

Hyaluronan Synthesis and Myogenesis

A REQUIREMENT FOR HYALURONAN SYNTHESIS DURING MYOGENIC DIFFERENTIATION INDEPENDENT OF PERICELLULAR MATRIX FORMATION*

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Background: The role endogenously synthesized hyaluronan plays in myogenesis is not yet known.

Results: Hyaluronan synthase genes were expressed during skeletal muscle growth and regeneration; inhibiting these synthases prevents myoblast differentiation and fusion.

Conclusion: Endogenous hyaluronan synthesis is required for myogenic differentiation.

Significance: The necessity for hyaluronan in myogenesis has implications when considering promoting muscle growth or regeneration.

Exogenous hyaluronan is known to alter muscle precursor cell proliferation, migration, and differentiation, ultimately inhibiting myogenesis *in vitro*. The aim of the current study was to investigate the role of endogenous hyaluronan synthesis during myogenesis. In quantitative PCR studies, the genes responsible for synthesizing hyaluronan were found to be differentially regulated during muscle growth, repair, and pathology. Although all *Has* genes (*Has1*, *Has2*, and *Has3*) were differentially regulated in these models, only *Has2* gene expression consistently associated with myogenic differentiation. During myogenic differentiation *in vitro*, *Has2* was the most highly expressed of the synthases and increased after induction of differentiation. To test whether this association between *Has2* expression and myogenesis relates to a role for *Has2* in myoblast differentiation and fusion, C2C12 myoblasts were depleted of *Has2* by siRNA and induced to differentiate. Depletion of *Has2* inhibited differentiation and caused a loss of cell-associated hyaluronan and the hyaluronan-dependent pericellular matrix. The inhibition of differentiation caused by loss of hyaluronan was confirmed with the hyaluronan synthesis inhibitor 4-methylumbelliferone. In hyaluronan synthesis-blocked cultures, restoration of the pericellular matrix could be achieved through the addition of exogenous hyaluronan and the proteoglycan versican, but this was not sufficient to restore differentiation to control levels. These data indicate that intrinsic hyaluronan synthesis is necessary for myoblasts to differentiate and form syncytial muscle cells, but the hyaluronan-dependent pericellular matrix is not sufficient to support differentiation alone; additional hyaluronan-dependent cell functions that are yet unknown may be required for myogenic differentiation.

Hyaluronan is an acidic polysaccharide composed of repetitive units of *N*-acetylglucosamine and glucuronic acid (1). Hyaluronan is classified as a glycosaminoglycan; however, unlike the glycosaminoglycan-containing proteoglycans, it lacks a protein core. The molecular mass of hyaluronan can vary from 20 kDa to 10 MDa, depending on the chain length, and different cellular functions are ascribed to these different molecular masses (2). Hyaluronan can be found associated with cells in a pericellular coat surrounding cells as well as mobile in solution. The physical properties, including the hygroscopic nature of hyaluronan, can convey mechanical support within connective tissue, which is especially important within synovial joints. Broadly, hyaluronan is linked to numerous tissue functions, including wound healing, inflammation, angiogenesis, and fibrosis (3).

In postnatal tissues, hyaluronan is found throughout the body; however, it is predominantly localized within the skin (~50% of total hyaluronan), skeleton (~30% of total hyaluronan), and skeletal muscle (~10% of total hyaluronan) (4). In rats, hyaluronan was found in all muscles that were examined, and it was located in the connective tissue layers surrounding myofibers (the endomysium, perimysium, and epimysium) (5, 6) and also within the mechanoreceptor spindle fibers (7). Hyaluronan is also associated with the functions of motility, migration, proliferation, and differentiation of cells from the mesenchymal lineage, including osteoblasts (8), chondrocytes (9), fibroblasts (10), and adipocytes (11), as well as the skeletal muscle-forming myoblasts (12–14).

The majority of studies examining the effect of hyaluronan on the differentiation and fusion of chicken-derived myoblasts concluded that myotubes were not as readily formed when myoblasts were cultured on a substratum consisting of hyaluronan compared with a collagen-I substratum (12, 14, 15). Subsequent studies suggested that myoblasts lose their hyaluronan-dependent pericellular matrix when fusing into myotubes and that this is necessary to allow membranes of adjacent myoblasts to fuse (16). Together, this work suggested that myoblast

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differentiation and fusion can be modulated by hyaluronan, where excess hyaluronan inhibits differentiation and a reduction in hyaluronan is required for fusion into syncytial muscle cells. A bimodal change in myoblast motility and decrease in the ability of newt myoblasts to differentiate has also been observed when cultured on a hyaluronan substratum (17). Although these studies suggest that exogenous hyaluronan can inhibit myoblast fusion, contrasting studies have found that a hyaluronan substratum coated on Chronoflex (polycarbonate polyurethane) can enhance myogenic differentiation and fusion compared with uncoated Chronoflex (18). Nevertheless, although these few studies have examined myoblast responses to exogenous hyaluronan, none have examined how endogenously synthesized hyaluronan influences myoblast behavior.

Hyaluronan synthase 1 (HAS1), 2 (HAS2), and 3 (HAS3) are the three enzymes found in mammals that synthesize hyaluronan (19, 20). The different synthases have been suggested to produce different size hyaluronan chains such that HAS2 can typically produce the largest chains, often greater than 2 MDa, whereas HAS1- and HAS3-synthesized hyaluronan chains are often shorter and range from 100 kDa to 2 MDa (21, 22). Based on mouse knock-out studies of the three isoforms, it is apparent that *Has2* is a crucial developmental gene. Cardiac morphogenesis of embryos lacking *Has2* expression was abnormal, and cardiac endothelial cell migration and transition into mesenchyme were prevented (23). *Has2*^{-/-} mice did not survive beyond embryonic day 10 (E10),² whereas the individual and combined *Has1*^{-/-} and *Has3*^{-/-} genotypes were not lethal. This indicates that *Has2* is crucial for development and cannot be compensated for by the other HAS enzymes.

Increased *Has2* expression has been observed in the longissimus dorsi muscles of sheep with the Callipyge mutation when compared with wild type (24). The longissimus dorsi muscles of Callipyge sheep display a high degree of hypertrophy compared with wild type, which appears to occur postnatally. *Has2* up-regulation occurs neonatally, prior to detection of hypertrophy, suggesting that *Has2* could be associated with the cause of hypertrophy in Callipyge muscles. Very recent studies have also reported increases in hyaluronan synthesis in a model of compensatory hypertrophy (25). Increased expression of *Has1* and *Has2* mRNAs was observed in this synergistic ablation model of hypertrophy, supporting the possibility of HAS2 mediating muscle growth and hypertrophy.

