear stress has been shown to both increase or decrease alkaline phosphatase activity in osteoblasts, depending on magnitude of shear stress, time of exposure, and even the roughness of the cell substrate surface [38-40]. Thus fluid shear stress effects on alkaline phosphatase activity may vary by cell type, as well as by duration and dose of shear and the extracellular environments. ATDC5 cells were more sensitive to shear stress than the RC cells, which may reflect species differences, differences in maturation state, or differences in culture conditions. Overall, the response to shear was similar in the two cell types, suggesting that reduced alkaline phosphatase activity may be a general property of growth plate chondrocytes.

Shear stress affected extracellular matrix production by RC and ATDC5 chondrocytes. We assess the effects on proteoglycan by measuring mRNA levels for aggrecan, which is the core protein of cartilage proteoglycan, and by measuring [^{35}S]-sulfate incorporation as an indicator of glycosaminoglycan sulfation. Both parameters were reduced by shear and this decrease was reduced during recovery, supporting the conclusion that RC cells and ATDC5 cells produce less cartilage matrix during exposure but once shear stress ceases, production of core protein and sulfation of associated glycosaminoglycans resumes. The observed decrease in [^{35}S]-sulfate incorporation may be due to decreased production of proteoglycan molecules, either due to reduced aggrecan expression as suggested by the reduction in aggrecan mRNA, or to decreased sulfation of existing glycosaminoglycans, but increased breakdown of proteoglycans may have played a role as well. It has been reported that mechanical stimuli can alter the expression of aggrecanases [41, 42], which can break down proteoglycan molecules and thus decrease the net amount of sulfate that would be incorporated into the cell layer if degradation rates were outpacing production rates. The effect of shear on aggrecanase production or activity has not yet been studied in our system.

Shear also affected the mRNA levels of other important matrix molecules. Our results suggest that exposure to shear initiated a reduction in collagen type II and COMP expression. COMP provides a three dimensional network throughout the cartilage extracellular matrix that can serve to organize and stabilize the collagen/proteoglycan aggregate composite hydrogel [43-45]. COMP has also been shown to be upregulated in articular chondrocytes by cyclic compression [46] and cyclic tension [47], indicating that this matrix molecule likely has an important role in responding to mechanical

stimulation. However, our results suggest that COMP may be regulated differently by laminar fluid shear stress, resulting in decreased expression.

Overall, our data suggest that growth plate chondrocytes reduce or delay their proliferation and differentiation under the shear stress culture conditions we studied. However, given that cells showed recovery towards control levels of [^{3}H]-thymidine incorporation and [^{35}S]-sulfate incorporation by 24 hours after shear suggest that the cells may eventually recover from inhibitory shear effects. Yokota et al. showed short exposure times (1 hour) at 5 dynes/cm^2 fluid flow were beneficial to a chondrocytic cell line whereas destructive responses were upregulated at 20 dynes/cm^2 [48]. The cells studied in our system were unresponsive to short periods of shear stress (2 hours, data not shown), and only showed inhibitory responses over the range of shear stresses applied at 24 hours of shear exposure, suggesting not all chondrocytes respond equally to the same stimuli or on the same time scale.

It is not well understood what shear stresses growth plate chondrocytes are exposed to in vivo and how they may differ from stresses in other cartilaginous tissue such as in articular cartilage or the nucleus pulposus of the intervertebral disc. The differences in matrix composition associated with endochondral development and the corrugated anatomy of the growth plate also make it difficult to model this important parameter. It has been reported that compressive forces can suppress longitudinal bone growth in rats, suggesting that growth plates are inhibited by this form of mechanical stimulation [49]. Moreover, recently characterized mineral tethers that can span the length of the growth plate [50] may serve to minimize the compression and loading that is experienced in this cartilage as compared to articular cartilage.

The consistent patterns observed in the ATDC5 cells as compared to primary rat resting zone chondrocytes indicate that ATDC5 cells are potentially a good model to study this cell type under mechanical loading in addition to its value in assessing growth plate chondrocyte response to biochemical regulation [51, 52]. The present study shows that growth plate chondrocytes can sense fluid shear; future studies can use these models to examine the underlying mechanisms involved.

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CHAPTER 5. INTEGRIN BETA 1 EXPRESSION PARTIALLY AFFECTS CHONDROCYTE REPONSE TO SHEAR STRESS

INTRODUCTION

Chondrocytes have shown that mechanical stimulation can be a significantly important parameter to their homeostasis and can result in either anabolic [1] or catabolic [2] effects on the extracellular matrix. A better understanding of the effects of mechanical stimulation on chondrocytes can offer potential advancements to many areas of study including cartilage tissue engineering via bioreactor technology [1, 3], endochondral development [4], fracture healing [5, 6], and the potentially divergent mechanotransduction pathways in normal versus osteoarthritic cartilage [7, 8].

In particular, fluid shear stress is one type of mechanical stimulation that has been shown to affect chondrocytes [1, 2, 9]. This may involve a number of potential mechanisms including changes in membrane potential, solute transport, or cellular deformation [10]. Integrin related stretch ion channels may also be involved [11], and integrins have been shown to potentially mediate many effects of mechanotransduction [12, 13]. Our previous work has shown that fluid shear stress is inhibitory on growth plate chondrocyte differentiation (see Chapter 4). While integrin Beta 1 blocking with antibodies or RGD peptides has been shown to prevent both mechanically stimulated increases [12, 14] and decreases [8] in cartilage matrix markers, it is not known if reduction of actual expression of integrin beta 1 itself can prevent the inhibition of differentiation previously observed by fluid shear in our system. Furthermore, conditional knock-out studies have shown that absence of integrin beta 1 in vivo results

in disorganized growth plate development in mouse embryos [15], suggesting a vital role of integrin beta 1 in growth plate orientation and differentiation. The objective of this study was to determine if shear regulates integrin mRNA expression in growth plate chondrocytes, and if silenced expression of a shear-regulated integrin could alter the corresponding effects of shear in chondrocytes.

METHODS AND MATERIALS

Cell Culture

Primary resting zone (RC) chondrocytes were obtained as previously described [16]. Briefly, chondrocytes from the resting zone (reserve zone) of the costochondral junction of 125-g male Sprague Dawley rats were cultured in DMEM containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 50 μg/ml ascorbic acid in an atmosphere of 5% CO_2 and 100% humidity at 37 ˚C. Confluent cultures of fourth passage cells were used for the experiments described below.

ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 ratio of DMEM/F12 media (Cellgro, Manassas, VA) containing 5% FBS, and 10 μg/ml human transferrin (Sigma Chemical Company, St. Louis, MO), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 3 x 10^{-8} M sodium selenite (Sigma). After reaching confluence cells were cultured with differentiation media, which is identical to maintenance media with the addition of 10 μg/ml bovine insulin (Sigma) and 50 μg/ml ascorbic acid [17, 18]. At 10 days post-confluence, cells were cultured for 24 hours in differentiation media supplemented with 20 mM phosphate (Pi) beyond media basal level

and 10% FBS based on a culture method previously shown by us to induce phenotypic similarity to resting zone chondrocytes in this cell line [19].

Application of Shear Stress

Shear stress was applied to the cells via a unidirectional cone-plate viscometer device as in previous experiments. Cultures were mostly exposed to a shear force of 6.5 dynes/cm^2 or in a few cases to 3.5 dynes/cm^2. A static control set of cultures was included in each experiment. Fourth passage RC cells were sheared at confluence and ATDC5 cells were sheared on day 11 post-confluence. Shear stress was applied for 24 hours for both cell types. Cells were cultured in 100mm x 20mm Petri dishes (BD Falcon, Franklin Lakes, NJ). Following shear stress application, cells were either harvested for assay immediately or were given fresh media and allowed up to 24 hours before harvesting.

Western Blot

Whole cell lysates were isolated by using RIPA lysis buffer (20mM Tris-HCL, 150mM NaCl, 5mM disodium EDTA, ddH$_2$O, Nonidet P-40) with homogenization in Wheaton glass tissue homogenizers and sonication, followed by the addition of Laemmli sample buffer containing 0.5% 2-mercaptoethanol and boiling for 5 minutes. Samples were separated by gel electrophoresis on 4-20% LongLife Mini Gels (NuSep, Lawrenceville, GA) and transferred to a nitrocellulose membrane using the iBlot™ Gel Transfer Device (Invitrogen, Carlsbad, CA). The membranes were blocked with 2% bovine serum albumin for one hour at room temperature. After removing the blocking solution, the membrane was rocked at 4ºC in 1° antibody overnight for Integrin β1 (Santa Cruz, Santa Cruz, CA) or GAPDH, (Millipore, Billerica, MA). The 1° antibody was

discarded and the membrane was washed in buffer three times for a total of 30 minutes. Then the correct HRP-conjugated 2° antibody (Jackson ImmunoResearch, West Grove, PA, or BioRad) was added to the membrane which rocked for one hour at room temperature, then washed again in buffer as before. Chemiluminescent bands were visualized using the Quantity One Versadoc software (BioRad) for Windows.

Silencing ATDC5 Integrin Beta 1 mRNA

To silence integrin β1 mRNA, ATDC5 cells were plated at 30,000 cells/cm^2 to achieve 70 percent confluence at 24 hours. The cells were then incubated using Mission® lentiviral particles (Sigma Aldrich, St Louis, MO) at a multiplicity of infection (MOI) of 7.5. After 18 hours, the media was changed to maintenance media for ATDC5 cells for 24 hours. Thereafter cells were then selected with 2.25μg/mL puromycin (Sigma Aldrich) in the maintenance media as successful incorporation of the plasmid should induce puromycin resistance. Silencing was verified using real-time PCR and Western Blot.

Cell Number

At harvest, cells were washed twice with DMEM and trypsinized (Invitrogen) for 10 minutes. Cells were resuspended in saline, and counted on a Beckman Coulter Z1 particle counter.

Alkaline Phosphatase Activity

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline]-specific activity was used as an indication of chondrocyte differentiation. Cells were harvested at 24 hours after shear by suspension in 0.05% Triton X-100. After three freeze-thaw cycles to lyse the cells, enzyme activity was measured in cell lysates and

normalized by protein content determined by using the Macro BCA Protein Assay Kit (Pierce, Rockford, IL).

[^{35}S]-Sulfate Incorporation

To determine the role of shear stress in mediating extracellular matrix production, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation as described previously [20, 21]. After shearing, samples were returned to static media conditions for 24 hours. Four hours prior to harvest, [^{35}S]-sulfate (Perkin Elmer, Waltham, MA) was added to the cultures. Cell layers were collected and dialyzed to remove any unbound [^{35}S]-sulfate. Radiolabeled [^{35}S]-sulfate incorporated into the cell layer was expressed as disintegration per minute normalized to protein levels for each sample.

mRNA Analysis with Quantitative Real-Time PCR

mRNA was extracted using the TRIzol Reagent Kit (Invitrogen). TRIzol was first added to the cells, then after standing at room temperature, chloroform was added to each sample. The samples were then centrifuged and upon discarding supernatant, isopropanol was added. After spinning the samples and discarding supernatant again, ethanol was added, samples were spun and the pellet was dissolved in DEPC H_2O. The RNA was quantified using the Nanodrop-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The samples were then used to make a cDNA library via reverse transcriptase using the RT Omniscript Kit (Qiagen, Valencia, CA) and random primers (Promega, Madison, WI). The samples were denatured at 65°C, then cooled to 4°C at which point the master mix (RT Omniscript Kit) was added, then reheated to 37°C.

Modulation of integrin mRNA levels by shear stress and lentiviral mRNA silencing was measured using quantitative real-time PCR. Primers were compatible for both the RC (rat) and ATDC5 (mouse) cells, and their sequences were: integrin β1, 5'-ATT ACT CAG ATC CAA CCA C-3' (sense) and 5'-TCC TCC TCA TTT CAT TCA TC-3' (antisense); integrin β3, 5'-ATA TGC CAC CTG CCT CAA C-3' (sense) and 5'-GCT CAC CGT GTC TCC AAT C-3' (antisense); integrin α2, 5'-ACT GTT CAA GGA GGA GAC-3' (sense) and 5'-GGT CAA AGG CTT GTT TAG G-3' (antisense); and integrin α5, 5'-ATC TGT CTG CCT GAC CTG-3' (sense) and 5'-AAG TTC CCT GGG TGT CTG-3' (antisense).

