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Journal of Cellular Biochemistry 112:1118–1129 (2011)

Journal of Cellular Biochemistry

The Presence of Extracellular Matrix Alters the Chondrocyte Response to Endoplasmic Reticulum Stress

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ABSTRACT

The objective of this study was to test the hypothesis that extracellular matrix (ECM) would alter the endoplasmic reticulum (ER) stress response of chondrocytes. Chondrocytes were isolated from calf knees and maintained in monolayer culture or suspended in collagen I to form spot cultures (SCs). Our laboratory has shown that bovine chondrocytes form cartilage with properties similar to native cartilage after 2–4 weeks in SCs. Monolayer cultures treated with ER stressors glucose withdrawal (–Glu), tunicamycin (TN), or thapsigargin (TG) up-regulated Grp78 and Gadd153, demonstrating a complete ER stress response. SCs were grown at specific times from 1 day to 6 weeks before treatment with ER stressors. Additionally, SCs grown for 1, 2, or 6 weeks were treated with increasing concentrations of TN or TG. Western blotting of SCs for Grp78 indicated that increased ECM accumulation results in delayed expression; however, Grp78 mRNA is up-regulated in response to ER stressors even after 6 weeks in culture. SCs treated with ER stressors did not up-regulate Gadd153, suggesting that the cells experienced ER stress but would not undergo apoptosis. In fact, SCs undergo apoptosis upon ER stress treatment after 0–1 day of growth; however, after 4 days and to 6 weeks, apoptosis in treated samples was not different than controls. Pro-survival molecules Bcl-2 and Bag-1 were up-regulated upon ER stress in SCs. These results suggest that presence of ECM confers protection from ER stressors. Future studies involving chondrocyte physiology should focus on responses in conditions more closely mimicking the in vivo cartilage environment. J. Cell. Biochem. 112: 1118–1129, 2011.

KEY WORDS: ER STRESS; CHONDROCYTE; EXTRACELLULAR MATRIX; APOPTOSIS; GRP78; BAG-1

Endoplasmic reticulum (ER) stress has been implicated in a number of disease processes, including diabetes [Cnop et al., 2008] and several cartilage-based diseases such as pseudoachondroplasia [Hashimoto et al., 2003; Hecht and Sage, 2006], multiple epiphyseal dysplasia [Hashimoto et al., 2003], and osteoarthritis (OA) [Nugent et al., 2009]. Additionally, induction of ER stress has been proposed for the targeted destruction of cancer cells [Backer et al., 2009; Healy et al., 2009]. Many physiological signals such as aggregation of mis-folded proteins or disruption of calcium homeostasis can induce ER stress, which elicits a specific cellular response in order to attenuate the stress. However, if the stress persists for too long or is too intense, apoptosis occurs through an ER-dependent mechanism [Nakagawa et al., 2000]. This well-characterized response has been shown to occur in both primary and immortalized rat chondrocytes in monolayer cultures [Yang et al., 2005].

Cells respond to impaired ER function by up-regulating chaperone proteins involved in maintaining proper protein folding and shuttling structural and matrix proteins through the ER. Glucose-regulated protein 78 kDa (Grp78/BiP) is a member of the heat-shock protein family of ER chaperones, and it has been demonstrated to be up-regulated under ER stress conditions. Grp78 modulates the ER stress response, acting as a first responder by preventing protein aggregation and targeting misfolded proteins for proteasomal degradation [Hendershot, 2004; Li and Lee, 2006; Lai et al., 2007]. Growth arrest and DNA-damage-inducible gene-153 (Gadd153/CHOP) is a stress-induced transcription factor [Wang et al., 1996a] that ultimately results in the down-regulation of Bcl-2

This work was performed at Department of Anatomy and Neurobiology, Northeastern Ohio Universities Colleges of Medicine and Pharmacy, Rootstown, OH.

Grant sponsor: NIH-Bag-1 proteins in chondrocyte a 1R15AG029659-01A1; Grant sponsor: Summa Ortho CY10 53320.

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Received 28 April 2010; Accepted 5 January 2011 • DOI 10.1002/jcb.23025 • © 2011 Wiley-Liss, Inc.

Published online 18 January 2011 in Wiley Online Library (wileyonlinelibrary.com).

and apoptosis [Matsumoto et al., 1996; McCullough et al., 2001]. Grp78 up-regulation during ER stress has been shown temporally to occur prior to the up-regulation of Gadd153 [Sato et al., 2000]. Additionally, overexpression of Grp78 attenuates the up-regulation of Gadd153 mRNA [Wang et al., 1996a]. This result implies that the cell attempts to abrogate the ER stress response prior to transcriptional inhibition of gene expression.

Bcl-2-associated athanogene-1 (Bag-1) has recently been shown to be down-regulated in response to ER stressors and to modulate the ER stress response in chondrocytes by delaying ER stress-induced down-regulation of collagen type II and apoptosis [Yang et al., 2007]. In addition, Bag-1 was shown to be up-regulated in coordination with Grp78 in advanced human osteoarthritis [Nugent et al., 2009]. Bag-1 is a multi-functional protein first described for its ability to bind to and enhance the anti-apoptotic capacity of Bcl-2 [Takayama et al., 1995]. Its other functions include interacting with Hsp70 to serve as a chaperone in the ER [Takayama et al., 1997] and activating Raf-1 to induce cell proliferation [Wang et al., 1996b]. These various functions all support a role of Bag-1 as a pro-survival molecule, whose expression levels may serve as an indicator of the chondrocyte capacity for withstanding stress.

Chondrocytes in particular experience a variety of stressors even under homeostatic cartilage conditions. Joint articular cartilage is an avascular tissue that experiences unique conditions of mechanical stress resulting from loading and oxidative and nutritional stress attributable to the lack of a direct vascular supply. Additionally, chondrocytes are the only cell type in cartilage, and they are responsible for the synthesis, maintenance, and turnover of the extracellular matrix (ECM). Secreted proteins such as cartilaginous ECM molecules are modified and transported through the ER. Therefore, chondrocytes may be uniquely capable of responding to and withstanding ER stress in vivo.

The study of chondrocytes in monolayer culture has provided the scientific community with information regarding potential cellular responses; however it is important to evaluate chondrocytes in an environment more similar to their setting in vivo. Previous work from this laboratory has characterized a model of bovine cartilage generated ex vivo using chondrocytes isolated from the knee articular cartilage of Holstein calves and maintained in a collagen gel [Nugent et al., 2010]. Here we present data using primary bovine chondrocytes in monolayer culture and within 3D cartilage, demonstrating that the presence of ECM alters the response to ER stressors through delayed Grp78 expression and inhibition of ER stress-induced apoptosis.

MATERIALS AND METHODS

CARTILAGE SAMPLES AND CULTURE METHODS

Knees of 1–3 weeks old Holstein calves were transported from the slaughterhouse (Mahan Packing Co., Bristolville, OH) in Betadine and on ice to maintain sterility and tissue viability. Articular cartilage was dissected from the femoral condyles and tibial plateaus and placed in phosphate buffered saline (PBS) with 100 μg/ml Primocin antibiotic/antimycotic (Invivogen) on ice. Cartilage was minced and digested in 4% collagenase type II (Worthington Biochemical) in Hank's Balanced Salt Solution (Gibco) with 100 μg/ml Primocin overnight at 37°C for chondrocyte isolation. For monolayer culture, chondrocytes were washed twice with PBS, then suspended in Ham's F-12 medium (Gibco) with 10% fetal bovine serum and plated at 1.33×10^5 cells/cm^2 into tissue culture (TC) flasks (Corning 430641). For spot cultures (SCs), collagen I solution (rat tail tendon, BD Biosciences) was prepared according to manufacturer's instructions at 2.7 mg/ml. Chondrocytes were suspended in the collagen I solution and plated as 300 μl "spots" into 100 mm^2 tissue culture dishes to form the SCs, with 5×10^6 cells per SC (1.67×10^7 cells/ml). SCs were incubated at 37°C for 10 min to allow collagen gel formation, then maintained in Opti-MEM I Reduced-Serum Medium (Gibco) with 100 μg/ml Primocin and 50 μg/ml ascorbate.

