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Integrin $\alpha 1 \beta 1$ participates in chondrocyte transduction of osmotic stress



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ABSTRACT

Background/purpose: The goal of this study was to determine the role of the collagen binding receptor integrin $\alpha 1 \beta 1$ in regulating osmotically induced $[Ca^{2+}]_i$ transients in chondrocytes.

Method: The $[Ca^{2+}]_i$ transient response of chondrocytes to osmotic stress was measured using real-time confocal microscopy. Chondrocytes from wildtype and integrin $\alpha 1$ -null mice were imaged *ex vivo* (in the cartilage of intact murine femora) and *in vitro* (isolated from the matrix, attached to glass coverslips). Immunocytochemistry was performed to detect the presence of the osmosensor, transient receptor potential vanilloid-4 (TRPV4), and the agonist GSK1016790A (GSK101) was used to test for its functionality on chondrocytes from wildtype and integrin $\alpha 1$ -null mice.

Results/interpretation: Deletion of the integrin $\alpha 1$ subunit inhibited the ability of chondrocytes to respond to a hypo-osmotic stress with $[Ca^{2+}]_i$ transients *ex vivo* and *in vitro*. The percentage of chondrocytes responding *ex vivo* was smaller than *in vitro* and of the cells that responded, more single $[Ca^{2+}]_i$ transients were observed *ex vivo* compared to *in vitro*. Immunocytochemistry confirmed the presence of TRPV4 on wildtype and integrin $\alpha 1$ -null chondrocytes, however application of GSK101 revealed that TRPV4 could be activated on wildtype but not integrin $\alpha 1$ -null chondrocytes. Integrin $\alpha 1 \beta 1$ is a key participant in chondrocyte transduction of a hypo-osmotic stress. Furthermore, the mechanism by which integrin $\alpha 1 \beta 1$ influences osmotransduction is independent of matrix binding, but likely dependent on the chondrocyte osmosensor TRPV4.

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1. Introduction

Articular cartilage forms a mechanically functional surface for articulating bones due to the complimentary physical properties of its three major components; interstitial fluid, proteoglycans and type II collagen [1]. Chondrocytes are the solitary cells of cartilage and are responsible for maintaining the extracellular matrix. Proteoglycans within the cartilage matrix contain fixed negative charges that are responsible for maintaining the resulting

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium; TRPV4, transient receptor potential vanilloid-4; GSK101, GlaxoSmithKline1016790A; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate buffered saline; CTRL, control; DIC, differential interference contrast.

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ionic imbalance and consequently, the elevated osmolarity of the interstitial fluid [3]. In addition to type II collagen, type VI collagen is found in articular cartilage and is most abundant in the pericellular matrix that encapsulates chondrocytes, forming the chondron [1,4]. The specificity of type VI collagen to the pericellular matrix suggests the importance of this collagen and its receptors in mediating structural interactions and signaling potentials between the chondron and its surroundings [4].

Integrins are transmembrane receptors for extracellular matrix components composed of an α and a β subunit. Upon binding to the matrix milieu, integrins activate via an 'outside-in' signaling mechanism enabling various intracellular molecular pathways and thus controlling a variety of cell responses [5,6]. Integrins have been shown to modulate the activity, function, and expression of various growth factor receptors, transporters, and the Cav1.2 calcium channel [7,8]. Within the integrin family, integrins $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ are two of the major collagen II receptors [5,9]. In addition, integrin $\alpha 1 \beta 1$ serves as the primary mediator of chondrocyte

adhesion to type VI collagen [5] and its expression is upregulated in the early stages of osteoarthritis disease [9,10]. Importantly, integrin $\alpha 1$ -null mice demonstrate increased subchondral bone density and cartilage degradation at a younger age compared to controls [9,11], suggesting a critical role for integrin $\alpha 1\beta 1$ in mediating chondrocyte–matrix interactions and cartilage remodeling occurring with spontaneous knee osteoarthritis.

Intracellular calcium ($[Ca^{2+}]_i$) transients are one of the first biological responses of chondrocytes to osmotic stress [12]. Chondrocytes possess the non-specific cation channel, transient receptor potential vanilloid-4 (TRPV4), an ion channel thought to be most critical in chondrocyte osmoregulation involving $[Ca^{2+}]_i$ transients [13,14]. Inhibition of TRPV channels (including TRPV4) by ruthenium red reduces the increase of $[Ca^{2+}]_i$ from the extracellular solution following a hypo-osmotic stress and the chondrocytes of *trpv4*^{−/−} mice have impaired $[Ca^{2+}]_i$ responses to osmotic stress [14,15].