Herein we examine *Has* expression during muscle development, postnatal regeneration following injury, dystrophic pathology, and *in vitro* myogenesis. We hypothesize that *Has2* may be the primary *Has* gene involved in syncytial muscle cell formation and muscle growth and repair. The requirement for hyaluronan synthesis during *in vitro* myogenesis is investigated with the hyaluronan synthesis inhibitor 4-methylumbelliferone and siRNA-mediated knockdown of *Has2* in C2C12 myoblasts induced to differentiate into syncytial muscle cells. We also contrast myogenic differentiation studies abolishing endogenously synthesized hyaluronan with those adding exogenous

hyaluronan in order to provide a comprehensive evaluation of the role hyaluronan and, more specifically, *Has2* play in the formation of muscle cells.

EXPERIMENTAL PROCEDURES

Mice—Mouse experiments were carried out in strict accordance with National Health and Medical Research Council NHMRC guidelines for the care and use of animals in research under the approval of the Animal Welfare Committee, Deakin University, ethics number A41/2009 and the Faculty of Veterinary Science AEC, University of Melbourne, approval number 081090. Six-week-old C57/Bl6J (Animal Resources Centre, Western Australia) female mice were mated with male mice after 1700 h, and conception was confirmed before 0900 h the next morning by observation of vaginal mucus. The morning of conception was designated as gestational age 0.5 days (E0.5). Timed embryos were acquired at E12.5, E13.5, E14.5, E15.5, E16.5, and neonatal (day of birth, 21 days) time points, whereby proximal hind limbs of embryonic ages E12.5–E14.5 or proximal hind limb muscles of older embryos (dissected away from hard tissue) were collected and stored in TRIzol at –80 °C until further processing.

Injections of notexin (obtained from Latoxan, France) into the tibialis anterior muscles of adult mice were performed as described previously (26). In brief, anesthetized C57BL/6 mice received a single intramuscular injection of 50 μl of 10 μg/ml notexin parallel along the tibia into the tibialis anterior muscle. Following notexin injection, at 1, 3, 5, 7, 10, and 14 days, the tibialis anterior muscles were sampled. Five mice were also sampled without notexin injection as an uninjured control. Tibialis anterior muscles were snap-frozen in liquid nitrogen in order to perform RNA extraction for quantitative PCR (qPCR) studies. The diaphragm muscles of C57BL10/*mdx* and C57BL10/*ScSn* mice at 2, 3, 6, 12, and 24 weeks of age were excised and snap-frozen to perform qPCR. Histology samples were obtained by freezing tragacanth-mounted muscles in liquid nitrogen-cooled isopentane and cutting 10-μm-thick sections with a cryostat onto glass slides.

Cell Culture and Reagents—C2C12 myoblast lines were obtained from the American Type Culture Collection and maintained in growth medium consisting of 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM). To induce differentiation as cells were approaching confluence, growth medium was replaced with differentiation medium containing 2% horse serum in DMEM. Differentiation medium was replenished every 24 h until the end point of the experiment.

4-Methylumbelliferone (4MU) was obtained from Sigma-Aldrich and dissolved in DMSO at a concentration of 1 mM and subsequently diluted out into medium to the described concentrations. 4MU treatments in differentiation experiments were performed by adding 4MU to the growth medium, which was then incubated with myoblasts for 24 h prior to the induction of differentiation, at which point the medium was removed and replaced with 4MU-containing differentiation medium that was subsequently replenished every 24 h until the end of the experiment.

² The abbreviations used are: En, embryonic day *n*; Pn, postnatal day *n*; qPCR, quantitative PCR; 4MU, 4-methylumbelliferone; HABP, hyaluronan-binding protein; MRF, myogenic regulatory factor.

TABLE 1
Oligonucleotide primers

Gene name	Gene ID	Forward (5'–3')	Reverse (5'–3')
<i>Bgn</i>	12111	CACCTGGACCACAACAAA	TCCGAATCTGATTGTGACCTA
<i>Cd44</i>	12505	CGCTACAGCAAGAAGGGCGAGT	GCAATGGTGGCCAAGGTGCTC
<i>Cdkn1a</i>	12575	GCAGACCAGCCTGACAGATTC	TTCAGGGTTTTCTCTTCAGAAAG
<i>Col1a1</i>	12842	CGGAGAAGAAGGAAAACGAG	CTTCACCAGGAGAACCTTTGG
<i>Dcn</i>	13179	CGCATCTCAGACACCAACAT	TTGGTGATCTTGTGTGCCATC
<i>Fn1</i>	14268	GAGCGCCCTAAAGATTCCAT	CTCCACTGTGCGCAATCTT
<i>Has1</i>	15116	TTCCACTGTGTGTCTGCAT	TGTACCAGGCCTCCAAGAAC
<i>Has2</i>	15117	GGGACCTGGTGAGACAGAAG	ATGAGGCAGGGTCAAGCATA
<i>Has3</i>	15118	TCCCCAAGTAGGAGGTGTTG	TTGCACACAGCCAAAGTAGG
<i>Hprt</i>	15452	GATTAGCGATGATGAACCAGGTT	TCCAAATCCTCGGCATAATGAT
<i>Hyal1</i>	15586	CCGTAATGCCCTACGTCCAG	GCCTGGCATGATTCTCTGGTA
<i>Hyal2</i>	15587	AGCCCGCAACTTTGTCAAGTTT	GAGTCCTCGGGTGTATGTGG
<i>Lama2</i>	16773	ACGCCAAAGATGATGAGGTC	GCACTTGGTCTCCCATTTGAT
<i>Myf5</i>	17877	CTGTCTGGTCCCAGAAAGAAC	AGCTGGACACGGAGCTTTTA
<i>Myf6</i>	17878	GGCTGGATCAGCAAGAGAAG	CCTGGAATGATCCGAAACAC
<i>Myod1</i>	17927	TACAGTGGCGACTCAGATGC	TAGTAGCGGGTGTCTAGCC
<i>Myog</i>	17928	AGTGAATGCAACTCCCACAG	ACGATGGACGTAAGGGAGTG
<i>Pcna</i>	18538	GGCCGTGAACCTCACCAGCA	CGTGCAAATTCACCCGACGGC
<i>Ppia</i>	268373	GGCCGATGACGAGCCC	TGCTTTTGGAACTTTGTCTGCAA
<i>Vcan</i>	13003	ACCAAGGAGAAGTTTCAGCA	CTTCCCAGGTAGCCAAATCA

Hyaluronan was obtained from Sigma-Aldrich (H5388) and was derived from rooster comb, for which the supplier expects an average molecular mass in the range of 1–4 MDa. Hyaluronan was reconstituted in PBS and added to culture medium at the described concentrations with dilutions of PBS as vehicle control.