INDUCTION OF ENDOPLASMIC RETICULUM STRESS

Primary bovine chondrocytes in monolayer were cultured for 4 days prior to ER stress treatment for 24, 48, 72, or 96 h with ER stressors: glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG). These concentrations have been shown by our laboratory to induce ER stress in primary and immortalized chondrocytes [Yang et al., 2005]. To determine the effect of an increasingly complex ECM on the chondrocyte ER stress response, SCs were cultured for 0–7 days, 2 weeks, or 6 weeks prior to treatment for 1 week with glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG). –Glu was achieved with glucose-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS). TN was suspended in dimethyl sulfoxide (DMSO) at a stock concentration of 20 mg/ml; TG was suspended in DMSO at a stock concentration of 1 mM. To determine the concentration of TN and TG that would induce ER stress after a complex ECM was already established, SCs were cultured for 1, 2, or 6 weeks prior to treatment for 1 week with increasing concentrations of TN (1, 2, 5, or 10 μg/ml) or TG (0.5, 1, 2, 5, or 10 μM). Control SCs (marked Ctrl throughout) were grown for the total length of time as treated SCs (i.e., SCs grown for 1 day prior to 1 week ER stress treatment had an associated control SC that was sacrificed after 8 days). To determine whether pharmacological treatments (TN and TG) were reaching chondrocytes surrounded by high amounts of ECM, 6-week-old SCs were treated with 5 μg/ml Actinomycin D (marked ActD). Additionally, since TN and TG are diluted with DMSO, 1-day-old SCs were treated with 0.5% DMSO for 1 week, to determine whether it caused any quantifiable effect (as measured by western blot and qRT-PCR, qRT-PCR not shown).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Tissue samples were fixed in 10% neutral buffered formalin (Fisher Scientific) and then processed and paraffin-embedded according to standard laboratory procedures. Paraffin blocks were sectioned at 10 μm and stained with 0.1% Thionin [Fisher Scientific, Király et al., 1996] for basic histological analysis. Primary antibody for Grp78 (Santa Cruz) immunofluorescence was applied at 1:50 concentration. Rabbit anti-goat AlexaFluor 488 secondary antibody was purchased from Molecular Probes (Invitrogen) and used at 1:200 concentration. In general, unmasking of epitopes was achieved using Chondroitinase ABC (Sigma) at 1:100 concentration for 20 min at 37°C. Primary antibody was incubated overnight at 37°C,

and secondary antibody was applied for 1 h at room temperature. Control slides were prepared by performing every step of the procedure except for the substitution of the primary antibody with blocking serum. Slides were counterstained with DAPI (Vectashield Hard Set Mounting Medium with DAPI, Vector Laboratories, Inc.) for immunofluorescence staining.

Histological sections stained with thionin were quantified across a time series using 255 grayscale, where 0 = black and 255 = white, to determine extracellular matrix accumulation over time. Images were background corrected and a standardized region of interest was generated. Bioquant Osteo II (v.8.00.20, Bioquant Image Analysis Corporation, Nashville, TN) software was used to generate average matrix density within the region of interest. Regions of interest were defined as any space between cells. For each field of view, approximately 15–20 regions of interest were quantified, each of which generates an average matrix density. Data represent the mean matrix density ± standard deviation of five fields of view for each of three independent experiments. Grp78 immunohistochemistry was quantified as percent positively stained cells, for which five fields of view were quantified. Data represent the mean ± standard deviation of three independent experiments. For apoptosis quantification, the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used, which generated a DAB reaction visualized by light microscope. Control slides were prepared by performing every step of the procedure except for the substitution of the primary antibody with blocking serum. Additionally, DNAse-treated slides were generated as a positive control for apoptosis quantification, applied at 1:100 for 15 min at room temperature. For each slide, 5 fields of DAB-positive and -negative chondrocytes were counted at 40× magnification and average percent positive cells quantified,

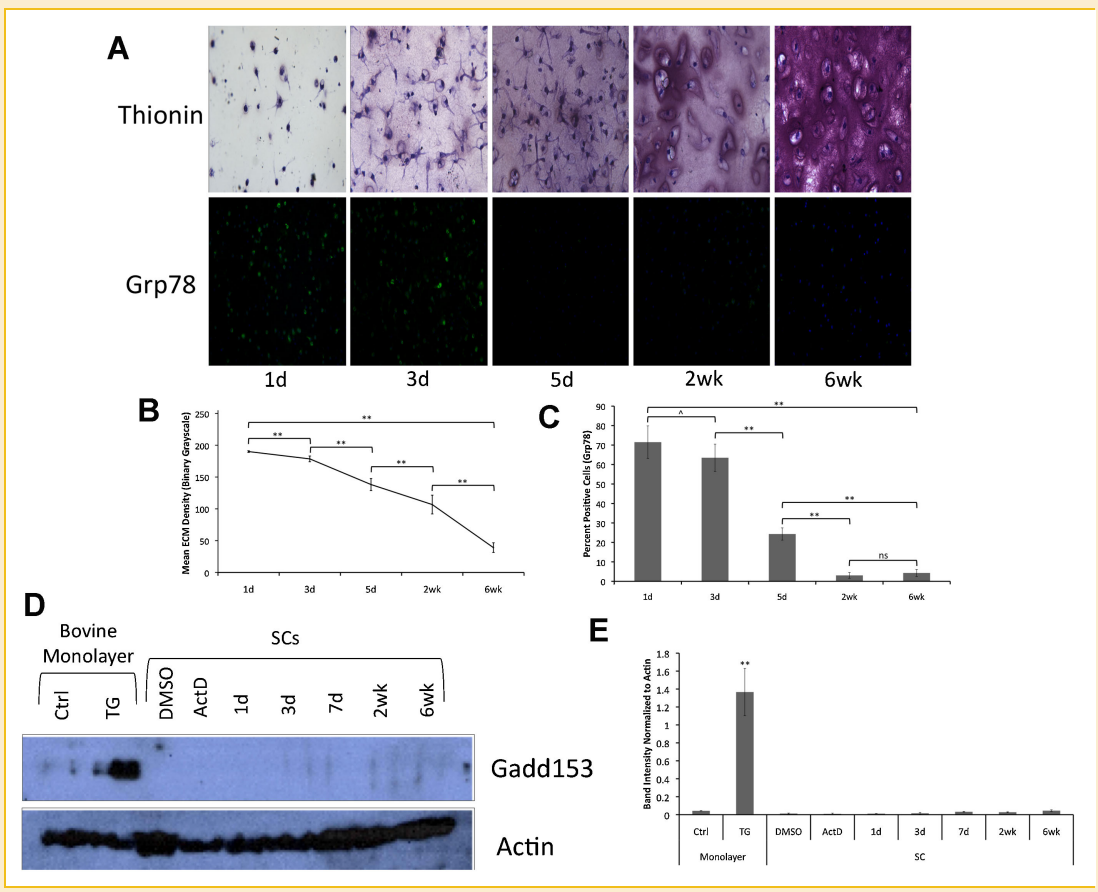

Fig. 1. A: Representative thionin histology (purple) and Grp78 (green, DAPI-positive nuclei are blue) immunohistochemistry of control SCs across a time series. SCs accumulated increased amounts of metachromatic ECM with time in culture (data quantified in B). Initial expression of Grp78 was high, but decreased after 3 days and remained low for 6 weeks in culture (data quantified in C). B: Histomorphometric analysis of ECM density for which representative images are shown in (A), in grayscale (0 = black, 255 = white). SCs accumulated increasingly dense ECM over time. Data represent the mean of five fields of view measured across each of three independent samples ± standard deviation (one-way ANOVA $^{**}P < 0.001$). C: Percent positive cell counts of Grp78 immunohistochemistry, of which representative images are shown in A. Grp78 expression was high at 1–3 days in culture, after which expression decreased at 5 days and further at 2 weeks, after which expression remained low. Data represent the mean of five fields of view measured across each of 3 independent samples ± standard deviation (one-way ANOVA $^{**}P < 0.01$, $^{\wedge}P = 0.052$, n.s., non-significant). D: Western blot analysis of Gadd153 protein expression in control SCs across time. Bovine chondrocytes in monolayer treated with control medium (Ctrl) and 0.5 μM thapsigargin (TG) were used as a positive control for Gadd153 expression. Control SCs did not up-regulate Gadd153 even at early timepoints, and DMSO (the solvent for TN and TG) did not cause ER stress. Actinomycin D (ActD), a positive control for ensuring that pharmacological agents could reach chondrocytes surrounded by dense ECM, did not cause ER stress (N = 3). E: Band intensities were quantified and normalized to Actin (see Materials and Methods Section). Data represent the mean ± standard deviation of three independent experiments (one-way ANOVA $^{**}P < 0.001$).