TRP channels form large molecular complexes that associate with the chondrocyte cytoskeleton [16] and the expression pattern of TRPV4 is similar to that of $\beta 1$ integrins [17] and chondrogenic markers such as type II collagen and aggrecan [18]. Interestingly, both *trpv4*-null and integrin $\alpha 1$ -null mice demonstrate an osteoarthritic phenotype, including full thickness loss of articular cartilage and an increase in subchondral bone thickness/volume at 12 months of age relative to controls, suggesting a common protective role against osteoarthritis by both TRPV4 and integrin $\alpha 1\beta 1$.

The possible interactions between integrins and TRPV4 and their influence upon chondrocyte $[Ca^{2+}]_i$ transients in response to osmotic stress are not very well understood. In this study we utilized integrin $\alpha 1$ -null mice to investigate the role of integrin $\alpha 1\beta 1$ in regulating osmotically induced $[Ca^{2+}]_i$ transients *ex vivo* and *in vitro*. Although wildtype and integrin $\alpha 1$ -null chondrocytes express TRPV4, chondrocytes from integrin $\alpha 1$ -null mice showed impaired $[Ca^{2+}]_i$ transient responses to osmotic stress and the TRPV4 agonist GSK1016790A (GSK101). These results suggest that integrin $\alpha 1\beta 1$ plays an important role in chondrocyte osmotransduction in a manner that is independent of matrix binding, and provide evidence of crosstalk between integrin $\alpha 1\beta 1$ and TRPV4.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Life and Environmental Sciences Animal Care Committee of the University of Calgary. Breeder pairs of heterozygous integrin $\alpha 1$ -null [19] mice were backcrossed onto the Balb/C background strain for ten generations. Genotype was confirmed by polymerase chain reaction of ear punch tissue as previously described [19]. Male or female skeletally mature integrin $\alpha 1$ -null ($n = 73$, age = 21 ± 2 weeks, mass = 30 ± 4 g; mean \pm sd) and wildtype ($n = 83$, age = 18 ± 3 weeks, mass = 29 ± 4 g; mean \pm sd) control mice were utilized for experiments.

2.2. Media preparation

For all *ex vivo* experimental procedures, murine femora were submerged in iso-osmotic (300 mOsm) wash medium (Dulbecco's Modified Eagle Medium (DMEM)-High Glucose) (Invitrogen; Grand Island, NY, USA). During isolation, *in vitro* chondrocytes were maintained in iso-osmotic (380 mOsm, to account for the loss of proteoglycan molecules [20–22]) feed medium (DMEM/F-12) (Invitrogen) containing 10% Fetal Bovine Serum and 2% Pen/Strep. During dye incubation and all confocal microscope imaging, *in vitro* chondrocytes were submerged in wash medium augmented with 1%

Kanamycin, 0.5% Fungizone and 0.1% Gentamycin. All media was adjusted to pH 7.4.

During confocal imaging, chondrocytes were presented with a hypo-osmotic (−50 or −100 mOsm) challenge, hyper-osmotic (+50 or +100 mOsm) challenge, or 10 nM GSK1016790A (GSK101, Sigma, Oakville, ON) a potent activator of TRPV4 [23]. Appropriate control experiments, iso-osmotic (final osmolarity 300 mOsm *ex vivo* or 380 mOsm *in vitro*) and GSK vehicular control (Dimethyl sulfoxide, Fisher Scientific, Ottawa, ON) ($1:10^6$), were also conducted. Medium osmolarity was adjusted using distilled water/sucrose, and confirmed using a freezing point osmometer (Advanced Instruments; Norwood, MA, USA).

2.3. Tissue preparation

Mice were weighed and euthanized prior to the hindlimbs being harvested and the femora isolated by microdissection. For *ex vivo* experiments, Krazy Glue was used on the anterior side of the femoral shaft to fix the intact femora to a glass coverslip with condyles facing up. The Krazy Glue was allowed to cure prior to placement of the femora inside a heated (37.0 ± 1 °C) perfusion chamber and submersion in wash media. Femora were imaged the same day as harvest. Cell viability assays were conducted to ensure >90% cell viability was maintained after this procedure.

For *in vitro* experiments, cartilage was harvested from the medial/lateral condyles of the left/right femora of eight mice and pooled in wash media. Enzymatic isolation of chondrocytes was achieved using sequential pronase (500 μ g/mL) and collagenase (Type II; 300 μ g/mL) application. Isolated chondrocytes were plated on glass coverslips at a density of ~ 700 cells/ μ L, incubated (37 °C) overnight in feed media and submerged in wash media in a heated (37.0 ± 1 °C) perfusion chamber the following day.