RNA Extraction, Reverse Transcription, and Quantitative PCR—RNA was extracted from developing proximal hind limb muscles as per the manufacturer’s protocol using TRIzol (Invitrogen), and equal amounts of total RNA (up to 1 μg) were reverse-transcribed with either the Superscript III reverse transcriptase kit (Invitrogen) or iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and relevant primers for the genes of interest. The Quant-iT OliGreen ssDNA assay kit (Invitrogen) was used to quantitate total cDNA as per the manufacturer’s protocol.

RNA was extracted from snap-frozen adult muscle tissue and cells by homogenizing in 1 ml of Tri-reagent, followed by the addition of chloroform to retrieve the RNA-containing aqueous layer. The aqueous layer was added in equal volume to a solution comprising one part 90% ethanol and one part SV lysis solution from the SV total RNA isolation system (Promega). The resulting solution was then passed through the SV columns and DNase-treated. The RNA was eluted from the columns, and 1 μg of RNA per sample was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) to produce cDNA. Real-time qPCRs were performed on a LightCycler 480 (Roche Applied Science), and the cycle thresholds (*Ct*) were calculated. Expression data were derived from the *Ct* and normalized to the housekeeper hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) for the *in vivo* studies and peptidylprolyl isomerase A (*Ppia*) for *in vitro* studies. These were the most appropriate housekeepers because they possessed the least variability across all samples within the study compared with other housekeepers tested, including glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β-actin (*Actb*), using the “Bestkeeper” Excel-based tool (27). Oligonucleotide primer sequences used are presented in Table 1 along with the PubMed Gene ID number for identification.

In histograms, qPCR data are displayed as the mean of normalized expression in order to show linear levels of transcripts of interest normalized to the housekeeper using the equation, normalized expression = $(A^x)/(B^y)$, where *A* represents the efficiency of amplification of the housekeeper, *B* is the efficiency of the gene of interest, *x* is the housekeeper *Ct*, and *y* is the gene of interest *Ct*, respectively.

Immunofluorescence—A biotinylated hyaluronan-binding protein (G1 domain of aggrecan) was kindly provided by Amanda Fosang (Murdoch Children’s Research Institute) and was used to label hyaluronan as described previously (28). Cultures and tissue sections were initially fixed in 4% formaldehyde containing 1% hexadecylpyridinium chloride monohydrate (Sigma-Aldrich) for 20 min at room temperature. They were then washed three times with PBS. The hyaluronan-binding protein (HABP) was diluted in PBS to 5 μg/ml and added to the cultures for 60 min at room temperature. After this incubation, samples were washed with three changes of PBS. The HABP was detected with an AlexaFluor-594 streptavidin conjugate diluted 1:500 in PBS (Invitrogen). Samples were counterstained with DAPI. Incubation with 200 units/ml hyaluronidase from *Streptomyces hyalurolyticus* for 3 h at 37 °C was performed to demonstrate the specificity of the binding protein for hyaluronan. For detection of laminin and desmin, the rat anti-laminin α-2 (4H8-2) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-desmin (DE-U-10) antibody (Sigma-Aldrich) were used.

The fusion index in differentiated C2C12 cultures, which is the percentage of nuclei contained within syncytial myotubes, was determined by immunofluorescence for desmin, a protein highly enriched in syncytial myotubes. Cells were fixed with 2% (w/v) paraformaldehyde, washed, and subsequently incubated with the primary antibody, mouse anti-desmin, and subsequently probed with an anti-mouse AlexaFluor-488 secondary antibody.

Transfection of C2C12 Myoblasts—C2C12 cells were transfected with 50 pmol of *Has2* Stealth Select RNAi siRNA (MSS236746; Invitrogen) in 50 μl of Opti-MEM and 1 μl of Lipofectamine 2000 (Invitrogen) for 6 h in serum- and antibiotic-free medium (0.5 ml in 24-well plates), and then an addi-

tional 0.5 ml of growth medium was added to each culture. Cells were cultured overnight before all medium was removed and replaced with normal growth medium (10% FBS). Controls were instead transfected with Stealth RNAi siRNA Negative Control Lo-GC (12935-200, Invitrogen).

C2C12 cells were transfected with the versican construct, which was kindly provided by Suneel Apte, by a similar method. A ratio of 0.5 μg of plasmid DNA to 1 μl of Lipofectamine 2000 in 50 μl of Opti-MEM was used to transfect each well of a 24-well plate by seeding 10,000 cells directly into the DNA and Lipofectamine 2000 mix-containing wells.

Creatine Kinase Activity Assay—Creatine kinase activity from lysed myoblast cultures was determined as described previously (29). In brief, cultures were lysed with 150 μl of lysis buffer containing 40 mM MES buffer, 50 mM Trizma base, 1% (v/v) Triton X-100, and protease inhibitors. Insoluble material in the cellular lysate was pelleted, and 10 μl of the supernatant was combined with 200 μl of CK-NAC reagent (Thermo Scientific). Creatine kinase activity was measured by the change in absorbance at 340 nm over 3 min (20-s intervals) at 37 °C. The change in absorbance was converted to activity in units/liter based on the molar absorption coefficient of NADH at 340 nm and normalized to DNA content.

Total DNA content was assayed using SYBR Safe DNA gel stain (10,000 \times) (Invitrogen) diluted 1:500 with water. Diluted SYBR Safe (1 μl) was combined with 19 μl of cell lysate or genomic DNA standards (0.78–100 $\mu\text{g}/\text{ml}$) into a black 384-well plate, and the SYBR fluorescence was quantitated on the Paradigm Detection Platform (Beckman Coulter).

Pericellular Exclusion Assay—The size of the pericellular matrix surrounding live C2C12 myoblasts was determined by a particle exclusion assay similar to that described previously (30, 57). C2C12 myoblasts transfected to express GFP were seeded at a density of 5,000 cells/well in 6-well plates, and after 2 days of either 4-methylumbelliferone or *Has2* siRNA, the assay was performed. Lyophilized sheep erythrocytes (Sigma-Aldrich) were resuspended in 10 ml of PBS, postfixed with 1.5% (w/v) paraformaldehyde in PBS, washed, and finally resuspended again in 10 ml of PBS. An aliquot (30 μl) of this cell suspension (containing $\sim 2 \times 10^7$ erythrocytes) was added to the cultures, and the erythrocytes were allowed to settle for ~ 10 min. The pericellular exclusion area was visualized by imaging the GFP-positive C2C12 cells and the red autofluorescent erythrocytes on an inverted microscope. Binary masks of these images were generated in ImageJ in order to measure the area occupied by the C2C12 cells plus the pericellular space excluding erythrocytes (from which the exclusion area was derived). The size of the myoblasts and the space between the cell membrane of the myoblast and the erythrocytes surrounding them were measured in a blinded fashion with 50 randomly selected cells measured per treatment group. Hyaluronidase digestion was also performed in cultures with 0.5 unit/ml hyaluronidase from *S. hyalurolyticus* (Sigma-Aldrich) in serum-free medium for 1 h prior to the exclusion assay as a control.