after which a percent different from control was quantified as $[(x_2 - x_1)/([x_2 + x_1]/2)] \times 100$, where x_2 = average percent positive cells from ER stress-treated samples, and x_1 = average percent positive cells from the associated untreated control sample for the respective time in culture. Data represent the mean ± standard deviation of three independent experiments. Images were obtained using Bioquant Osteo II software and background exposure was normalized to the associated minus-primary control slide, with any additional staining considered positive.

WESTERN BLOTTING

In monolayer studies, media were removed from TC flasks and cells were washed twice with PBS prior to scraping and suspension in radioimmunoprecipitation assay (RIPA) buffer (1× PBS, 1% Nonidet P-40 [Roche], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with 100 mM phenylmethanesulfonylfluoride (PMSF), 1 mg/ml aprotinin, and 100 mM sodium orthovanadate. In order to isolate total cellular lysate, chondrocytes in SCs were disrupted from their ECM by incubation in 4% collagenase for 1 h at 37°C, then washed twice in PBS prior to suspension in RIPA buffer with protease inhibitors (as indicated above). Protein concentration was obtained using Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA) and read against a standard curve. Thirty micrograms of protein were suspended in an equal volume of Novex Sample Buffer with 10× NuPage Sample Reducing Agent (Invitrogen) and loaded onto 8–16% Tris-Glycine (Invitrogen) gels along with 5 μl of MagicMark XP Western Protein Standard (Invitrogen) and run at 125 V in Novex Tris-Glycine SDS Running Buffer (Invitrogen). Gels were transferred onto 0.45 μM polyvinylidene fluoride (PVDF) membranes at 25 V for 2 h in Novex Tris-Glycine Transfer Buffer (Invitrogen).

Membranes were blocked for 1 h in milk with 0.1% Tween-20, then washed once in tris-buffered saline (TBS) with 0.1% Tween-20 prior to incubation in primary antibody overnight at 4°C. Primary antibody concentrations were as follows: mouse monoclonal Gadd153 (Santa Cruz) 1:500; goat polyclonal Grp78 (Santa Cruz) 1:7,000; goat polyclonal β-Actin (Santa Cruz) 1:6,000. After primary antibody incubation, membranes were washed four times in TBS with Tween. Goat anti-mouse and donkey anti-goat secondary antibodies (Santa Cruz) were applied at 1:10,000 concentration for 1.5 h at room temperature. Membranes were washed five times in TBS with Tween and once in TBS without Tween. Protein expression was visualized using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) onto CL-Xposure Film (Thermo Scientific). Relative band intensities were generated on Kodak Molecular Imaging Software Standard Edition v.5.0.1.27. Band intensity of actin was subtracted from experimental bands; data represent the mean ± standard deviation of three independent experiments.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME RT-PCR

RNA was extracted using TRIZOL (Molecular Research Center, Inc., Cincinnati, OH) phenol-choloroform and precipitated with isopropanol according to manufacturer's instructions. RNA was quantified and purification determined on a Nanodrop® ND-1000 Spectrophotometer. Two micrograms of RNA was treated with DNAse I Amplification Grade (Invitrogen) for 15 min at room temperature with 2 μl 10× DNAse I Reaction Buffer, and the reaction was stopped with 2 μl of 25 mM EDTA for 10 min at 65°C. RNA was then reverse transcribed using Taq® Reverse Transcriptase reagents (Applied Biosystems) according to the manufacturer's instructions and run on a Bio-Rad DNA Engine® PTC 0200 thermocycler at 25°C for 10 min, 37°C for 60 min, 95°C for 5 min, and 4°C for 30 min. Seventy-five microliters water was added to a 25 μl reaction to generate 10 ng/μl concentration. Forty nanograms of cDNA were loaded onto MicroAmp Fast 96-well Reaction Plates (4346907, Applied Biosystems), covered with Adhesive Covers (AB 4311971), and reacted with 100 nM forward and reverse primers, along with 12.5 μl Sybr Green per 25 μl reaction. Primer sets are as follows: bovine Bag-1 forward primer: 5′CCAGCAGCCCACCTTGTCT3′; bovine Bag-1 reverse primer: 5′GAAAGCACCCAAAGGTCCAA3′; bovine Grp78 forward primer: 5′AAGATGTTCGGAAGGACAACAGA3′ bovine Grp78 reverse primer: 5′GCCCGTTTGGCCTTTTCT3′; bovine collagen II forward primer: 5′GCATTGCCTACCTGGACGAA3′ bovine collagen II reverse primer: 5′GAACCTGCTGTTGCCCTCAG3′; bovine aggrecan forward primer: 5′TTCACCTGTAAAAAGGGCACAGT3′ bovine aggrecan reverse primer: 5′CAGGGCATTGATCTCGTATCG3′; rat 18s rRNA forward primer: 5′AGTCCCTGCCCTTTGTACACA3′ rat 18s rRNA reverse primer: 5′GATCCGAGGGCCTCACTAAAC3′; bovine ADAMTS-5 forward primer: 5′AGCTGTGCGGTGATTGAAGA3′ bovine ADAMTS-5 reverse primer: 5′CCAAGCAGATGTCCAATTTCG3′; bovine Bcl-2 forward primer: 5′TGTGGATGACCGAGTACCTGAA3′ bovine Bcl-2 reverse primer: 5′AGCCTCCGTTGTCCTGGAT3′. PCR cycles were as

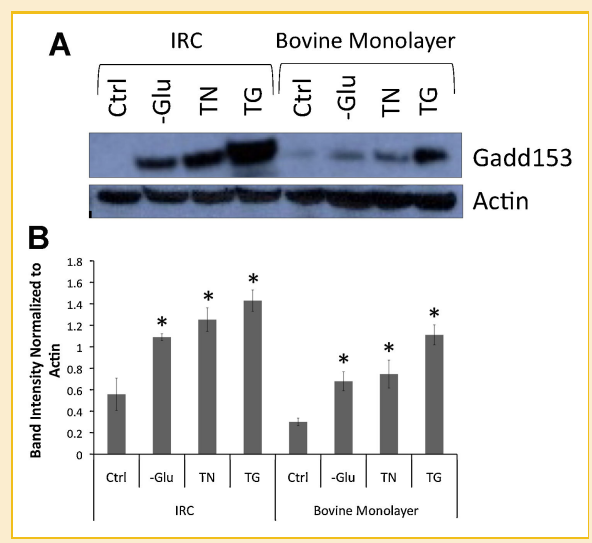

Fig. 2. A: Up-regulation of Gadd153 protein by immortalized rat chondrocytes (IRC) and primary bovine chondrocytes in monolayer, visualized by Western blot. Chondrocytes were treated for 48 h with control medium (Ctrl), glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated (N = 3). B: Quantification of band intensities of three independent experiments normalized to Actin (see Materials and Methods Section). Bars represent mean ± standard deviation of three independent experiments; one-way ANOVA analyzation of experimental samples to the associated control (*$P < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

follows: 95°C for 10 min, (95°C for 15 s, 60°C for 1 min) 40×, followed by a dissociation stage (95°C for 15 s, 60°C for 20 s) 2×. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001] normalized to 18s rRNA and calibrated to a control sample at each time-point. Data represent the mean ± standard deviation of three independent samples.