The chondrocytic mRNA regulation by shear stress was quantitatively defined using real-time PCR performed on collagen type II, aggrecan, and COMP mRNA expression ATDC5 cells at 12 hours after shear. The primer sequences used were as follows: collagen type II, 5'-TGG AGC AGC AAG AGC AAG G-3' (sense) and 5'-GTG GAC AGT AGA CGG AGG AAA G-3' (anti-sense); aggrecan, 5'-GGT CTG TGC CAT CTG TGA GG-3' (sense) and 5'-CCC AGT CCA GCC GAG AAA TG G-3' (anti-sense); COMP, 5'-AAT ACG GTG ATG GAA TGT GAT G- 3' (sense) and 5'-TCT CGG AGC AGA CTA CGC-3' (anti-sense); and GAPDH, 5'-TTC AAC GGC ACA GTC AAG G-3' (sense) and 5'-TCT CGC TCC TGG AAG ATG G-3' (anti-sense).

Statistical Analysis

All individual shear stress experiments were analyzed with Student's T-test to compare the static control to shear group. Quantitative experiments with more than two groups were analyzed with ANOVA followed by Bonferroni's modification of Student's T-test. Differences in means were considered to be statistically significant if the P value

was less than 0.05. The cone-plate devices allow for up to 8 simultaneously sheared samples and thus experiments generally have sample size of n = 8 per shear group and also n = 8 for static control. In some cases treatment-to-control ratios from independent experiments for wild type (WT) and silenced cells were calculated to compare effects of shear dose in populations with altered integrin beta 1 expression.

RESULTS

Many integrin subunits were significantly upregulated at the end of 24 hours of shear stress in ATDC5 cells with comparable trends in primary resting zone chondrocytes. The mRNA expression of integrin subunits alpha 2 and alpha 5 was significantly higher in ATDC5 cells at the end of shear, but became comparable to control levels by 12 static hours following the end of shear (Figure 5.1). Resting zone chondrocytes exhibited a similar trend although not statistically significant. Expression of integrin beta 1 mRNA was regulated by shear stress consistently in both cell types. Levels were significantly higher in both ATDC5 cells and resting zone chondrocytes at the end of 24 hours of shear. In both cell types, however, beta 1 mRNA levels were comparable to control by 12 hours following the end of shear stress. Integrin beta 3 showed a similar trend upwards at the end of shear, although not significantly different at that time or by 12 hours later.

As integrin beta 1 mRNA levels exhibited a consistent increase in both cell types during shear stress, it was hypothesized that silencing mRNA expression of integrin beta 1 would alter the response of ATDC5 cells under shear. To test this hypothesis, a clone of ATDC5 cells was established for permanent reduced expression of beta 1 mRNA.

Levels of beta 1 mRNA were shown to be significantly reduced in 3 of 5 tested clones targeted for silencing with one clone (clone 3) showing the greatest reduction in mRNA expression at 23% the expression level of wild type ATDC5 cells (Figure 5.2A). The effects of the lentiviral delivery particles alone or a nonspecific plasmid sequence were not shown to alter integrin beta1 mRNA levels. When assessed for reduction in protein expression, clone 3 also showed the greatest reduction in integrin beta 1 protein expression at 38% expression level of wild type ATDC5 cells (Figure 5.2B). Clone 3 and wild type ATDC5 cells were used in remaining experiments for comparison to response to shear stress to determine if integrin beta 1 expression modulates response to fluid shear.

Cell number, alkaline phosphatase activity, and [^{35}S]-sulfate incorporation were all reduced at 24 hours after the end of shear stress (Figure 5.3A-C) as observed previously. ATDC5 cells silenced for integrin beta 1 expression were also comparably reduced in cell number and the trend was consistent over multiple experiments (Figure 5.2A). Similarly, the reduction of alkaline phosphatase activity (Figure 5.2B) and [^{35}S]-sulfate incorporation (Figure 5.2C) was not statistically different in integrin beta 1 silenced ATDC5 cells compared to wild type over multiple experiments.

Our previous work showed that mRNA of chondrocytic markers could be down regulated by 6.5 dynes/cm^2 shear, and it was hypothesized that integrin beta 1 expression might mediate these effects. First, to determine if silencing integrin beta 1 had altered baseline expression of the markers of interest mRNA levels were assessed from wild type and silenced ATDC5 cells (clone 3) from samples cultured and harvested in parallel. Baseline levels of aggrecan and cartilage oligomeric matrix protein were not significantly

altered by silencing, but collagen type II mRNA levels were increased in silenced ATDC5 cells (Figure 5.4A).

Shear stress induced a reduction of aggrecan mRNA in both wild type and integrin beta 1 silenced ATDC5 cells at 12 hours after shear, however this observation was not seen when done at a lower level of shear stress 3.5 dynes/cm^2 shear stress (Figure 5.4B). Collagen type II in WT cells was reduced at 12 hours after shear at 6.5 dynes/cm^2 comparable to previous findings. However it was not significantly reduced in silenced integrin beta 1 cells, or in either cell type at lower shear (Figure 5.4C). Similarly, COMP mRNA levels were reduced in wild type cells but were not in silenced cells, nor in either cell type at lower shear treatment (Figure 5.4D). Wild type and silenced cultures were sheared in independent experiments due to limited sample size in the cone-plate device, and baseline fluctuations are observed due to variability between independent experiments. For this reason, absolute value comparisons should only be made between shear and static pairs shown together.