Statistical Analysis—GraphPad Prism software was used for statistical analysis. Student's *t* tests were performed when comparing a single treatment with control. All histograms represent the mean and the S.E. One-way analysis of variance was used to

compare multiple treatments with control, and two-way analysis of variance was used when additional variables were considered. A 95% confidence interval was accepted, where $p \leq 0.05$ was deemed significant and is denoted on histograms with an *asterisk*. The Relative Expression Software Tool was used to analyze statistical differences between *Ct* values from qPCR (31).

RESULTS

Hyaluronan Synthase Gene Expression Is Regulated during Myogenesis—C2C12 myoblasts were induced to differentiate and fuse into multinucleate myotubes over a period of 72 h in differentiation medium. During this time, mRNA was extracted, and *Has* transcript levels examined by qPCR (Fig. 1A). *Has1* expression was at a maximum in undifferentiated nearly confluent myoblasts and decreased as differentiation and fusion proceeded. *Has2* expression demonstrated an inverse trend, where expression was lowest in undifferentiated myoblasts but increased as differentiation and fusion proceeded. *Has3* expression did not appear to fluctuate throughout the time course.

Has mRNA expression was examined in the developing (E12.5–E15.5), neonatal, and postnatal (P10, P21, and adult) mouse limb muscles (Fig. 1B). *Has1* transcript appeared to have increased at E13.5 through birth in comparison with E12.5. *Has2* transcript displayed a sharp increase, peaking at E14.5, and decreased subsequently to very low levels in adult muscle. *Has3* transcript demonstrated a peak in neonatal muscle compared with developing and adult muscle. Low levels of all three transcripts were seen in adult muscle relative to neonatal (*Has3*) and developing muscle (*Has1* and *Has2*), indicating potential roles in muscle development. *Has2* was the most highly expressed gene relative to housekeeper, however, and showed the most dramatic increase at E14.5, coincident with the approximate onset of secondary myogenesis.

Notexin is frequently used as a model of postnatal muscle regeneration because it consistently and reproducibly generates whole muscle changes associated with muscle breakdown, necrosis, inflammation, myoblast growth, differentiation, and the regeneration of syncytial muscle cells (32), processes that can be distinguished temporally in this model. *Has1*, *Has2*, and *Has3* gene expression were all increased following notexin injection compared with uninjured control (day 0; Fig. 1C). Both *Has1* and *Has3* expression followed a strikingly similar trend, with a large peak at day 1, which is typically coincident with large numbers of neutrophils and increased cytokine expression in the regenerating muscle. *Has2* expression, however, increased progressively following notexin injection, coincident with increasing myoblast fusion and myotube maturation with a peak around day 10.

The expression of *Has* genes was also examined in the diaphragms of *mdx* mice, a model of human dystrophinopathies (Fig. 1D). Compared with the wild type (C57BL10/ScSn or BL10), *mdx* mice demonstrated significantly increased mRNA levels of *Has1* at 3 weeks of age and *Has2* at 6 and 12 weeks of age. No significant differences in *Has3* expression between genotypes were observed.

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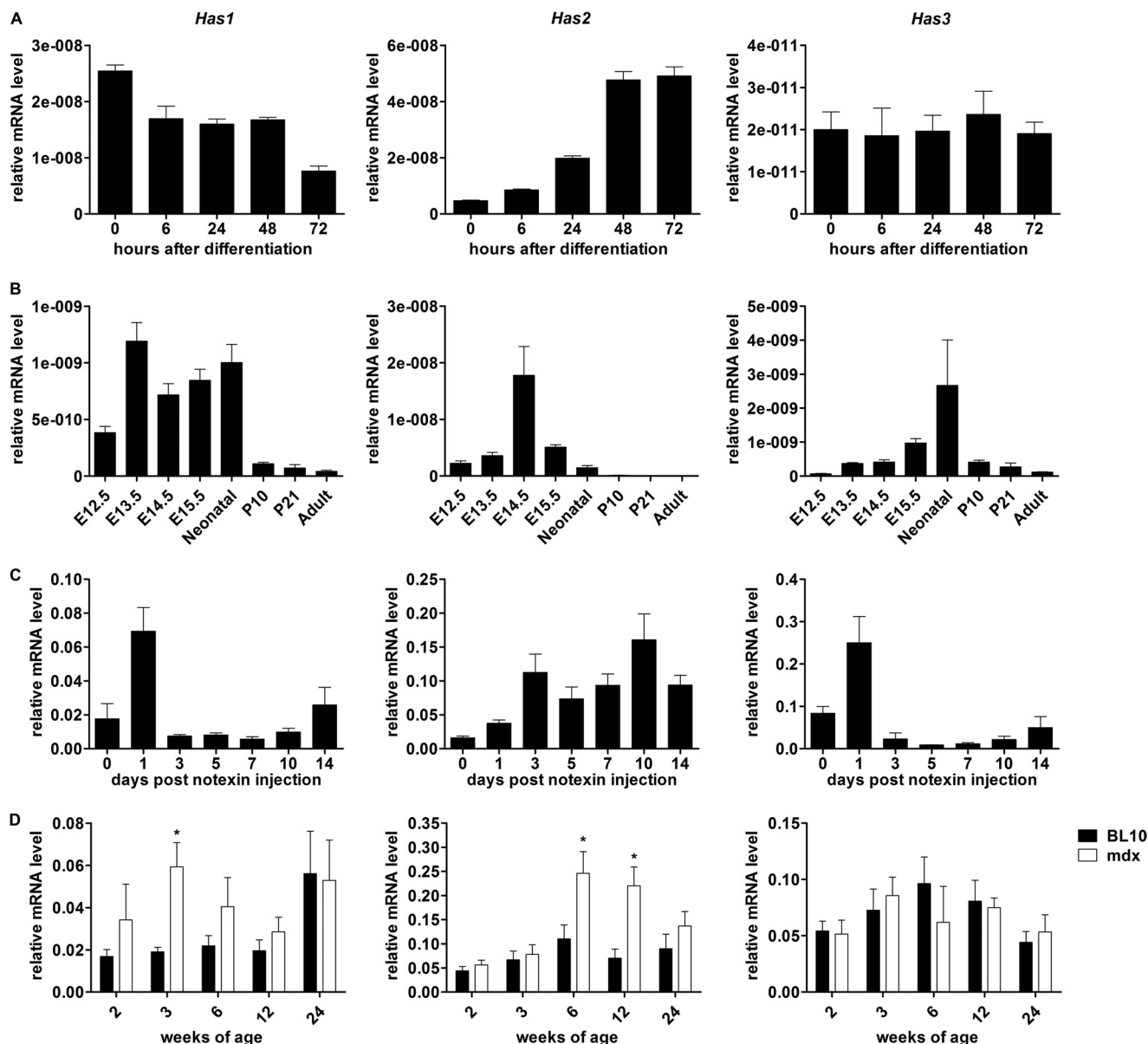


FIGURE 1. **Has gene expression during skeletal muscle growth and remodeling.** Mean normalized expression of relative mRNA levels of *Has1*, *Has2*, and *Has3* in differentiating C2C12 myoblasts after the addition of differentiation medium (A), developing and adult limb muscles (B), notexin-injured tibialis anterior (C), and *mdx* and wild type BL10 diaphragms (D). *, $p < 0.05$ for comparisons between genotypes in D ($n = 8$). Error bars, S.E.