STATISTICAL ANALYSIS

Data are reported as mean ± standard deviation. N numbers of independent experiments were considered as cells/SCs that were prepared from separate animals. Significance was assessed using one-way analysis of variance. Student–Newman–Keuls or Tukey post hoc tests were performed depending on whether the distribution of the pertinent data was consistent with normality.

RESULTS

To confirm the accumulation of ECM over time, SCs were histologically sectioned and stained with thionin, a metachromatic dye that visualizes proteoglycan content. Bovine chondrocytes in SCs accumulated metachromatic ECM with increased time in culture (Fig. 1A, Thionin, data quantified in Fig. 1B). To determine the level of ER stress marker Grp78 expressed by SCs under homeostatic conditions, control SCs were analyzed immunohistochemically. SCs exposed only to control medium up-regulated Grp78 for the first several days in culture, in coordination with the secretion of high amounts of matrix molecules through the ER. After 3 days in culture, Grp78 expression decreased and remained low throughout 6 weeks in culture (Fig. 1A, Grp78, data quantified in Fig. 1C). SCs in control culture did not up-regulate the terminal transcription factor Gadd153 (Fig. 1D, data quantified in Fig. 1E), a result suggesting that, while the chondrocytes were experiencing ER stress as evidenced by Grp78 up-regulation, they would not terminate in ER stress-induced apoptosis. Neither the transcriptional inhibitor actinomycin D (ActD) nor DMSO (in which TN and TG were suspended; see Materials and Methods Section) up-regulated Gadd153 (Fig. 1D,E). These studies demonstrated that control SCs up-regulated an ER stress-associated chaperone protein in coordination with high amounts of secreted matrix molecules through the ER, but the cells did not up-regulate Gadd153, the transcription factor associated with ER stress-induced apoptosis.

Immortalized rat chondrocytes (IRCs) were used as a positive control to determine whether bovine chondrocytes also respond to the ER stressors glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG). IRCs and bovine chondrocytes

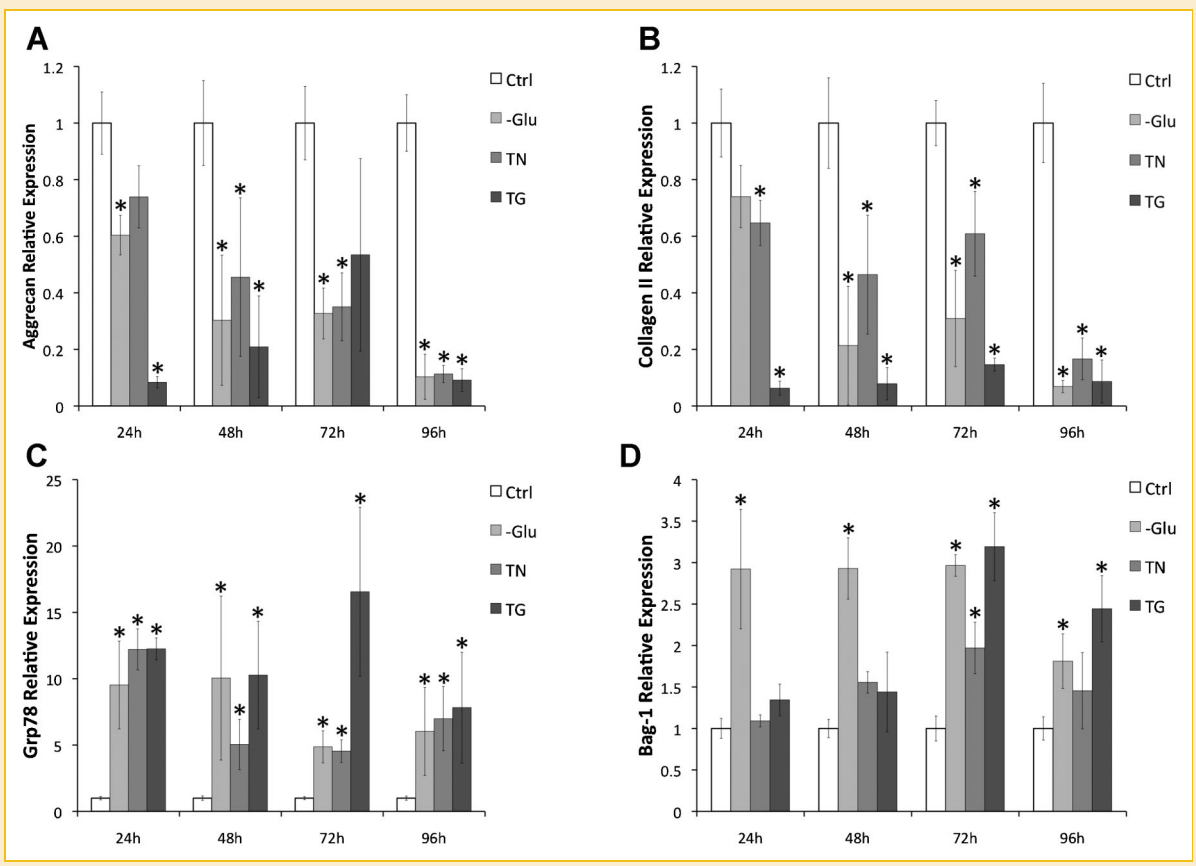

Fig. 3. Quantitative real-time RT-PCR of bovine chondrocytes in monolayer treated for 24, 48, 72, or 96 h with control medium (Ctrl), glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated. Relative expression of (A) Aggrecan, (B) Collagen type II, (C) Grp78, and (D) Bag-1 mRNA. Data represent the mean ± standard deviation of three to five independent experiments, one-way ANOVA of experimental samples to the associated control ($^{*}P < 0.05$).

in monolayer both responded to ER stressors by up-regulation of the transcription factor Gadd153 (Fig. 2A, data quantified in Fig. 2B).

Bovine chondrocytes in monolayer responded to ER stressors –Glu, TN, and TG by down-regulating the mRNA coding for the extracellular matrix molecules aggrecan and collagen type II (Fig. 3A,B), determined by quantitative RT-PCR. Glucose withdrawal and TN treatment resulted in a progressive down-regulation in both aggrecan and collagen type II mRNA transcripts over time, while TG treatment resulted in a more rapid decrease in steady-state mRNA levels by 24 h, which persisted through 96 h. In contrast to aggrecan and collagen II mRNA, the ER stress-associated chaperone Grp78 was up-regulated at the mRNA level following treatment of bovine chondrocytes with ER stressors (Fig. 3C). Previous studies in this laboratory with immortalized rat chondrocytes showed that ER stress resulted in an inhibition of Bag-1 expression [Yang et al., 2007]. However the current study using primary bovine chondrocytes in monolayer culture demonstrated up-regulation of Bag-1 mRNA in response to ER stress (Fig. 3D). Glucose withdrawal resulted in high Bag-1 mRNA levels at 24 h, which persisted through 72 h before falling slightly at 96 h. TN and TG treatment resulted in slower up-regulation of Bag-1, not increasing until 48 h, peaking at 72 h, and then dropping again at 96 h. These studies demonstrated the capacity for primary bovine chondrocytes to respond to ER stressors in terms of mRNA coding for matrix proteins and markers of ER stress and survival.

To determine the effect of ER stress treatment on ECM composition, SCs were treated with ER stressors for 1 week after the time of growth indicated by the left column (Fig. 4A, data quantified in Fig. 4B). Treatment with ER stressors glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) results in the loss of metachromatic ECM in SCs through 2 weeks in culture, but not after 6 weeks in culture (Fig. 4A,B). This loss of metachromasia most likely was not the result of up-regulation of the aggrecanase ADAMTS-5, since this mRNA was not up-regulated in bovine chondrocytes exposed to ER stressors in monolayer culture (Fig. 4C).