All chondrocytes were incubated with the calcium sensitive fluorescent dyes: Fura Red AM (60 M) and Fluo-4 AM (15 M) (Invitrogen) for 40 min and washed for 20 min immediately prior to imaging. The $[Ca^{2+}]_i$ transients were measured using an adaptation of a previously described ratio imaging technique: fluorescence of Fluo-4 AM divided by that of Fura Red AM [14,24].

2.4. Laser scanning microscopy

Ex vivo experiments were performed on an upright confocal laser scanning microscope (Olympus Fluoview FV1000, Shinjuku-ku, Tokyo, Japan) using a LUMPlanFI 40 \times /0.80 W dipping objective lens (Olympus). *In vitro* chondrocytes were imaged with an inverted confocal laser scanning microscope (LSM 7 Duo; Carl Zeiss Canada Ltd., Toronto, ON) fitted with an incubation chamber using an Aplanachromat 40 \times /1.1 NA water immersion objective lens (Carl Zeiss). All chondrocytes were excited using an argon ion laser (488 nm), and fluorescence emission was recorded at 505–550 nm (Fluo-4) and at greater than 650 nm (Fura Red) with the pinhole opened to allow collection of the fluorescence over the entire diameter of the cells (10 μ m). Images (512 \times 512 pixels) were recorded every 3.6 s for 12 min (Fig. 1A and B).

2.5. Data analysis and statistics

Cells were identified using a custom MATLAB program by manually circling individual cells during the first scan of an experiment. The average fluorescence ratio of Fluo 4/Fura Red was calculated for each cell of each image over the 12 min experiment (Fig. 1C–E). Calcium transients were defined as events when the following two conditions were met: [1] both dyes responded at a given time and [2] the magnitude of the ratio of Fluo4/Fura Red response was >3.5 standard deviations above background noise. For all tests, chondrocytes experiencing transient changes in