Localization of Hyaluronan in Skeletal Muscle Tissue and Cultured Myoblasts—We used the previously described hyaladherin G1 domain of aggrecan to investigate the presence and localization of hyaluronan in normal and notexin-injured muscle as well as in cultured C2C12 myoblasts. A biotinylated G1 domain, herein referred to as HABP, bound to hyaluronan in tissue and culture samples and was detected with streptavidin fluorescence and co-stained with DAPI and laminin to highlight nuclei and the endomysium of myofibers. As expected, hyaluronan appeared to localize to the extracellular spaces surrounding myofibers in normal uninjured muscle. HABP fluorescence co-localized with laminin in the endomysium (Fig. 2A) but was also present in the perimysium and epimysium (data not shown). Control sections incubated with hyaluronidase displayed no specific fluorescence, highlighting the specificity of HABP fluorescence for

detection of hyaluronan. Concordant with previous descriptions, we also found intense HABP reactivity within muscle spindle fibers (7) but also intermittently throughout nerve bundles, which could be easily determined by their unique histological appearance (Fig. 2B).

In notexin-injured muscle, however, intense HABP reactivity was observed 3 days after notexin injection in necrotic fibers, which were being invaded by numerous inflammatory cells, and was also present in the surrounding mononucleated cells (Fig. 2C, top). Following the formation of nascent myotubes 7 days after notexin injection, HABP reactivity was present around the centrally nucleated nascent myotubes as well as surrounding the mononucleated cells between fibers, indicating that the mononucleated myoblasts that will subsequently fuse into myotubes are likely to be surrounded by a hyaluronan-containing matrix (Fig. 2C, bottom).

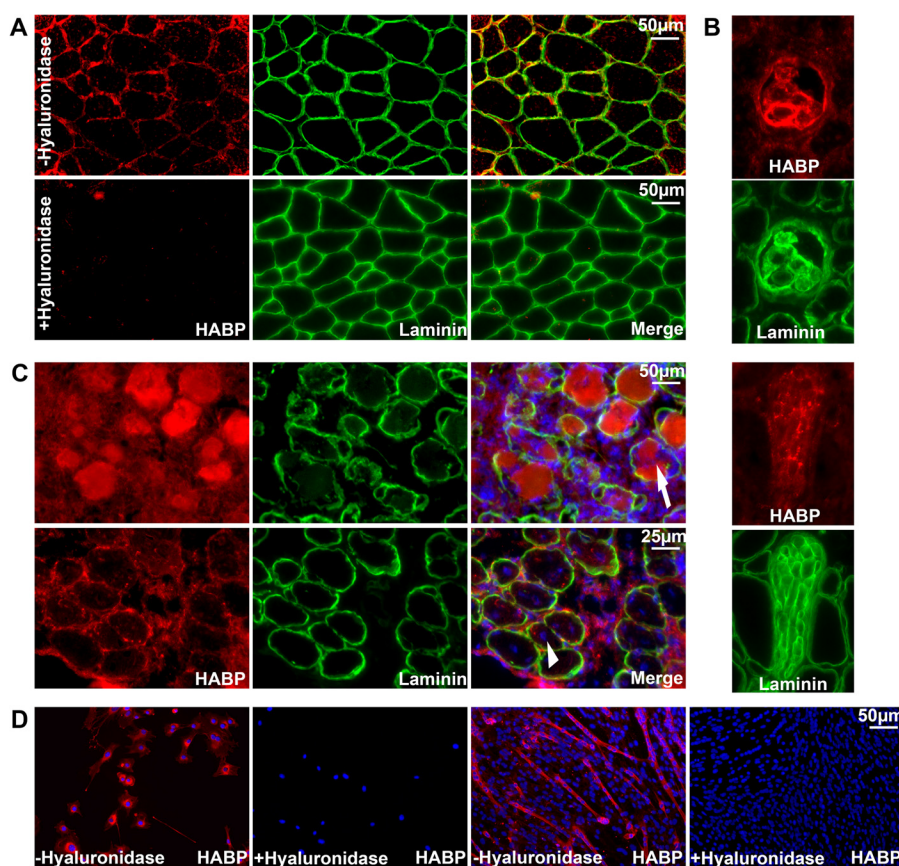


FIGURE 2. Localization of hyaluronan in skeletal muscle tissue and cultured myoblasts. Hyaluronan was detected by biotinylated HABP probed with red fluorescent conjugated streptavidin. Laminin α -2 immunofluorescence was also performed in green to highlight the endomysium surrounding muscle fibers (A–C). A, localization of hyaluronan or laminin surrounding the fibers in normal adult muscle without (top) or with hyaluronidase pretreatment (bottom) to demonstrate the specificity of the reactivity for hyaluronan. B, strong HABP reactivity in muscle spindle fibers (top) and nerve bundles (bottom). C, localization of hyaluronan in muscle following notexin injection at 3 days (top) in necrotic fibers invaded by inflammatory cells (indicated with an arrow) and 7 days (bottom) surrounding the central nuclei of nascent myotubes (indicated by an arrowhead). D, cell-associated hyaluronan was detected in myoblasts (left) and myotubes (right).

Hyaluronan was indeed associated with mononucleated myoblasts in culture. HABP reactivity in C2C12 cultures was found associated with myoblasts; this was subsequently lost by incubation with hyaluronidase (Fig. 2D, left). Hyaluronan was also associated with long multinucleate myotubes formed by culturing myoblasts to confluence and switching to a low serum differentiation-inducing medium (Fig. 2D, right).

Knockdown of *Has2* during *In Vitro* Myogenesis Reduces Hyaluronan Production and Abolishes the Pericellular Matrix—All three HAS proteins are expressed by myoblasts in skeletal muscle (25), but the consistent relationship observed herein between increased *Has2* transcript expression and myogenesis both *in vivo* and *in vitro* suggested that *Has2* is intimately involved in the process by which myoblasts differentiate and fuse to form syncytial muscle cells. To test this hypothesis, we performed *Has2* knockdown in C2C12 myoblasts that were induced to differentiate. The subsequent knockdown during the period of differentiation was monitored by qPCR alongside changes in cell-associated hyaluronan and the pericellular matrix to determine the efficacy of *Has2* knockdown and whether this subsequently induced hyaluronan loss.