To determine whether the presence of ECM affected the mRNA transcript levels of ECM molecules upon ER stress, bovine

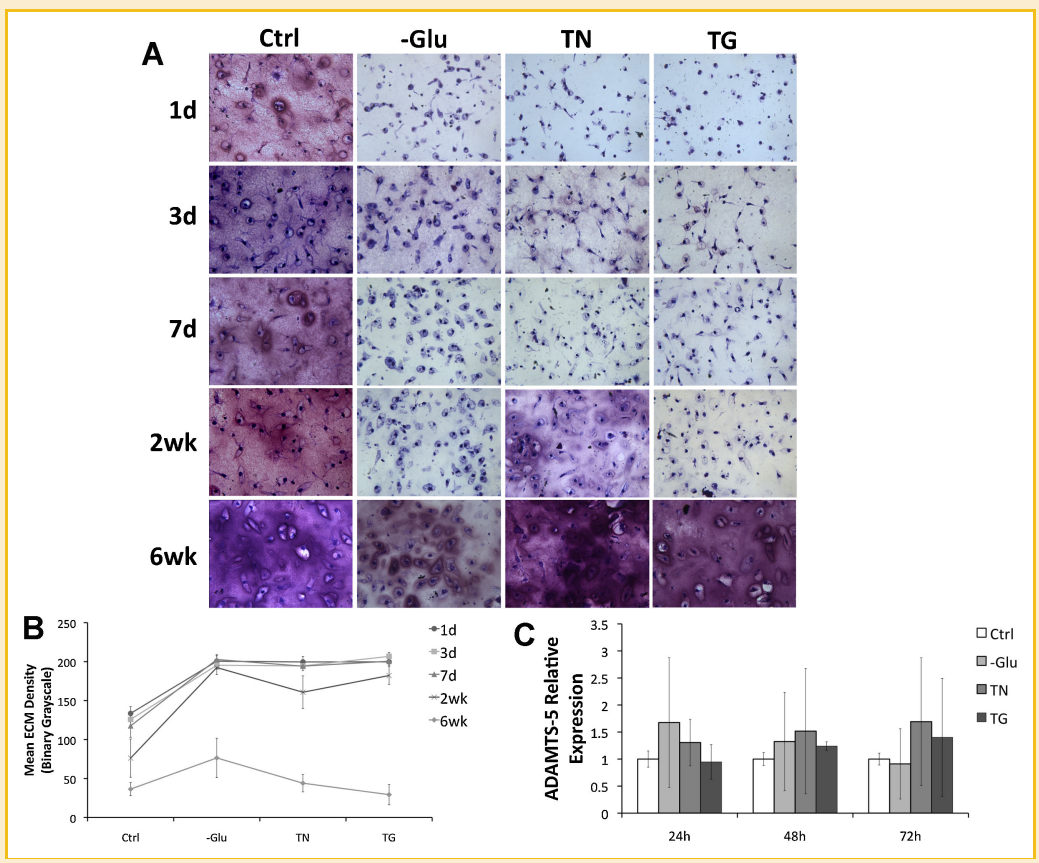

Fig. 4. A: Representative thionin histology of control and ER stress-treated SCs across time. SCs lose metachromatic ECM with ER stress treatment, except after 6 weeks of control growth. SCs grown in control medium for the time period indicated by the left column, then treated for 1 week with control medium (Ctrl), glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated (N = 3–6). B: Histomorphometric analysis of ECM density for which representative images are shown in (A), in grayscale (0 = black, 255 = white). SCs accumulated increasingly dense ECM over time, and ER stress treatment resulted in loss of ECM density at all timepoints except at 6 weeks. Data represent the mean of five fields of view measured across each of three independent samples ± standard deviation. C: Quantitative real-time RT-PCR showing the relative expression of ADAMTS-5 in bovine chondrocytes in monolayer treated for 24, 48, or 72 h with control medium, glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated. Data represent the mean ± standard deviation of three independent experiments. ADAMTS-5 expression is not significantly different from controls.

chondrocytes in SCs were grown for different lengths of time and then analyzed after treatment for 1 week with ER stressors. Bovine chondrocytes in SCs down-regulated mRNA for the ECM molecules aggrecan and collagen type II in response to ER stress, even after 6 weeks in culture (Fig. 5A,B). Moreover, mRNA transcript levels for the ER stress-associated molecules Grp78 and Bag-1 were also determined. Chondrocytes in SCs up-regulated the ER stress-associated chaperone Grp78 at each time in culture to varying degrees (Fig. 5C). Chondrocytes in SCs up-regulated Bag-1 after 4 days or more in culture (Fig. 5D). However, the anti-apoptotic molecule Bcl-2 was up-regulated at the earlier times analyzed (in comparison to Bag-1) upon exposure to glucose withdrawal (Fig. 5E).

To determine whether bovine chondrocytes responded similarly to ER stressors in monolayer culture and within a 3D matrix, chondrocytes in each of these settings were exposed to ER stressors and analyzed for Grp78 and Gadd153. Bovine chondrocytes in monolayer culture treated with TN or TG, and SCs grown for 1 week prior to treatment for 1 week with low (1 μg/ml) or high (10 μg/ml) concentrations of TN, or low (0.5 μM) or high (10 μM) concentrations of TG, all up-regulated Grp78 at the protein level (Fig. 6A, data quantified in Fig. 6B). Additionally, bovine chondrocytes in monolayer up-regulated the terminal transcription factor Gadd153 upon ER stress treatment. However, chondrocytes in SCs did not up-regulate Gadd153 even after treatment with high concentrations of ER stressors (Fig. 6A,B). These data demonstrated a differential