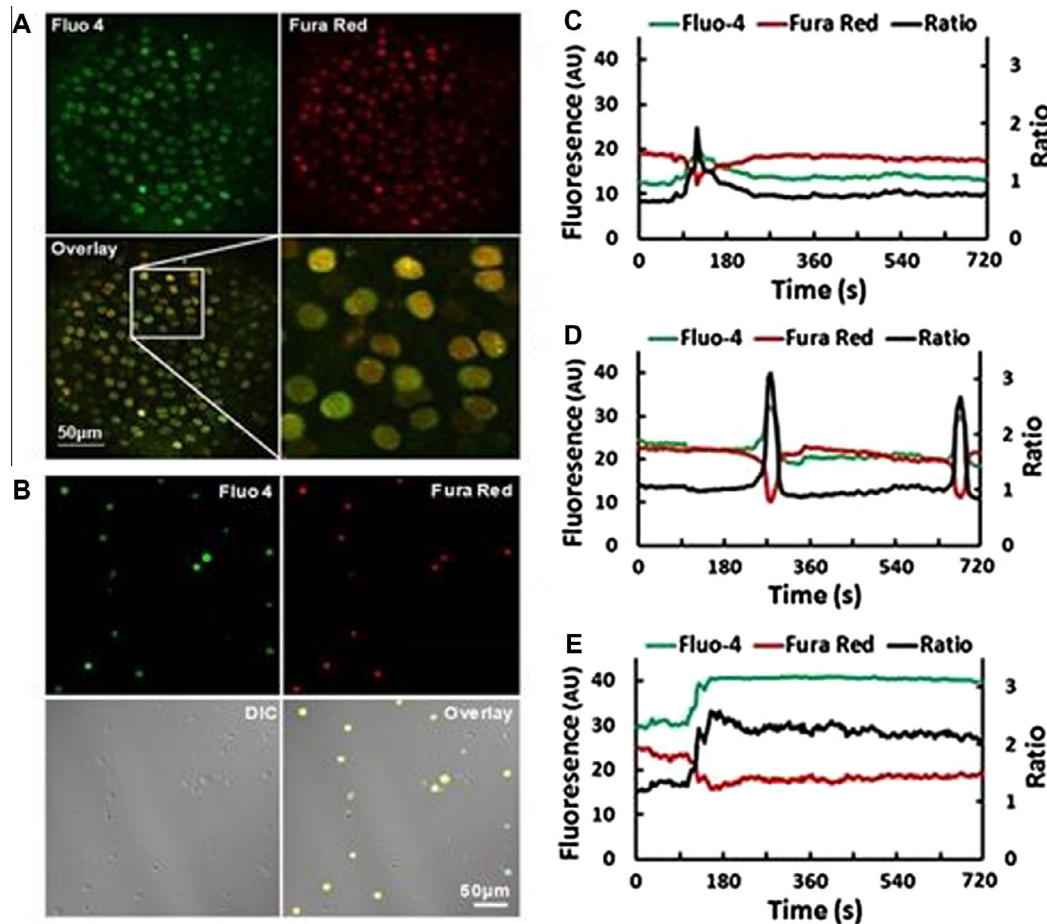


Fig. 1. Murine chondrocytes loaded with Ca^{2+} sensitive dyes *ex vivo* (A) and *in vitro* (B) responded with single (C), multiple (D) and GSK (E) $[\text{Ca}^{2+}]_i$ transients. Chondrocytes were incubated with Fluo 4 (green) and Fura Red (red). Fluo 4 and Fura Red fluorescence are overlayed and the boxed area is magnified to demonstrate resolution (A). A differential interference contrast (DIC) image of isolated chondrocytes is shown separately and overlayed with Fluo 4 and Fura Red fluorescence (B). Traces are from *in vitro* integrin $\alpha 1$ -null chondrocytes stimulated with 280 mOsm hypo-osmotic media (C and D) and a wildtype chondrocyte stimulated with 10 nM GSK101 (E) with fluorescence in arbitrary units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$[\text{Ca}^{2+}]_i$ were reported as a percentage of the total number of chondrocytes imaged. The percentage of cells responding with single, multiple or GSK transients was analyzed using a Chi-square (χ^2) test. Multiple regression analysis was applied to the results of the percentage of cells responding as well as the type of $[\text{Ca}^{2+}]_i$ transient (single/multiple) observed in the responding cells. Multivariate analysis of variance with Tukey's post hoc comparison was performed on the parametric data describing the characteristics of the $[\text{Ca}^{2+}]_i$ transients with osmolarity, genotype and matrix as factors.

2.6. Immunocytochemistry

Murine femora were fixed in 4% paraformaldehyde and decalcified (CalEX, Fisher Scientific, Ottawa, ON) prior to being submerged in 25% sucrose overnight. Samples were embedded in optimal cutting temperature compound and flash frozen. Sagittal sections (12 μm) were cut, washed (phosphate buffered saline (PBS)) and blocked (normal goat serum and Triton X (Sigma, Oakville, ON)) before going through sequential wash (PBS and Tween 20 (Sigma, Oakville, ON)) and antibody application steps. A primary antibody for TRPV4 (1:200, Abcam, Cambridge, MA), the secondary antibody Alexa Fluor[®] 647 (1:500, Invitrogen) and the nucleic acid stain Hoescht 33342 (0.1 mM, Invitrogen) were applied to all sections. After staining, sections were mounted in ProLong[®] Gold (Invitrogen,) and coverslipped.

Slides were imaged using an oil immersion lens (40 \times , 1.4 N.A.) on an LSM 7 DUO (Carl Zeiss) confocal microscope in either differential interference contrast (DIC) or channel scanning confocal modes; Hoescht (excitation 405 nm, emission 429–684 nm) and Alexa Fluor[®] 647 (excitation 633 nm, emission 638–755 nm). At least 30 cells located in the superficial and middle/deep zones of the cartilage were identified.

3. Results

3.1. Control experiments

Control experiments revealed that withdrawal and perfusion of iso-osmotic media caused significantly ($p < 0.001$) more chondrocytes to respond with $[\text{Ca}^{2+}]_i$ transients *in vitro* (52% wildtype, 74% integrin $\alpha 1$ -null) compared to *ex vivo* (8% wildtype, 8% integrin $\alpha 1$ -null) (Fig. 2A and B), with the effect of genotype not reaching statistical significance.

3.2. Osmotic affect

Application of 50 or 100 mOsm of hypo-osmotic stress resulted in a 1.8 or 1.9-fold increase in the percentage of *ex vivo* wildtype cells responding with $[\text{Ca}^{2+}]_i$ transients ($p = 0.05$, $p = 0.037$), however hyper-osmotic stress had no effect (Fig. 2A). Similarly, a