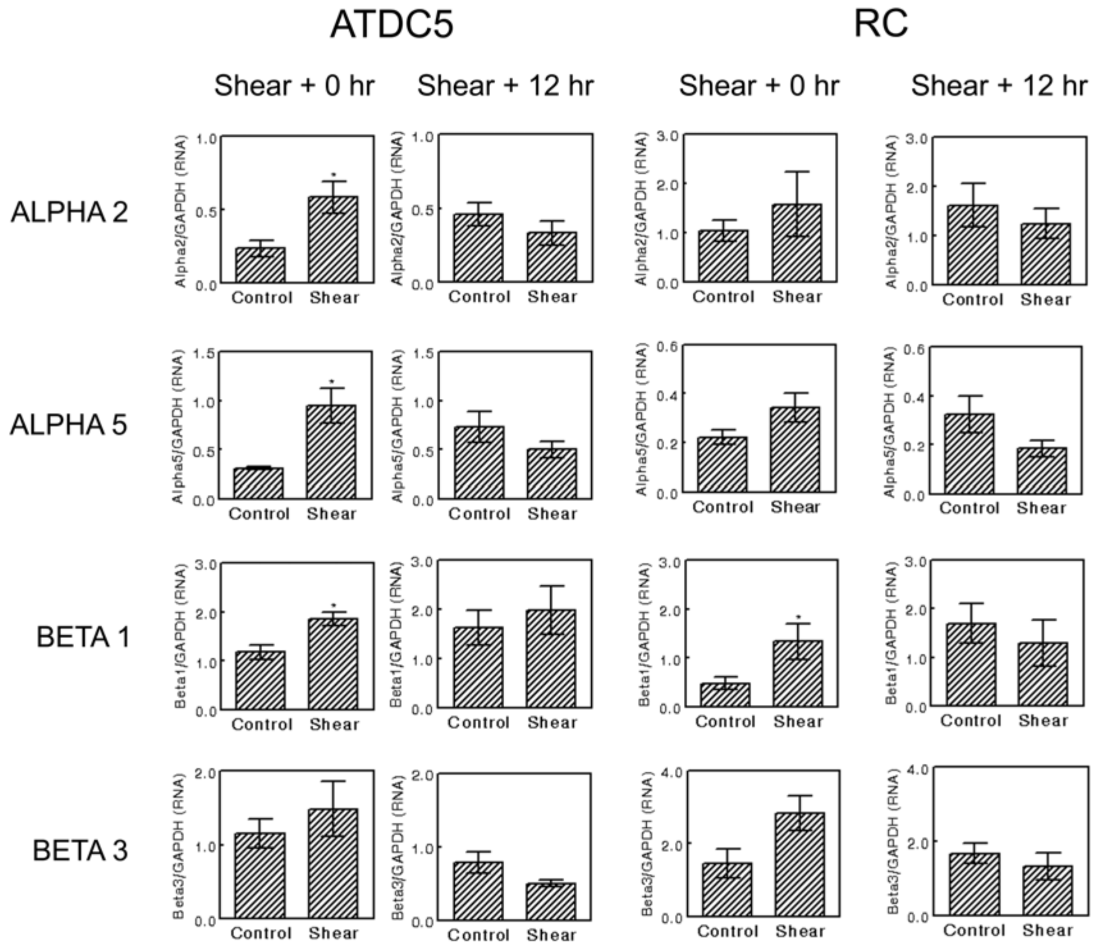

Figure 5.1. Shear stress increases mRNA of some integrin subunits during shear. ATDC5 cells (first and second columns) or resting zone chondrocytes (RC, third and fourth columns) were exposed to 6.5 dynes/cm^2 for 24 hours and then harvested immediately (Shear + 0 hr) or after 12 hours of static culture following shear treatment (Shear + 12 hr). Real-time PCR was used to assess changes in mRNA levels of integrin alpha 2 (first row), alpha 5 (second row), beta 1 (third row), and beta 3 (fourth row) at these time points. * $P < 0.05$ vs. static control.