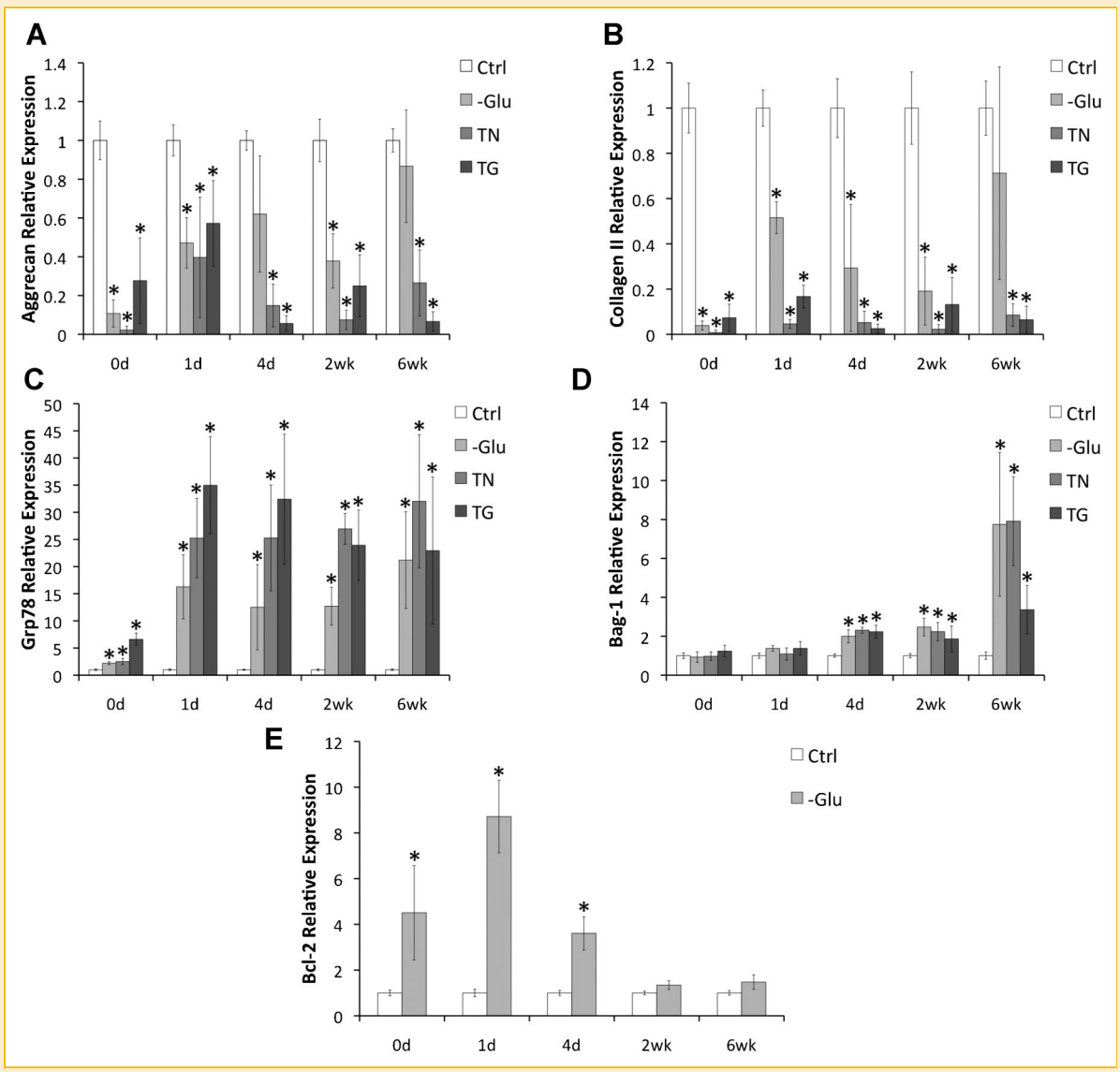

Fig. 5. Quantitative real-time RT-PCR of bovine chondrocytes in SCs grown for the length of time indicated by the *x*-axis, then treated for 1 week with control medium (Ctrl), glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated. Relative expression of (A) Aggrecan, (B) Collagen type II, (C) Grp78, (D) Bag-1, and (E) Bcl-2 mRNA (Bcl-2 analyzed in Ctrl and –Glu treated samples only). Data represent the mean ± standard deviation of three independent experiments; one-way ANOVA of experimental samples to the associated control ($^*P < 0.05$).

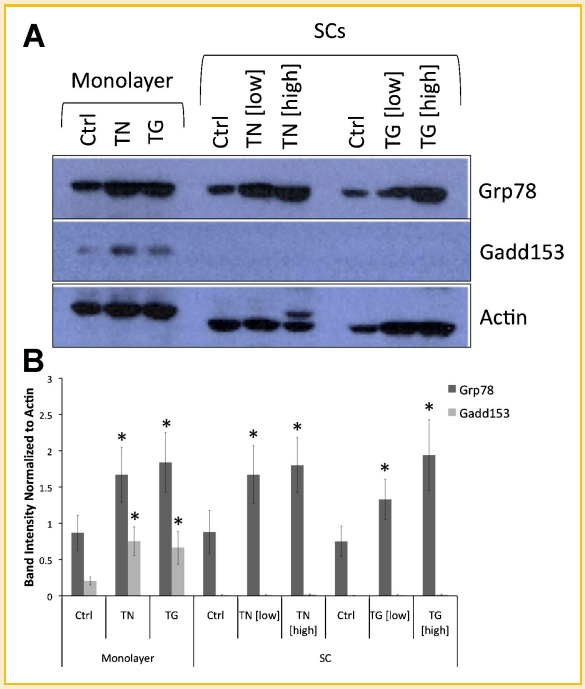

Fig. 6. A: Western blot visualizing protein expression levels of Gadd153 and Grp78 in monolayer and in SCs. Bovine chondrocytes in monolayer up-regulate both Gadd153 and Grp78 upon ER stress treatment. SCs surrounded by a complex ECM up-regulated Grp78, but did not up-regulate Gadd153 in the presence of ER stressors even at high concentrations. Primary bovine chondrocytes in monolayer (Monolayer) were treated for 48 h with control medium (Ctrl), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated. SCs were grown for 1 week in control medium, then subjected to control medium (Ctrl), 1 μg/ml tunicamycin (TN [low]), 10 μg/ml tunicamycin (TN [high]), 0.5 μM thapsigargin (TG [low]), or 10 μM thapsigargin (TG [high]) ($N = 3$). B: Quantification of band intensities of three independent experiments normalized to Actin (see Materials and Methods Section). Bars represent mean ± standard deviation of three independent experiments; one-way ANOVA of experimental samples to the associated control ($^{*}P < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

response of bovine chondrocytes within a three-dimensional matrix to ER stress.

In order to determine the effect of increased time in culture (and therefore increased matrix accumulation) on the chondrocyte response to ER stress, SCs were grown for 1, 2, or 6 weeks prior to treatment with increasing concentrations of TG. In contrast to the mRNA data, SCs grown for 2 weeks or longer require higher concentrations of TG in order to up-regulate Grp78 protein than at earlier time-points (Fig. 7A, data quantified in Fig. 7B). Despite the up-regulation of Grp78 mRNA after 6 weeks of growth seen in Figure 5C, its translation into protein is shown here not to occur except at higher concentrations of TG.

Apoptosis levels were quantified by TUNEL assay in SCs to determine whether an increasingly complex ECM affected the level of ER stress-induced apoptosis. Bovine chondrocytes in SCs are protected from ER stress-induced apoptosis after 4 days in culture (Fig. 8A). SCs treated with ER stressors immediately after formation (0 day) or after 1 day in culture (1 day) underwent much higher levels of apoptosis than controls. However, after 4 days and through 6 weeks of growth, ER stress treatment did not induce apoptosis to levels different from control levels (Fig. 8A). These data demonstrated that chondrocytes residing in a complex ECM respond differently to ER stressors than chondrocytes with little or no ECM. Representative images from quantified sections of control and –Glu-treated SCs are shown in Figure 8B (arrows indicate examples of TUNEL-positive cells, not all positive cells are indicated).

DISCUSSION

Tissue and/or organ cultures are increasingly used as models for hypothesis-driven basic science research. For example, Zheng et al. [2009] used vascular smooth muscle cells in organ culture to study changes in tensile stress through variation in phenotype of these cells. There are a multitude of models for cartilage tissue engineering being generated in attempts to create surgically implantable constructs as a treatment for degenerative joint diseases [Moutos and Guilak, 2009; Ng et al., 2009; Ragetly et al., 2010]. However, it is less common to utilize these models to examine differential responses to experimental conditions when cells are surrounded by a complex and highly organized 3D matrix as compared to monolayer culture. Applying an established model of bovine cartilage generated ex vivo [Nugent et al., 2010] toward answering fundamental questions of chondrocyte physiology is an important step forward in understanding the complex microenvironment of chondrocytes residing in joint articular cartilage in vivo.

The ER stress response is a specific, well-characterized set of pathways activated by cells in order to alleviate the stress and survive. The importance of ER stress in disease processes is supported by experimental data, including in cardiovascular diseases [Minamino and Kitakaze, 2009], neurological dysfunction [Cho et al., 2009], and connective tissue diseases [Boot-Handford and Briggs, 2010]. Our laboratory has previously described the ER stress response in primary and immortalized rat chondrocytes in monolayer culture [Yang et al., 2005]. In order to determine whether primary bovine chondrocytes respond differently to ER stressors while encapsulated within an organized 3D matrix, they were first characterized for their response in monolayer culture. Bovine chondrocytes in monolayer culture exhibited the typical ER stress response through the up-regulation of ER stress markers Grp78 and Gadd153 and down-regulation of extracellular matrix molecules aggrecan and collagen type II. One surprising difference to previous studies was the up-regulation of Bag-1, where it was previously shown to be down-regulated in response to ER stress in immortalized rat chondrocytes [Yang et al., 2007]. It is possible that the Myc oncogene responsible for immortalizing the rat chondrocytes [Horton et al., 1988] alters the Bag-1 response. While c-Myc has been shown to repress the ER stress response, its effect on Bcl-2 and/or Bag-1 expression is unknown [Barsyte-Lovejoy et al., 2004]. Bag-1 is a powerful pro-survival molecule and has been shown to act as a chaperone protein through interaction with Hsp70 and shuttling proteins to the proteasome for degradation [Takayama et al., 1997; Elliot et al., 2007]. This function, in addition to its anti-apoptotic properties, is consistent with up-regulation of Bag-1 under ER stress conditions, as shown here.