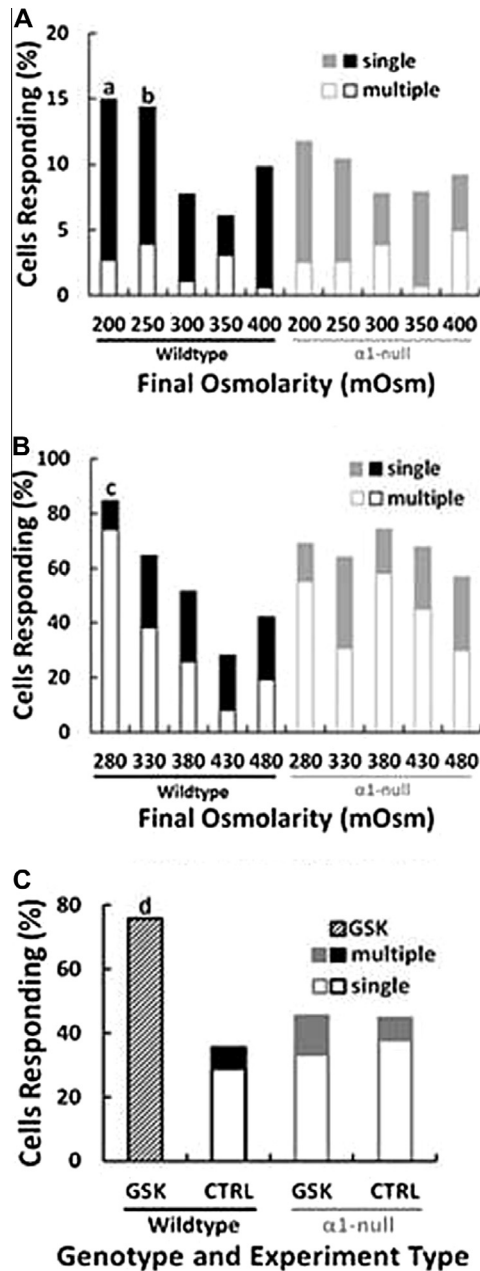


Fig. 2. The $[Ca^{2+}]_i$ response of wildtype chondrocytes to hypo-osmotic stress and the TRPV4 activator GSK is absent from integrin $\alpha 1$ -null chondrocytes. The percentage of wildtype (black) and integrin $\alpha 1$ -null ($\alpha 1$ -null) (gray) chondrocytes responding with single, multiple or GSK $[Ca^{2+}]_i$ transients. *Ex vivo* (A) and *in vitro* (B, C) transients are expressed as a function of final osmolarity (starting osmolarity = 300 mOsm *ex vivo* and 380 mOsm *in vitro*), GSK101 or corresponding vehicular control (CTRL). *a* = Significantly different from *ex vivo* wildtype 300 mOsm, $p < 0.05$; *b* = significantly different from *ex vivo* wildtype 300 mOsm, $p = 0.05$; *c* = significantly different from *in vitro* wildtype 380 mOsm, $p < 0.01$; *d* = significantly different from wildtype control, $p < 0.01$. $N \geq 3$ independent experiments giving $n \geq 25$ cells for *in vitro* experiments and $n \geq 139$ cells for *ex vivo* experiments for each bar.

100 mOsm hypo-osmotic stress applied *in vitro* resulted in a 1.6-fold increase in wildtype cells responding ($p = 0.003$), with hyper-osmotic stress having no effect (Fig. 2B). Together these results suggest that chondrocyte $[Ca^{2+}]_i$ response to hypo-osmotic stress is independent of attachment to the extracellular matrix. Interestingly, the induction of an osmotic stress (hypo- or hyper-) had no effect on the percentage of integrin $\alpha 1$ -null cells responding with $[Ca^{2+}]_i$ transients *ex vivo* or *in vitro* (Fig. 2A and

B), suggesting that integrin $\alpha 1\beta 1$ is necessary for osmoregulation and that this mechanism is independent of attachment to the extracellular matrix.

Both hypo- and hyper-osmotic stress delayed the $[Ca^{2+}]_i$ transients after withdrawal and infusion of the medium by 57 s ($p = 0.04$) or 122 s ($p < 0.001$), respectively relative to iso-osmotic controls (Fig. 3A). Additionally, the delay in $[Ca^{2+}]_i$ transients observed after inducing a hyper-osmotic stress was significantly greater ($p = 0.003$) than hypo-osmotically stressed cells (Fig. 3A).

3.3. Extracellular matrix effect

On average, the percentage of chondrocytes responding during *ex vivo* experiments was 6 times smaller than for *in vitro* experiments ($p < 0.001$) (Fig. 2). Furthermore, of the cells that responded, a larger proportion were single $[Ca^{2+}]_i$ transients *ex vivo* (average 73%) compared to *in vitro* (average 40%) ($p = 0.007$) (Fig. 2).

To determine how the presence of the extracellular matrix influenced the characteristics of chondrocyte $[Ca^{2+}]_i$ transients, *ex vivo* and *in vitro* comparisons were made. $[Ca^{2+}]_i$ transients observed in *ex vivo* chondrocytes were more than twice as large but 4.3 s shorter than those *in vitro* ($p < 0.001$, $p = 0.006$, respectively) (Fig. 3B and C). The rise time of $[Ca^{2+}]_i$ transients was approximately half the duration of the total transient suggesting that the calcium waves were symmetrical about their peak (data not shown). Finally, $[Ca^{2+}]_i$ transients in *ex vivo* chondrocytes were delayed by 113 s after withdrawal and infusion compared to *in vitro* chondrocytes ($p < 0.001$) (Fig. 3D).