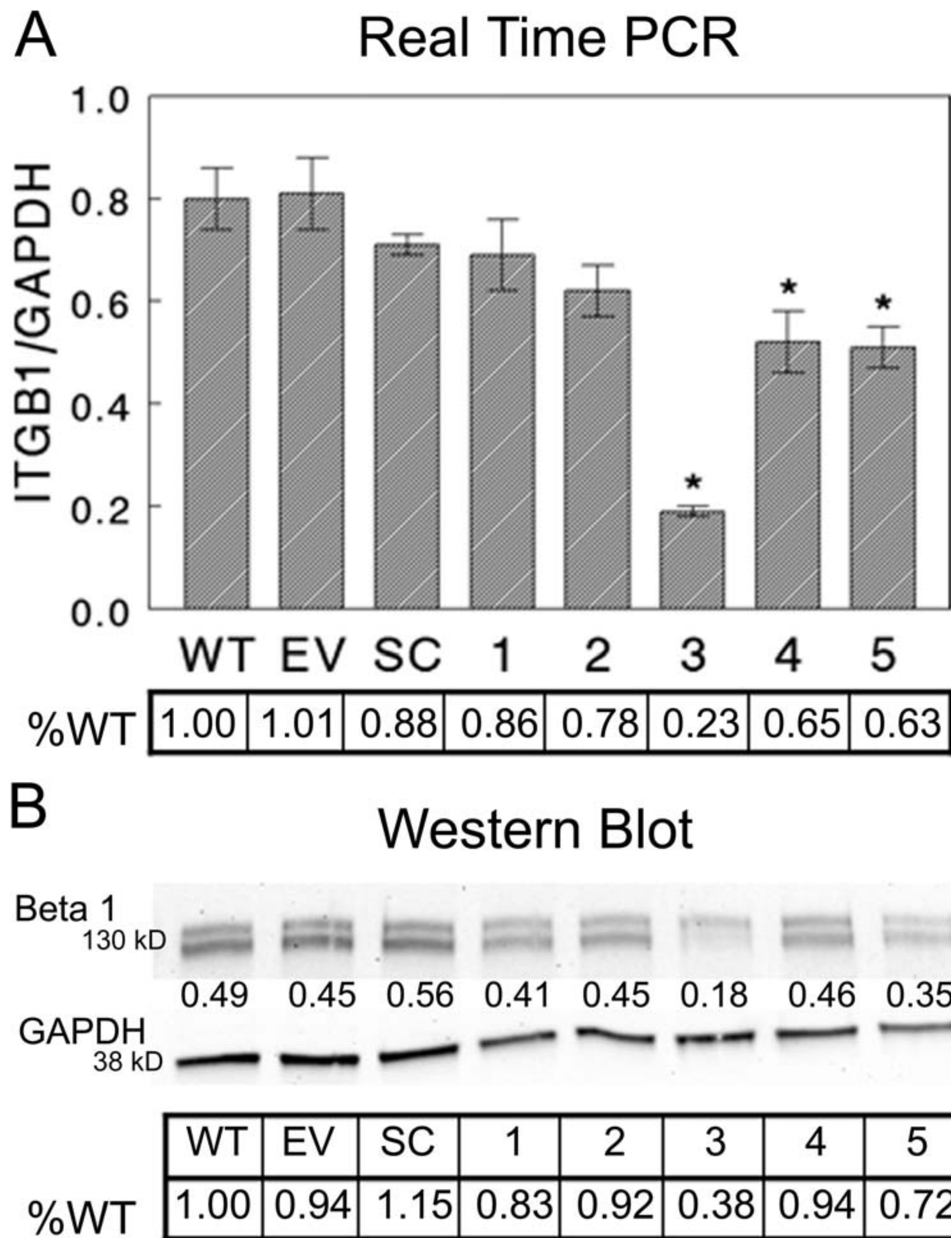

Figure 5.2. Verification of silencing of integrin beta 1 in ATDC5 cells. ATDC5 cells were either not transduced (WT), treated with empty viral particles without a plasmid (EV), a scrambled sequence plasmid not targeting beta 1 (SC), or one of five plasmids introducing a sequence of shRNA targeted for degradation of shRNA (1-5). (A) Real-time PCR was performed on parallel cultures with these treatments and were assessed for expression levels of integrin beta 1 (ITGB1). Percentage of WT value is shown in table below graph. * $P < 0.05$ vs. WT. (B) Western blot was performed on cultures also grown in parallel with same treatments as in (A) and assessed for intensity of Beta 1 expression and GAPDH as a control. Value of the intensity of the beta 1 bands (normalized by corresponding GAPDH intensity) is shown below beta 1 bands and lower table indicates percentage of WT.

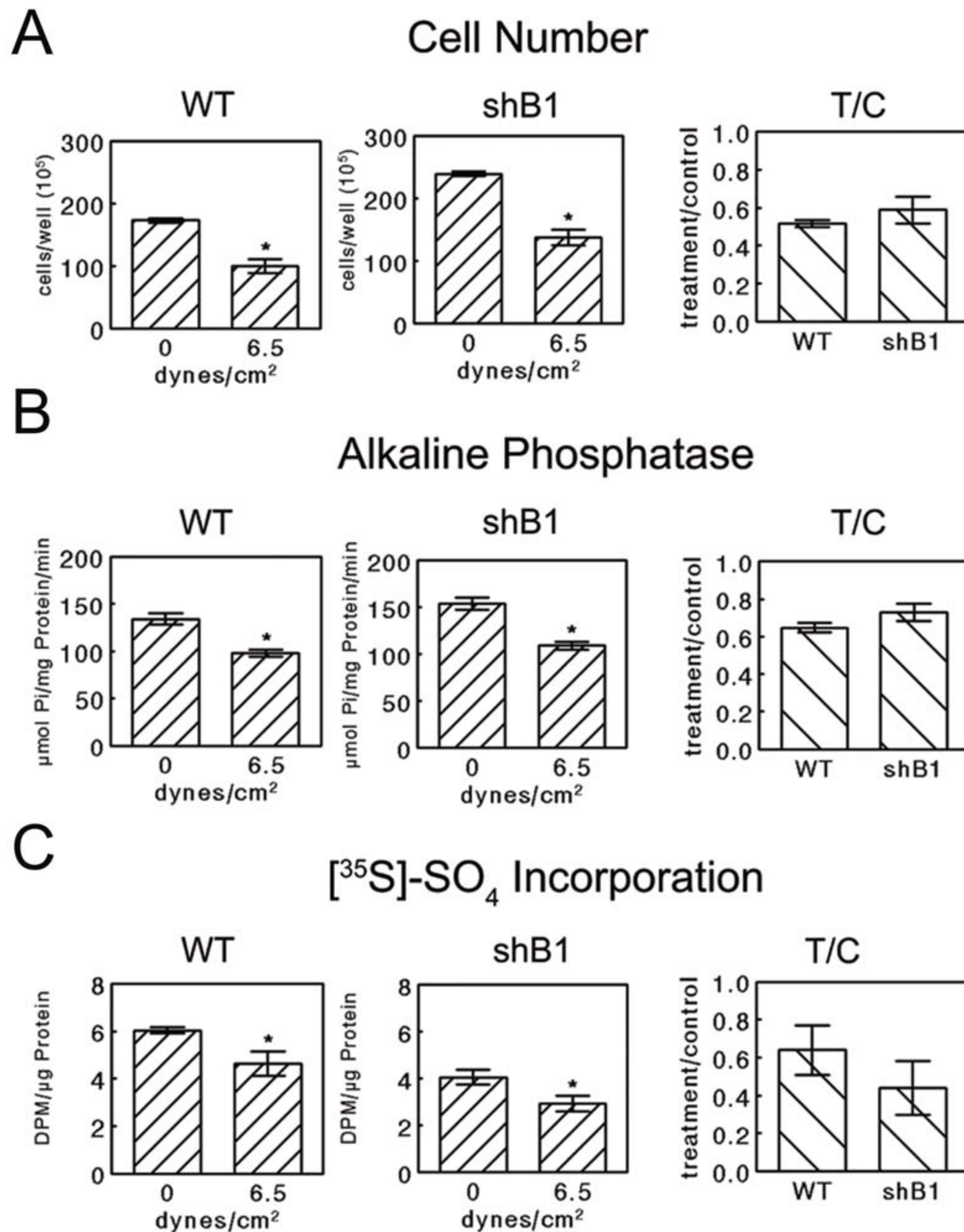

Figure 5.3. Integrin beta 1 silencing does not alter shear induced effects on cell number, alkaline phosphatase specific activity, or [^{35}S]-sulfate incorporation. WT and beta 1 silenced ATDC5 cells (shβ1, clone 3 from 5.2A and 5.2B) were exposed to 24 hours of shear stress at 6.5 dynes/cm^2 and then allowed to return to static culture for 24 additional hours and harvested for cell number (A), alkaline phosphatase activity (B), and [^{35}S]-sulfate incorporation (C). An individual experiment of WT and shβ1 are shown separately with a graph of average treatment/control ratios over three independent experiments shown on far right for each assay. * P < 0.05 vs. Static Control.