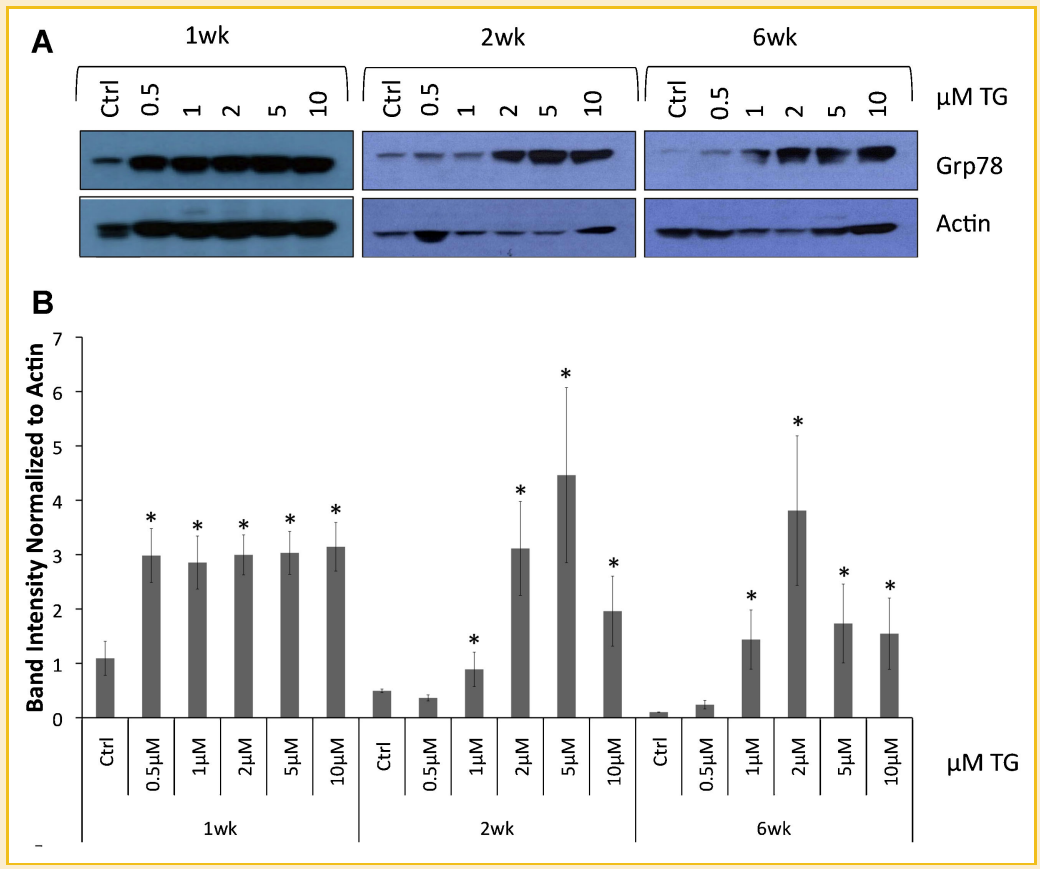

Fig. 7. A: The presence of a complex ECM prevented ER stress-induced up-regulation of Grp78 protein except at higher concentrations than those applied in monolayer, visualized via Western blot. SCs were grown in control medium for the time period indicated, then treated for 1 week with control medium (Ctrl) or thapsigargin (TG) at the indicated concentrations (N = 3). B: Quantification of band intensities of three independent experiments normalized to Actin (see Materials and Methods Section). Bars represent mean ± standard deviation of three independent experiments; one-way ANOVA of experimental samples to the associated control (*$P < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The data presented in this study suggest that ER stress may be occurring in chondrocytes under conditions of active synthesis and secretion of matrix molecules, as evidenced during the first several days in 3D control culture. This result suggests that ER stress may be a normal response of chondrocytes during phases of active synthesis and transport of proteins through the ER. In fact, it has been shown that mild ER stress is a regular occurrence in highly secretory cells such as the insulin-generating beta cell and is efficiently modulated by the UPR [Kennedy et al., 2010]. Our laboratory has previously linked the up-regulation of a large secreted molecule (collagen type VI) with ER stress in the pathology of osteoarthritis [Nugent et al., 2009]. The fact that chondrocytes in control SCs in this study up-regulated the chaperone protein Grp78 under phases of active synthesis but not Gadd153, a transcription factor associated with ER stress-induced apoptosis, suggests that while the chondrocytes are experiencing ER stress, they will not terminate in apoptosis. Therefore the chondrocytes under these conditions restored homeostasis by re-creating the proper ECM environment and altering chaperone expression to deal with the stress on the ER caused by the increased throughput.

In the presence of ER stressors, bovine chondrocytes in SCs responded in ways both similar and different to bovine chondrocytes in monolayer culture. Chondrocytes in SCs down-regulated mRNA coding for matrix molecules aggrecan and collagen type II, and up-regulated mRNA for the ER stress marker Grp78, even after 6 weeks in culture (and therefore after extensive ECM formation). In this instance, chondrocytes in SCs responded similarly to chondrocytes in monolayer, and the presence of ECM did not affect gene expression. Comparing these data to protein expression at 2 and 6 weeks in culture, Grp78 protein was not up-regulated until SCs were treated with higher concentrations of ER stressors than monolayer. This result suggests that there are post-transcriptional controls over Grp78 protein expression. In fact, it has been shown that an increase in Grp78 mRNA does not necessarily result in increased protein expression, and that Grp78 translation efficiency is increased upon ER stress [Gülow et al., 2002]. In addition, chondrocytes in SCs did not up-regulate Gadd153 after exposure to ER stressors at any time-point, an observation suggesting that they were protected from transitioning into a terminal apoptotic mode. Kinetically, it has been shown that up-regulation of chaperone proteins occurs very quickly in response to ER stressors in a time series profile of protein expression [Mintz et al., 2008]. This quick response is presumably in place to try to alleviate stress on cells prior to initiating apoptotic pathways.