Genotype had no effect on the characteristics of chondrocyte $[Ca^{2+}]_i$ transients.

3.4. TRPV4

To assess whether loss of integrin $\alpha 1\beta 1$ might affect TRPV4 levels and/or expression, immunocytochemistry was conducted. Immunostained full-thickness sections of cartilage showed similar expression of TRPV4 in wildtype and integrin $\alpha 1$ -null chondrocytes, in terms of both staining intensity and punctate pattern (Fig. 4).

To determine if TRPV4 was functional as well as present on integrin $\alpha 1$ -null and wildtype chondrocytes, the selective agonist of TRPV4, GSK101 was utilized to stimulate *in vitro* chondrocytes and the resulting $[Ca^{2+}]_i$ transients were measured. The addition of 10 nM GSK101 resulted in a 2-fold increase in the percentage of wildtype chondrocytes responding with $[Ca^{2+}]_i$ transients ($p = 0.004$), however GSK101 had no effect on the percentage of integrin $\alpha 1$ -null chondrocytes responding (Fig. 2C). Interestingly, the GSK101 $[Ca^{2+}]_i$ transients of wildtype chondrocytes occurred within seconds and lasted throughout the 9 min experiment (Fig. 1E). In contrast, $[Ca^{2+}]_i$ transients produced in the presence of GSK101 in integrin $\alpha 1$ -null chondrocytes were similar to the single or multiple transients produced by vehicular control or osmotic challenge (Fig. 2C). Thus, the $[Ca^{2+}]_i$ transient characteristics of integrin $\alpha 1$ -null chondrocytes in the presence of GSK101 suggest that chondrocytes were responding to infusion/withdrawal and not the presence of GSK101.

4. Discussion

This study shows that integrin $\alpha 1\beta 1$ plays a critical role in chondrocyte $[Ca^{2+}]_i$ transient response to hypo-osmotic stress, and that integrin $\alpha 1\beta 1$ exerts this influence in a ligand-independent manner. Previous studies on isolated bovine chondrocytes suspended in alginate hydrogels supplemented with Arg-Gly-Asp, an adhesion protein found on type VI collagen and other molecules