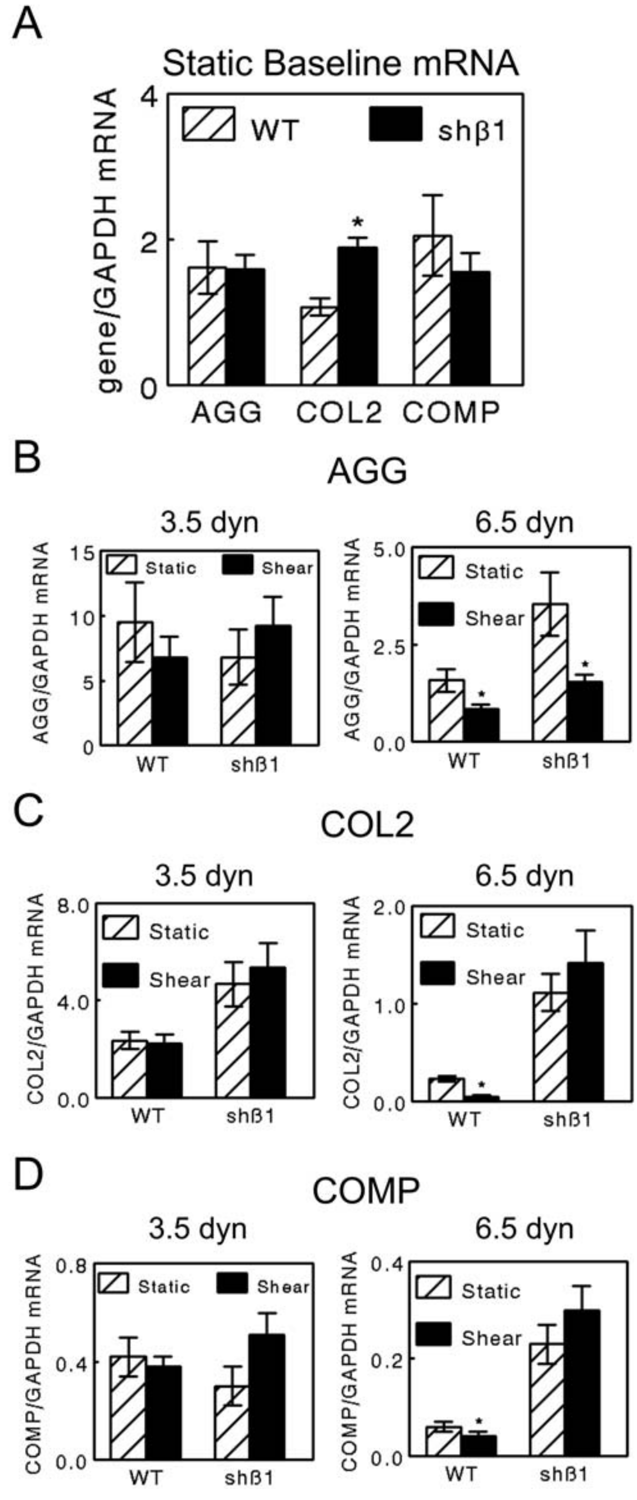

Figure 5.4. Silencing beta 1 blocks dose-dependent, shear-induced mRNA reduction of collagen type II and COMP. (A) Baseline mRNA levels of aggrecan (AGG), collagen type II (COL2), and cartilage oligomeric matrix protein (COMP) were measured

(Figure 5.4 continued) from WT and beta 1 silenced (shβ1) ATDC5 cells cultured in parallel to determine if silencing of beta 1 altered baseline levels of these markers of interest. * $P < 0.05$ vs. WT for each gene. WT or shβ1 cells were sheared for 24 hours at 3.5 dynes/cm^2 or 6.5 dynes/cm^2 and returned to static culture for 12 hours and then harvested for mRNA levels using real-time PCR and measured for AGG (B), COL2 (C), and COMP (D). * $P < 0.05$ vs. static control for same cell type.

DISCUSSION

Our results show that integrin subunits are upregulated by fluid shear stress in our system with integrin beta 1 being consistently higher in both primary resting zone chondrocytes and the ATDC5 culture model. This finding together with the consistent decrease in chondrocytic mRNA, sulfate incorporation, and alkaline phosphatase activity caused by shear suggest that after 24 hours of shear stress the cells reduce metabolic maintenance of chondrocytic markers and perhaps reallocate resources towards a potential increase in integrin expression. This may be important as the cells likely need more focal adhesion formation under shear stress to remain attached [22].

Silencing of integrin beta 1 did not significantly alter the magnitude of shear-induced decrease of cell number, alkaline phosphatase activity, or sulfate incorporation. This suggests that the mechanisms resulting in these decreases are not dependent on integrin beta 1 and likely occur through other pathways or phenomena experienced by the cells during shear. Shear-induced reduction of chondrocytic mRNA in wild type cells was dependent on shear level for aggrecan, collagen type II, and COMP as seen by the absence of significant reduction at a lower level of shear. Aggrecan mRNA levels were still reduced by high shear in beta 1 silenced cells, suggesting beta 1 is not involved in shear-induced reduction of aggrecan. These results corroborate the similar results of the

[^{35}S]-sulfate incorporation data and strongly suggest that fluid shear causes a decrease in proteoglycan production that is not mediated by integrin beta 1.

However, mRNA levels for collagen type II and COMP in sheared silenced cells were very similar to static control. This suggests that lack of beta 1 expression blocks the shear-induced reduction of mRNA of these genes observed in WT cells, or that the rates of occurrence of these responses is altered such that it was not seen at the time point measured. It may be possible that shear stress induces additional motion and spreading of the cellular membrane which may influence the kinetic rates of integrin receptor interaction with ligand proteins in the matrix [23]. Interestingly, both collagen type II [24] and COMP [25] are capable of ligand binding with integrin beta 1 when paired with the appropriate dimeric alpha subunit. Our baseline comparison of silenced versus wild type cells indicate when integrin beta 1 is silenced there is a resulting increase in collagen type II mRNA. This may be caused by a reduction of integrin-associated intracellular and autocrine signals [26] that might serve as a negative feedback system [27] to the cell to inform when sufficient levels of collagen type II are already present in the matrix. If the cell does not receive these signals, it may result in a net increase in basal expression of collagen type II. If shear stress did increase integrin signaling by increasing kinetic receptor interaction, then sheared wild type cells may sense more collagen type II in the matrix at a faster rate and decrease levels of mRNA, whereas the silenced cells may not correctly sense the levels collagen type II present correctly due to diminished presence of receptor and thus the cells would not reduce levels of mRNA during shear. Future work should determine the response to shear at multiple time points to delineate if silencing

beta 1 resulted in a truly blocked effect or an alteration of a rate either via acceleration or deceleration.