Transfections with the *Has2* and negative control siRNA were performed 3 days before the myogenic cultures were induced to differentiate; mRNA was collected at the time of and

after induction of differentiation for qPCR analysis. Transfection with *Has2* siRNA significantly down-regulated *Has2* mRNA compared with negative control siRNA immediately prior to the induction of differentiation (72 h after transfection; 0 h after differentiation), with an 89% decrease in transcript levels (Fig. 3A). This decrease was maintained through to 24 h after differentiation.

In *Has2* siRNA-treated cultures, there was also a clear reduction in the reactivity of HABP associated with myoblasts and myotubes at 0 and 48 h after differentiation, respectively (Fig. 3B), indicating that the knockdown of *Has2* led to a decrease in hyaluronan accumulation that persisted throughout the time course. 4MU is a well documented inhibitor of hyaluronan synthesis; it depletes cellular UDP-glucuronic acid, one of the substrates for hyaluronan synthesis, and can also directly reduce hyaluronan synthase gene transcript levels (33) with little to no effect on proteoglycan synthesis (34). Treatment with 4MU, at a concentration of 1 mM in the growth medium 24 h prior to inducing differentiation and also in the differentiation medium reduced cell-associated HABP reactivity in both the undifferentiated and differentiated cultures, but to a greater degree than *Has2* siRNA. Together, this suggested that both treatments had reduced the synthesis of hyaluronan, yet 4MU was the most effective, almost completely ablating the detection of hyaluronan with the HABP.

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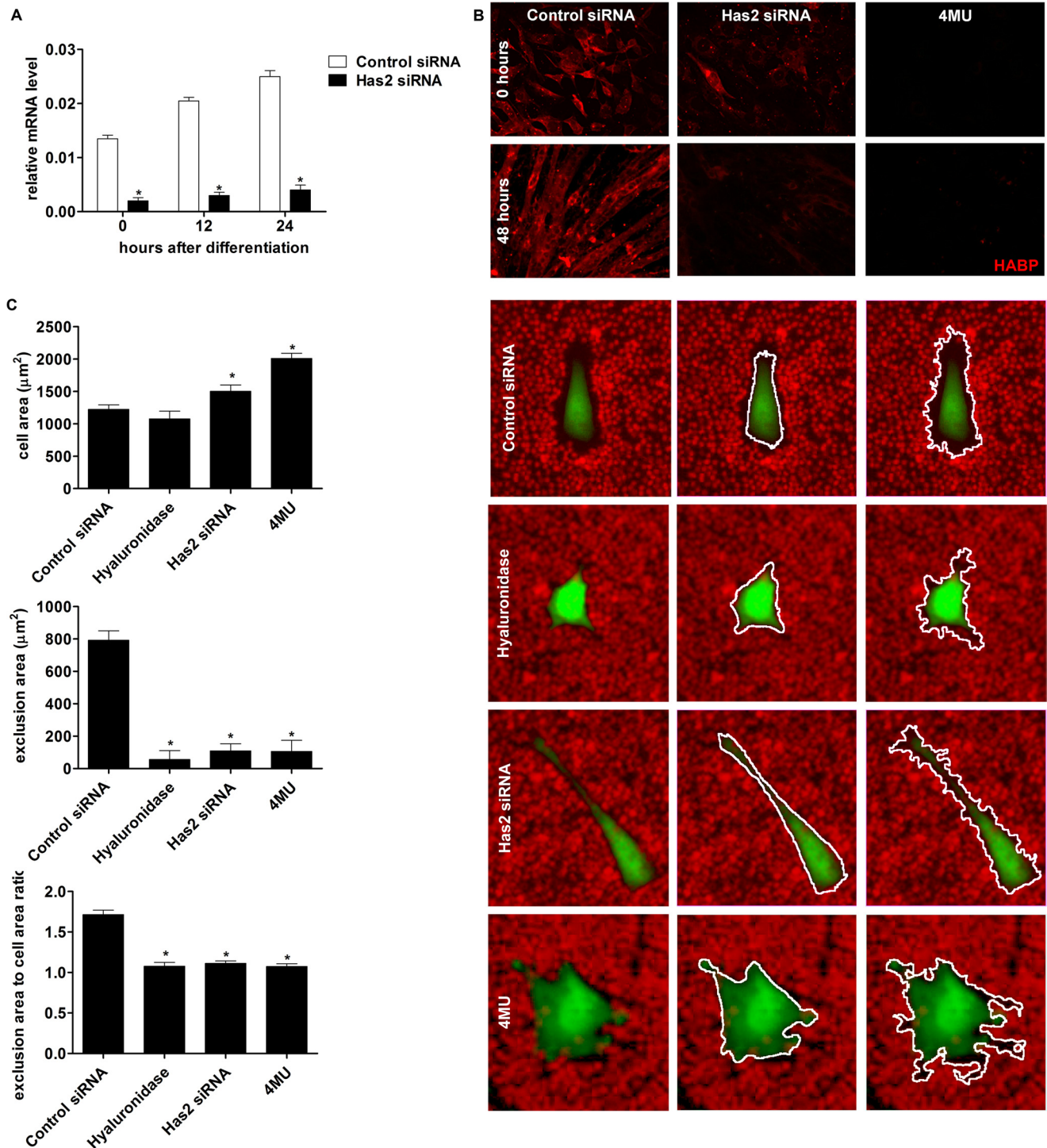


FIGURE 3. Knockdown of *Has2* and reduction in levels of hyaluronan. *A*, relative mRNA levels of *Has2* following C2C12 transfection of *Has2* siRNA and induction of differentiation. An 89% reduction in *Has2* mRNA compared with control was achieved at day 0, and similar magnitude reductions persisted for 24 h following the addition of differentiation medium. Statistical analysis was performed by the Relative Expression Software Tool. * $p < 0.05$, $n = 4$. *B*, HABP reactivity in control and *Has2* siRNA- and 4MU-treated C2C12 cells in nearly confluent undifferentiated cultures (0 h after the addition of differentiation medium) and 48 h after the addition of differentiation medium. Representative images of HABP immunofluorescence with *Has2* siRNA and 4MU treatment demonstrated a decrease in hyaluronan content compared with control; in the vast majority of 4MU-treated cells, there was a near absence of HABP reactivity. *C*, representative images of the pericellular erythrocyte exclusion assay, demonstrating reduced area of the exclusion zone surrounding myoblasts with hyaluronidase, *Has2* siRNA, and 4MU treatment compared with control. An increase in cell size and altered morphology with *Has2* siRNA and 4MU is also evident. *White outlines* highlight the binary masks generated for the area measured of the cell (*middle*) and exclusion area (*right*). Quantitation of the areas measured for the cells and the exclusion areas alone and the ratio of the exclusion area plus cell size divided by the cell size are presented below. * $p < 0.05$, $n = 4$. Error bars, S.E.