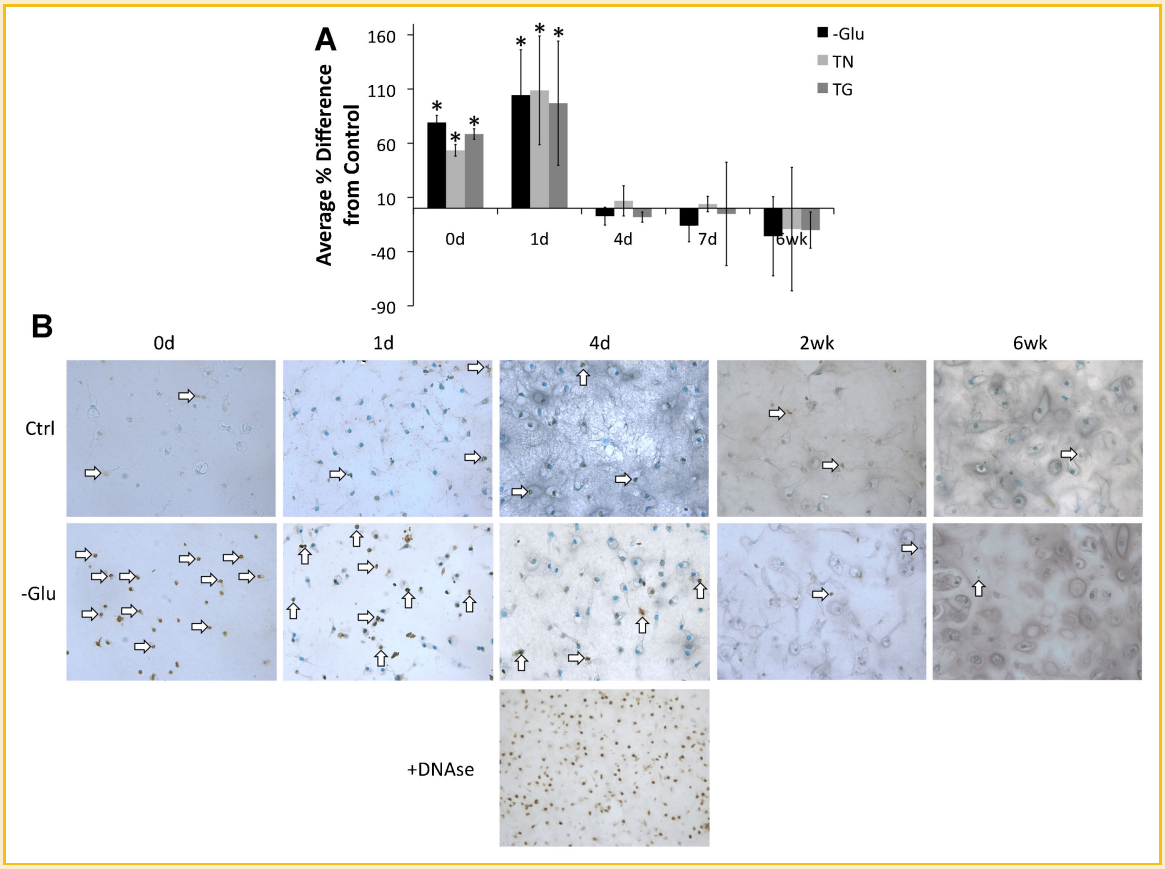

Fig. 8. The presence of a complex ECM conferred protection from ER stress-induced apoptosis. A: SCs were grown in control medium for the time period indicated by the *x*-axis, then treated for 1 week with control medium, glucose withdrawal (–Glu), 1 μg/ml tunicamycin (TN), or 0.5 μM thapsigargin (TG) where indicated. Apoptotic cells were quantified for five fields of view across three independent experiments following histological TUNEL assay; data represent the mean % apoptosis different from control (see Materials and Methods Section); one-way ANOVA analyzation of experimental samples to the associated control ($^{*}P < 0.01$). B: Representative images from the TUNEL-stained histological sections that were quantified in (A). SCs were grown in control medium for the time period indicated, then treated for 1 week with control medium (Ctrl) or glucose withdrawal (–Glu) as indicated. Arrows represent examples of counted cells (not all TUNEL-positive cells are marked with arrows). As a positive control, DNAse was applied (+DNAse, see Materials and Methods Section).

ER stress resulted in the loss of metachromatic ECM in SCs except after 6 weeks of culture. We hypothesized that chondrocytes were up-regulating the aggrecanase ADAMTS-5 in response to ER stress, but this was not the case. It is possible that disrupted homeostasis is preventing the chondrocytes from maintaining their extracellular matrix, and that after 6 weeks of growth the SCs had accumulated enough matrix that the loss was not easily visualized histologically. However, it may also be possible that a certain level of matrix accumulation is protective against ER stress-induced ECM degradation. The loss of metachromatic ECM may be the result of normal turnover, and the down-regulation of matrix molecules as shown by RT-PCR is preventing the accumulation of new ECM. Loss of metachromatic ECM is characteristic of osteoarthritis [Yagi et al., 2005]. However, this loss is often attributed to the up-regulation of aggrecan degrading enzymes such as ADAMTS-5, which was not the case here [Echtermeyer et al., 2009]. Future studies will analyze culture media for biochemical products under control and ER stress conditions to determine whether the level of matrix breakdown is more than or similar to control cultures.

The expression of Bag-1 in response to ER stress in SCs was interesting to compare across different lengths of time in culture: there was no detectable up-regulation of Bag-1 until after 4 days of growth prior to ER stress treatment. Four days of growth is the same time at which chondrocytes in SCs are protected from ER stress-induced apoptosis. We hypothesize that the up-regulation of Bag-1 is aiding in that protection, as it binds to Bcl-2 and enhances the effectiveness of this anti-apoptotic protein. Bcl-2 mRNA was up-regulated early, after 0–4 days of growth prior to ER stress treatment. This suggests that chondrocytes up-regulated Bcl-2 quickly to protect against apoptosis (unsuccessfully) while up-regulation of Bag-1 occurred more slowly, but the synergistic action between these molecules, in addition to other protections afforded by the presence of ECM, was enough to protect cells against ER stress-induced apoptosis after 4 days of growth. It has been shown in photoreceptor cells that Bag-1 expression alone was not enough to prevent apoptotic retinal degeneration, but the co-expression of Bcl-2 and Bag-1 synergistically prevented apoptosis [Eversole-Cire et al., 2000]. To determine whether or not the up-regulation of Bcl-2

and Bag-1 are protecting cells from ER stress-induced apoptosis, future work can test this hypothesis using siRNA knock-down of Bcl-2 and Bag-1 (alone and in combination) and determining whether this protection is still afforded after ER stress treatment. Knock-down of Bag-1 has been shown to induce apoptosis in immortalized chondrocytes [Yang et al., 2007], and further studies would require measuring basal levels of apoptosis that Bag-1 knock-down would cause in primary bovine chondrocytes prior to seeding into the collagen gels.

The specific mechanism by which the ECM alters the chondrocyte response to ER stressors needs to be explored in detail. An ideal experiment would be to allow chondrocytes to grow and maintain a fully mature ECM, somehow interrupt the associations (and therefore the inside-out and outside-in signaling) between the chondrocyte and the ECM, and subsequently determine the resulting cellular response to ER stressors. Another route for examining the mechanisms by which the ECM alters the chondrocyte ER stress response would be to determine whether the specific signaling pathways associated with chondrocyte-matrix binding under homeostatic conditions are somehow linked to the ER stress pathway by altering the expression of chondrocyte-specific integrins. Active integrins in adult articular chondrocytes (with their binding molecule in parentheses) include: $\alpha1\beta1$ (collagens II and VI), $\alpha10\beta1$ (collagen II), $\alpha5\beta1$ (fibronectin), $\alpha3\beta1$ (secondary collagen and fibronectin receptor), NG2 (collagen VI), and CD44 (hyaluronic acid) [Loeser, 2000]. It would be possible to knock down systematically the expression of each of these and determine whether or not its absence, alone or in combination with another integrin, affects the chondrocyte ER stress response in a similar way as described in this study.

Finally, it is important to fully elucidate the specific ER stress pathway(s) utilized by bovine chondrocytes, and whether it differs between monolayer cultures and SCs. Under homeostatic conditions, Grp78 is bound to the ER-lumenal surface of three ER membrane-associated molecules: inositol-requiring enzyme-1 (IRE-1), pancreatic ER eukaryotic translation initiation factor (eIF)-2α kinase (PERK), and activating transcription factor-6 (ATF6). Upon accumulation of misfolded proteins, Grp78 dissociates from these membrane-bound molecules and binds to the accumulated proteins in order to perform its functions while IRE-1, PERK, and ATF-6 translocate to other cellular compartments in order to influence gene transcription to attenuate the stress. Future studies will determine whether one of these pathways is more heavily utilized by bovine chondrocytes, whether this is altered by the presence of ECM, and whether the specific stressors used here (–Glu, TN, and TG) act through a preferred pathway in these cells.

The work described in this study emphasizes the importance of experimental paradigms that mimic the environment in vivo. Monolayer studies in vitro can tell us much about the biochemistry of specific signaling pathways. However, in order to draw conclusions about the physiological response of cells residing in all types of tissues in vivo, more comprehensive studies using model systems of higher levels of complexity than monolayer culture such as the system described here or live animals should be employed.

ACKNOWLEDGMENTS

The authors would like to thank Mahan's Packing Company in Bristolville, Ohio for supplying all bovine knee samples.

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