Our work confirms that chondrocytes do respond to shear stress dose dependently and that shear inhibits markers of differentiation and increases potential for integrin expression. Many of the observed effects of shear stress were not altered by a disruption in integrin beta 1 expression, suggesting that integrin beta 1 is not involved in those responses. However, shear induced decrease of the integrin ligands collagen type II and COMP may be mediated by interaction with integrin beta 1, and reduction of integrin beta 1 expression prevents the shear-induced decrease.

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CHAPTER 6. CONLUSIONS AND FUTURE PERSPECTIVES

This work has shown new information regarding chemical and mechanical modifiers of chondrocyte differentiation, more specifically regarding chondrocytes that are at a resting zone stage of differentiation or have gained sensitivity to $24,25(OH)_2D_3$. The growth plate provides a spectrum of chondrocytes at uniquely staged phases of differentiation. Understanding the factors that uniquely coordinate cellular activity at different stages of endochondral activity can ultimately open up better opportunities for cartilage regeneration and treatment of pathologies such as osteoarthritis.

Interactive Coordination of Inorganic Phosphate and $24,25(OH)_2D_3$

The results from specific aim 1 of this work has provided evidence that inorganic phosphate and $24,25(OH)_2D_3$ may have a careful interplay in advancing and resisting end-stage differentiation in growth plate chondrocytes. Building upon this work may offer many opportunities for understanding natural regulation of growth plate differentiation as well as potentially identifying new targets or pathways for therapeutic intervention in cartilage related pathologies such as arthritis. Our data suggest that inorganic phosphate is a cue for advanced differentiation towards mineralization competency and hypertrophic apoptosis indicated by the increase in alkaline phosphatase activity, collagen X mRNA expression, and markers of apoptosis. The hormone $24,25(OH)_2D_3$ showed the capacity to offset apoptosis and induce proliferation and restore a Pi-induced decrease in proteoglycan production.

To better characterize the interplay of Pi and 24,25$(OH)_2D_3$, it would be good to test extended time courses of varied interaction with Pi and 24,25$(OH)_2D_3$ exposure. For example, cells could be treated for 48-96 hours with Pi before testing if 24,25$(OH)_2D_3$ can rescue them from a more prolonged induction of apoptosis. Also, cells could be treated concurrently with Pi and 24,25 together to see if that blocks initiation of apoptosis. Also, varying lengths of exposure to 24,25 following induction of apoptosis would help determine how long a treatment of 24,25 would be needed to potentially act as a therapeutic.

A provisional patent to use 24,25$(OH)_2D_3$ as an anti-apoptotic for the treatment of osteoarthritis was filed based on the initial findings of our work and also some related work by Jennifer Hurst-Kennedy also within our lab. To further verify if 24,25$(OH)_2D_3$ could serve in this capacity it would be important to test it in experiments directly relevant to arthritis. First, it should be determined if 24,25$(OH)_2D_3$ promotes other activity that prevents cartilage degradation and/or promotes matrix synthesis. This would involve determining if destructive enzymes are down-regulated such as matrix metalloproteinases, and testing if inhibitors of matrix destruction are upregulated such as tissue inhibitors of metalloproteinases (TIMPs). Further work could be done to quantify how much matrix protein production is induced or recovered in normal and stressed conditions (such as induced apoptosis). The effect of 24,25 on mineralization of cells could also be of interest, although well characterized models of mineralization would need to be established.

To be more pertinent to arthritis therapy development, studies involving articular cartilage (cells or organ culture) should be performed, including human derived sources

as well. Also, in vivo animal studies would need to be performed in an appropriate animal model(s) to test for reduction or recovery from induced osteoarthritis. Endpoints might include histology evaluation following treatment with recovery time, as well as measurements of reduction in pain assessed by willingness to bear more weight on effected limbs. Also, if $24,25(OH)_2D_3$ were to be injected in combination with other arthritis treatments such as hyaluronic acid, it should be tested how the metabolite reacts in the presence of HA and what else may or may not be appropriate as part of the delivery vehicle. It should also be tested what levels of $24,25(OH)_2D_3$ are found in synovial fluid of both normal and arthritic articular joints in animal models (and in humans if possible). It may be necessary to also characterize how well $24,25(OH)_2D_3$ would remain within the joint space if injected locally, how much would leak into the blood stream and any effects of this, and finally how well the hormone would diffuse into chondrocytes at different depths within the articular cartilage tissue. It is probably less likely that $24,25(OH)_2D_3$ would remedy the causes of rheumatoid arthritis as it involves a strong autoimmune component, however, this should be tested for any efficacy if osteoarthritis treatment appears to be successful.

Characterization of Shear Stress on Growth Plate Chondrocytes

Initially, it was suspected that shear stress may be a parameter that could promote increased differentiation of growth plate chondrocytes through the stages of endochondral ossification. However, over the range of shear stress we studied and over the time points which were assessed it appears that fluid shear stress dose dependently inhibits or delays

cellular differentiation and proliferation. It may be possible that different levels or time of shear exposure may indicate an increase in differentiation. Furthermore, additional time points of assessment should be included to determine how quickly the cells begin responding to shear stress, as well as how long after cessation of shear are cells still exhibiting some alteration caused by this treatment.

It would be beneficial to determine more information about protein level changes at the matrix. Perhaps quantitative histology or ELISA of sheared cultures could be performed to determine if expression of collagen type II, type I, and COMP had changed. It should be assessed if catabolic enzymes that degrade the integrity of matrix proteins such as matrix metalloproteinases (MMP-13) and aggrecanases (ADAMTS5) are upregulated by shear which may influence the breakdown of the matrix. Western blot may also be another method although probably less desirable to try to quantify the alteration of proteins expressed after shear.