Hyaluronan is essential for the formation of the pericellular matrix, which is particularly large and well described in chondrocytes. The pericellular matrix is largely composed of hyalu-

ronan stabilized by proteoglycans and is capable of excluding cells and other particles from the area that it occupies. It is typically the first point of contact between a cell and the exter-

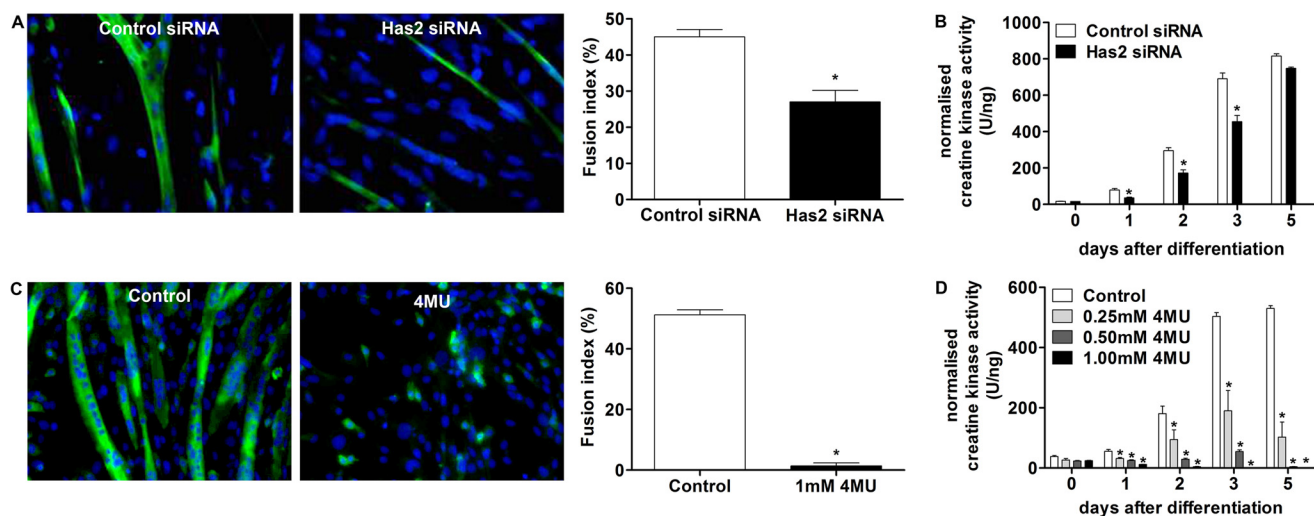


FIGURE 4. **Has2- and 4MU-mediated loss of hyaluronan inhibits myogenic differentiation.** *A*, desmin immunofluorescence for fused cultures (24 h) following both control and *Has2* siRNA transfection and the quantitated fusion index on the right. *B*, creatine kinase activity demonstrating reduced differentiation with *Has2* siRNA. A greater inhibition of differentiation was observed with 4MU treatment by fusion index (*C*) and creatine kinase activity (*D*). *, $p < 0.05$, $n = 4$. Error bars, S.E.

nal environment. Previous reports have suggested that *Has2* is critical for the formation of the cell-associated matrix in human articular chondrocytes (35). Therefore, we investigated whether *Has2* knockdown affects the maintenance of the myoblast pericellular matrix because this could relate to the ability of myoblasts to come into close contact with one another and fuse into syncytial muscle cells.

The pericellular matrix was visualized using the pericellular erythrocyte exclusion assay in C2C12 cells transfected with *Has2* or control siRNA or treated with 1 mM 4MU. Representative images of the cells and the exclusion zones are presented in Fig. 3C, with the *binary mask outline* highlighted for the myoblast (*middle*) and the exclusion zone (*bottom*). Control myoblasts demonstrated a moderate sized exclusion zone on average $800 \mu\text{m}^2$ in area; however, *Has2* siRNA- and 4MU-treated cells had on average an area less than $100 \mu\text{m}^2$. The hyaluronidase-treated control group also demonstrated an almost complete lack of exclusion zone with an average area less than $100 \mu\text{m}^2$.

Interestingly, both *Has2* siRNA- and 4MU-treated myoblasts demonstrated atypical cell morphology, with increased numbers of particularly slender membrane extensions and overall increased cell size as shown in the representative images. There was a significant increase in the area measured for the *Has2* siRNA-treated cells, and this was further exaggerated in the 4MU treatment group. When considering the ratio of the size of the exclusion area plus cell size to cell size alone, in order to account for differences in cell size, the decrease in exclusion area was maintained. An average ratio of 1.6 was observed for control myoblasts, indicating an exclusion area equivalent to 60% of the cell size, whereas these ratios were only marginally higher than 1.0 for cells treated with hyaluronidase, *Has2* siRNA, and 4MU, indicating almost no pericellular matrix relative to cell size. Together with HABP detection of hyaluronan, this finding suggested that knockdown of *Has2* reduced hyaluronan and ablated the hyaluronan-dependent pericellular matrix, as did 4MU treatment.

Has2 Knockdown and Loss of Hyaluronan Inhibits Myogenesis—The fusion index, which is the proportion of myoblasts that had fused 24 h after differentiation in *Has2* siRNA-treated cultures, was significantly reduced compared with control (Fig. 4A). The fusion index measurement is normalized and expressed as a proportion of the total number of cells and thus is not biased by potential differences in cell density, which did not appear to exist in the cultures examined. Creatine kinase activity (a marker of myogenic differentiation), which is normalized per μg of genomic DNA to account for differences in cell density, was significantly decreased in *Has2* siRNA-transfected cultures compared with control cultures at 1, 2, and 3 days after induction of differentiation (Fig. 4B). This inhibition would best be described as a delay in differentiation to the maximum extent possible for these cultures, because by 5 days after differentiation, creatine kinase activities were not significantly different. This could be due to the acute nature of siRNA-mediated knockdown; however, it would not be possible to address whether differentiation would remain inhibited if the siRNA was replenished due to the challenges involved in transfecting differentiating myogenic cultures. Nevertheless, these data demonstrated that loss of the *Has2* gene does impair differentiation and therefore is required during this process.