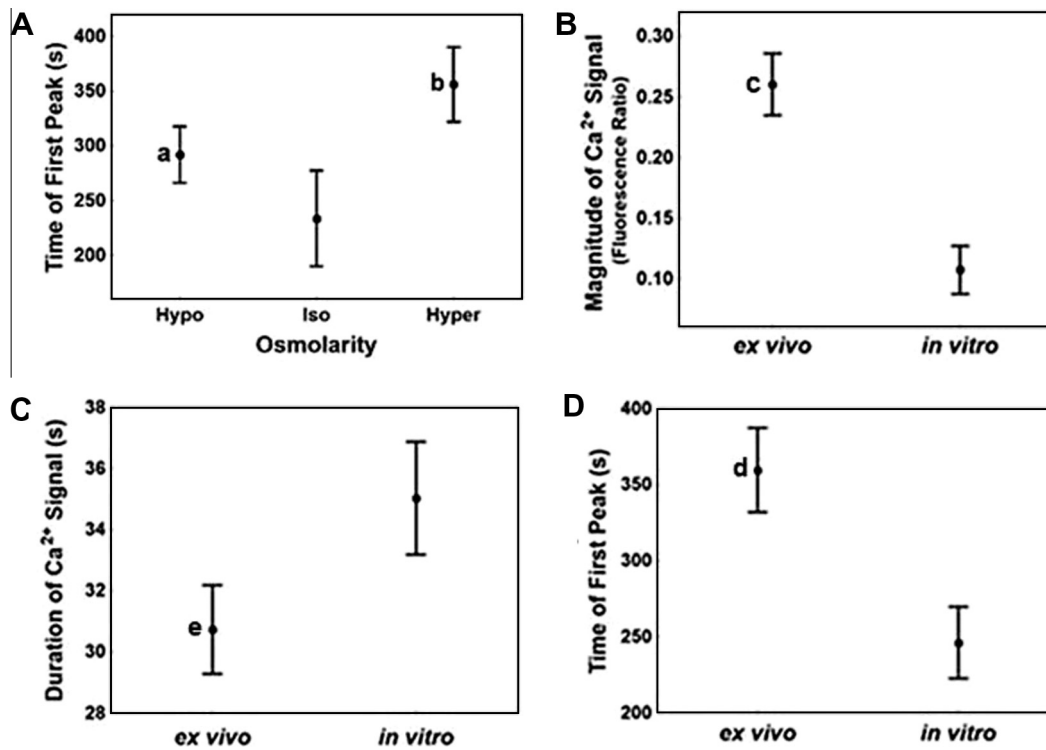


Fig. 3. The $[Ca^{2+}]_i$ transient response of chondrocytes is delayed with hyper- compared to hypo-osmotic stress and is larger, shorter and delayed *ex vivo* compared to *in vitro*. The time of first peak (A and D), magnitude (B) and duration (C) of $[Ca^{2+}]_i$ transients are expressed as a function of final osmolarity (Hypo-, Iso-, Hyper-) (A) or experiment type (*ex vivo* or *in vitro*) (B–D), where magnitude is a dimensionless quantity expressed as a fluo-4/fura red fluorescence ratio. Hypo = combined -50 and -100 mOsm, Iso = combined 300 (*ex vivo*) and 380 (*in vitro*) mOsm, Hyper = combined $+50$ and $+100$ mOsm, *ex vivo* and *in vitro* = combined iso-, ± 50 and ± 100 mOsm. Error bars represent 0.95 confidence intervals and significance is defined as: *a* = significantly different from all other osmolarity data points, $p < 0.05$; *b* = significantly different from all other osmolarity data points, $p < 0.01$; *c* = significantly different from *in vitro*, $p < 0.001$; *d* = significantly different from *in vitro*, $p < 0.01$; *e* = significantly different from *in vitro*, $p < 0.001$. $N \geq 12$ (A) and $N \geq 30$ (B–D) independent experiments giving $n \geq 63$ cells (A) and $n \geq 181$ cells (B–D) for each data point.

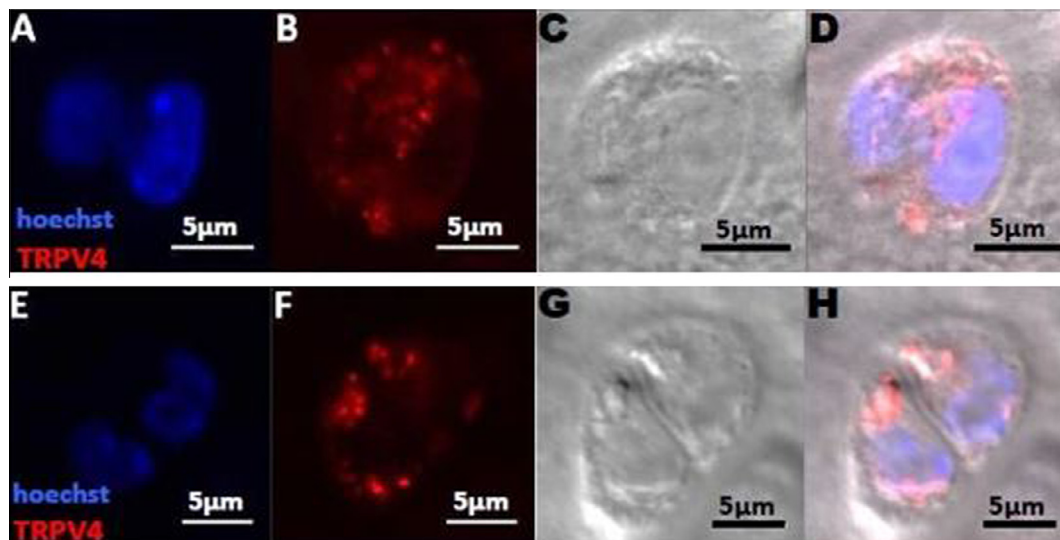


Fig. 4. TRPV4 protein expression is similar on wildtype and integrin $\alpha 1$ -null chondrocytes. Immunocytochemistry images of a wildtype chondrocyte (A–D) and an integrin $\alpha 1$ -null chondrocyte (E–H). Images are of fluorescence alone (A, B, E, F), DIC (C and G) or DIC overlaid with fluorescence (D and H). TRPV4 is labeled red and nuclei labeled blue. TRPV4 is seen as the bright red punctate dots outside of the nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the cartilage matrix, identify integrin–matrix adhesion via a Ca^{2+} -dependent mechanism as a key regulator of chondrocyte Ca^{2+} response to fluid flow [25]. The apparent insensitivity of

integrin $\alpha 1$ -null chondrocytes to osmotic stress both *ex vivo* and *in vitro* would suggest an important new role for integrins in the osmotransduction of chondrocytes.