Although we tested viability of the cells after shear, it would be good to characterize this more fully. Studies have shown that fluid shear stress can induce apoptosis and full characterization of this would be absolutely necessary if this system were to be used further, including time course and intensity of apoptosis induced over various levels and exposure periods of shear stress. However, some assays for apoptosis would be very challenging in this system. DNA fragmentation assay cannot be performed without contaminating the device with radioactive isotopes which would need to be pulsed into the cultures prior to our shear treatment. As many of the better apoptosis assays can be very costly they are often done on small volume culture sizes, however samples in this system must be cultured in large Petrie dishes to be compatible

with the cone-plate setup. Caspase-3 activity might be an option to test this activity in cultures following shear. However, this would require significant dilution of the large Petrie dish culture collection to accommodate the smaller volumes testable with a single kit. Assessment of Bax and Bcl-2 by western blot may be an option. Furthermore, MTT could be another assessment of viability although this assay arguably not strictly a measurement of apoptosis so much as assessing the level of mitochondrial activity. Live/dead staining may be possible if the microscope can accommodate the large Petrie dish. This would also be interesting to assess if cells are equally viable throughout the culture dish or if variations in shear at regions of the plate alter the viability in that region, including at increasing levels of shear.

As the shear stress model did not turn out thus far to be a method of studying guided increase of differentiation, it may be more productive to alter the perspective and develop protocols for the device suitable for the study shear induced apoptosis or stress, such as may be observed in osteoarthritis. This would still hinge on future results characterizing if apoptosis is induced by the system or if differentiation actually is induced under different conditions. As mentioned above, Aim 1 future work suggested characterizing the potential use of 24,25 to limit or treat occurrences during arthritis. If the shear system could be characterized as a good model of physiologically relevant shear induction of apoptosis or death, then coordinated studies involving $24,25(OH)_2D_3$ could determine if this hormone could also rescue chondrocytes from this inducer of death. This along with Pi-induced apoptosis would be two relevant forms of stress to cells in osteoarthritic cartilage and would lend more credence to the potential therapeutic benefit of this molecule as a drug.

To further characterize mechanotransduction in growth plate chondrocytes, other methods of culture or mechanical stimulation could be tested (potentially requiring acquisition of additional equipment) to expand the scope of what our system was able to study. Chondrocytes can benefit from three-dimensional culture and perhaps another system could be used to mechanically stress the cells in another way in a 3-D culture perhaps in alginate or another hydrogel. Other devices could be purchased or custom-made that could apply different forms of mechanical loading to the cells such as cyclical strain, compression, or hydrostatic pressure. Chondrocytes have shown varied responses to different kinds of mechanical loading and another type may prove more relevant to growth plate chondrocytes specifically. Finally we have focused on using resting zone chondrocytes or $24,25(OH)_2D_3$-sensitive cells in these studies, and similar experiments could be performed with chondrocytes from the proliferative zone of the growth plate.

Integrin Mediated Mechanism of Mechanotransduction

More work should be done to characterize how integrins may be involved in mechanotransduction in the growth plate. First, more evidence could be tested to verify that integrin beta 1 is truly involved in the effects of shear that we observed. This could include studies blocking beta 1 with antibodies and/or RGD peptides or also inducing transient silencing with siRNA. These methods could also be implemented in primary cells and not just the cell line model. It is also important to determine if specific alpha subunits are important to pair with beta 1 to mediate mechanotransduction. If alpha subunits prove to be important for mechanotransduction, then interesting studies can be

performed to show that receptor specificity is may be important for translation of mechanical cues. Each integrin receptor pair generally has one molecule or class of molecules that it binds to with the greatest affinity, but there is some level of ligand promiscuity between different integrin receptors. Furthermore, some of these molecules, such as COMP, can alter conformation in different conditions (such as during calcium binding) and this alters which integrin receptor has greater affinity for the molecule. Using experimental designs exploiting integrin specificity may provide greater understanding about how ligand/receptor interactions may be involved in translation of mechanical signals. Other experiments could apply shear to cells cultured on a surface coated with specific proteins to also exploit some of these analyses.

Other studies could focus on deciphering intracellular mechanisms of integrin-mediated mechanotransduction. Autocrine factors and signaling cascades have been shown to be activated mechanically via integrins and additional studies could use inhibitors to test if blocking specific steps of suspected pathways are involved. Integrins interact on the intracellular side of the membrane with other molecules such as vinculin, talin, focal adhesion kinase, and cytoskeletal components. Many studies could target methods of disruption of these specific players to determine their involvement in cell signaling also.

Successful Prioritization and Considerations Moving Forward

There is potentially much more valuable knowledge that could be gleaned by continuing with these studies. Some aspects should be considered when contemplating

what work may be most valuable or effective in the acquisition of productive knowledge. First, regarding the therapeutic effects of $24,25(OH)_2D_3$, it should be strongly considered what would validate this for potential clinical use and proof of efficacy in humans. If it turns out that $24,25(OH)_2D_3$ is not efficacious in treating osteoarthritis, it may actually be indicative that part of the etiology of this disease is caused by a loss of sensitivity of articular chondrocytes to this hormone. This would be very laborious to determine the mechanism, but may be very valuable and of great clinical impact to determine this mechanism.

There are a number of challenges to consider when using the cone-plate system as we did. The greatest challenge was the limited amount of sample size. It took a great amount of time to simply characterize the effects of different levels of shear on a few chondrocytic markers in two cell types. Then to start considering the effects of time by harvesting samples at the end of shear or 24 hours later expands the work greatly again. Furthermore, it would be quite valuable to consider more time points especially during shear and after the end of shear, but as can be observed this gets exponentially larger very quickly. It would be advisable to only choose one or two levels of shear when expanding to several time course measurements and the most important assays.

Compounding this problem is the fact that multiple parameters cannot be expanded within a single experiment (due to limited number of cone-plates) so several repeats of experiments at the same parameters must be performed before obtaining a reliable sense of the average response (typically by observing treatment-to-control ratios between several independent experiments for only one combination of parameters). If significantly more studies were to be pursued using the cone-plate device it may be

advisable to invest in additional devices so the studies could be completed much more quickly. The other challenge with collecting data over such a long term is that variation in the cone plates or the associated equipment (incubators, vacuum pumps, new batches of media sera, etc.) can be much more significant over long periods of time. When experiments can be repeated over a few weeks versus a few months these long-term variables are less influential. Finally, more and more literature is arguing that fluid shear stress on monolayer culture (while it is pertinent and often more convenient) may not be the most physiologically relevant form of mechanical stimulation for testing responses by chondrocytes. These factors should be carefully evaluated at the onset to ensure that time-intensive experiments have the greatest probability of productivity, especially compared to other projects that might be able to accumulate data at a faster rate.