The characteristics of integrin-mediated chondrocyte $[Ca^{2+}]_i$ signaling are important to consider within the context of TRPV4 – the dominant channel by which chondrocytes sense and respond to an osmotic stress [14]. Immunocytochemistry confirmed a similar expression pattern for TRPV4 on both wildtype and integrin $\alpha 1$ -null chondrocytes, both in terms of the punctate pattern and intensity of staining within cells. To further explore the relationship between integrin $\alpha 1\beta 1$ and TRPV4, we presented *in vitro* chondrocytes from wildtype and integrin $\alpha 1$ -null mice with the TRPV4 agonist, GSK101 and measured the resulting $[Ca^{2+}]_i$ transients. When presented with GSK101, over twice as many wildtype cells responded with $[Ca^{2+}]_i$ transients compared to vehicular controls. GSK101 transients occurred within seconds and lasted throughout the entire 9 min experiment. Both of these phenomena were absent in integrin $\alpha 1$ -null chondrocytes. Taken together, the results above may suggest that integrin $\alpha 1\beta 1$ has a high position in the hierarchy of osmotic signal transduction, similar to TRPV4, and/or some influence on TRPV4.

Although the levels of this channel are similar in both genotypes, we provide evidence that its activity is decreased in the integrin 1-null chondrocyte suggesting that integrin $\alpha 1\beta 1$ directly participates in osmotic signal transduction. Further evidence supporting a role for TRPV4 in integrin-mediated mechanotransduction are the findings that anti-integrin $\alpha 2$ antibody reduces the TRPV4-mediated response to hypotonicity and that TRPV4 coimmunoprecipitates with the integrin $\alpha 2$ subunit in cultured dorsal root ganglion neurons of mice and rats [17].

Our finding that hypo-osmotic stress induces $[Ca^{2+}]_i$ transients in chondrocytes both *in vitro* and *ex vivo* agrees well with previous studies [14,26]. In agreement with previous *ex vivo* studies [14] but in contrast to *in vitro* studies [27], we measured no effect of 50 mOsm or 100 mOsm of hyper-osmotic stress on $[Ca^{2+}]_i$ transients *ex vivo* or *in vitro*. A recent study showed porcine chondrocytes exposed to >140 mOsm of hyper-osmotic stress responded with $[Ca^{2+}]_i$ transients *in vitro* [27]. These data together with the present study may suggest there is a minimum threshold requirement for a hyper-osmotic response to occur *in vitro*. It is important to note that the osmolarity of the iso-osmotic control media used by Erickson et al. was 310 mOsm compared to 380 mOsm – the surface zone maximum and deep zone minimum osmolarity measured in human tissue – in our study.

Additionally, we provide a parallel comparison of *ex vivo* and *in vitro* measurements in osmotically stressed chondrocytes from the same species for the first time enabling us to measure the effect of the extracellular matrix in decreasing the percentage of chondrocytes responding with $[Ca^{2+}]_i$ transients and limiting the characteristics of those signals to single $[Ca^{2+}]_i$ transients. The observed calcium peaks of *ex vivo* experiments were larger in magnitude and both shorter in duration and delayed compared to *in vitro* experiments. The duration of the calcium peaks we observed *ex vivo* agree well with previously reported *ex vivo* studies utilizing compression-stimulated lapine chondrocytes or osmotically-stimulated murine chondrocytes [14,28] and the magnitude and delay of the $[Ca^{2+}]_i$ transients we observed was comparable to the previously reported murine study [14]. Together these observations suggest that chondrocytes utilize contrasting signaling modalities in response to the type of stress (osmotic or mechanical) applied in addition to when surrounded by/isolated from the extracellular matrix. As such, the $[Ca^{2+}]_i$ transient response may be modulated by cytoskeleton/extracellular matrix interactions perhaps involving ligand binding by integrins.

In conclusion we have shown that integrin $\alpha 1\beta 1$ is necessary for chondrocytes to transduce a hypo-osmotic stress into an $[Ca^{2+}]_i$ transient, and that this influence is independent of its adhesion to the extracellular matrix. We suggest that the influence of integrin $\alpha 1\beta 1$ on chondrocyte transduction of osmotic stress is made

via a mechanism that likely involves TRPV4. Furthermore, we show that preservation of chondrocyte/extracellular matrix interactions *ex vivo* influences both the number of cells responding and the signaling modalities harnessed by the chondrocyte. Together, these observations suggest a critical role for integrins in chondrocyte osmotransduction and thus in the homeostasis of articular cartilage. Further analysis of integrin $\alpha 1\beta 1$ expression, activation and function, particularly in relation to TRPV4 and other ion channels on the chondrocyte, will provide new insights into healthy chondrocyte function, as well as the degenerative processes involved in the onset and progression of osteoarthritis.

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