To test whether inhibition of myogenic differentiation could be linked to loss of hyaluronan, the effect of 4MU on differentiation was examined. As demonstrated in Fig. 3, 4MU treatment abolished hyaluronan synthesis; 4MU treatment also abolished myogenic differentiation. In 4MU-treated cultures, almost no myotubes were observed by immunofluorescent staining of desmin (Fig. 4C). Myoblasts were treated with 1 mM 4MU for 24 h in growth medium prior to the induction of differentiation as well as in the differentiation medium, which was replenished every day. Interestingly, although very few desmin-positive syncytia could be seen, there were numerous mononucleated desmin-positive cells, indicating that although 4MU prevents fusion, some degree of the myogenic program relating to the regulation of desmin expression is conserved. Creatine

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kinase activity from 4MU-treated cultures was significantly reduced at 1, 2, 3, and 5 days after differentiation at a variety of concentrations compared with vehicle control (Fig. 4D). These observations indicated that loss of hyaluronan synthesis resulting from *Has2* knockdown was highly likely to have caused inhibition of myogenic differentiation and furthermore suggested that endogenous hyaluronan synthesis by myoblasts is essential to myogenic differentiation and fusion, because the formation of myotubes and increases in creatine kinase activity could not occur with 4MU treatments at concentrations that prevented hyaluronan synthesis. In these experiments, 4MU was consistently replenished with medium changes to maintain inhibition of hyaluronan synthesis; however, if 4MU-containing medium was washed out and cultures were returned to control medium, fusion was able to occur eventually (results not shown), which suggested that 4MU completely blocks fusion and that this effect is reversible when hyaluronan synthesis can be regained by removal of 4MU.

Hyaluronan Synthesis Inhibition Induces Alterations in Expression of Myogenic and Extracellular Matrix Transcripts—Across this same time course of differentiation for which *Has2* knockdown was performed, we investigated transcriptional changes relating to hyaluronan, myogenesis, and the extracellular matrix in order to provide insights into the downstream effects of decreased *Has2* transcript levels and loss of *Has2*-mediated hyaluronan synthesis (Fig. 5).

Genes related to hyaluronan anabolism, catabolism, and receptors were examined. Transcripts for the *Has1* and *Has3* genes were not significantly altered with *Has2* siRNA, suggesting that there is no compensation for loss of *Has2* by hyaluronan anabolic mechanisms. Hyaluronidase-2 (*Hyal2*) in conjunction with *Cd44* is capable of catabolizing high molecular weight hyaluronan produced by HAS2 that subsequently undergoes endocytosis from the cell surface (2, 36). No significant differences in *Hyal2* expression were observed; however, *Cd44* expression was significantly decreased at the time of induction of differentiation (72 h after siRNA transfection). No differences in the expression of the less well characterized hyaluronan receptor gene, hyaluronan-mediated motility receptor (*Hmmr*), also known as RHAMM, were observed (results not shown). *Hyal1* has also recently been suggested to enhance myogenic differentiation by degrading chondroitin sulfate and allowing myoblast fusion (37); no changes in *Hyal1* transcripts were observed with *Has2* siRNA treatment.

Transcripts for the proliferative marker, proliferating cell nuclear antigen (*Pcna*), and the cell cycle inhibitor p21 (*Cdkn1a*) were not significantly altered in the *Has2* siRNA group (Fig. 5). This suggested that *Has2* knockdown did not affect proliferation or the cessation of proliferation necessary for myogenic differentiation. Of the four myogenic regulatory factors (MRFs) that control myogenic differentiation, only *Myf6* (Herculin/MRF4) and *MyoD1* were significantly down-regulated by *Has2* siRNA; *Myog* and *Myf5* transcripts were not significantly altered. This coordinated down-regulation of a specific subset of MRFs (*Myf6* and *MyoD*) is probably the result of loss of *Has2*-mediated hyaluronan synthesis and is consistent with the observation that *Has2* knockdown inhibits myogenic differentiation.

Because hyaluronan is an important part of the extracellular matrix, we also investigated how loss of *Has2* and hyaluronan synthesis influenced extracellular matrix transcripts, including interstitial, basement membrane, and proteoglycan components. Although transcripts for the myofiber basement membrane protein laminin α -2 (*Lama2*) were not significantly changed with *Has2* knockdown, the interstitial proteins collagen α -1 (*Col1a1*) and fibronectin (*Fn1*) were significantly up-regulated with *Has2* knockdown at 0 and 12 h compared with control. Biglycan (*Bgn*) transcripts followed a similar trend, with significant up-regulation at 0, 12, and 24 h, and the other proteoglycans examined, versican (*Vcan*) and decorin (*Dcn*), followed a different trend, only significantly up-regulated at 12 and 24 h. Expression changes that were altered at 0 h relative to differentiation (72 h after siRNA transfection), *Col1a1*, *Fn1*, and *Bgn*, would appear to be the direct result of *Has2* knockdown, whereas the changes in *Vcan* and *Dcn* expression, which only occurred after the initiation of differentiation, were probably the results of inhibited or delayed myogenesis and not an immediate response to *Has2* and hyaluronan loss.

We also examined the changes in gene expression caused by 4MU along the same time course of differentiation (Fig. 6). Although 4MU is suggested to act primarily by limiting the availability of UDP-glucuronic acid to cells for glycosaminoglycan synthesis, it was reported that 4MU can cause decreases in transcripts for hyaluronan synthase genes (33). Similarly, we found that 4MU significantly decreased transcripts for *Has2* but interestingly increased transcripts for *Has3*. *Hyal1* mRNA was also significantly increased with 4MU treatment; however, *Hyal2* and *Cd44* were not.

Although no MRFs were significantly altered, *Pcna* and *Cdkn1a* were both significantly different at 24 h of differentiation in the opposite directions. 4MU increased *Pcna* and decreased *Cdkn1a*, suggesting that 4MU-treated myoblasts were more likely to be proliferative and unable to exit the cell cycle compared with control myoblasts. Matrix transcripts were also altered; *Col1a*, *Fn*, *Bgn*, and *Dcn* were all significantly down-regulated at certain points with 4MU treatment compared with control, suggesting an inability to produce matrix components.

Restoration of the Pericellular Matrix to Hyaluronan Synthesis-blocked Myoblasts Does Not Rescue Myogenic Differentiation—We examined whether the addition of high molecular mass hyaluronan from rooster comb (which, according to the supplier, is expected to be in the range of 1–4 MDa), which corresponds closely with the molecular mass of hyaluronan produced by HAS2, could in any way compensate for *Has2* knockdown or loss of hyaluronan by 4MU and allow myogenic differentiation.

Hyaluronan was added to cultures in the growth medium for 24 h prior to inducing differentiation and also in the differentiation medium. The addition of exogenous hyaluronan by itself mildly inhibited differentiation at 24 and 48 h; there was significant reduction in creatine kinase activity at 24 and 48 h with both 0.1 and 0.25 mg/ml hyaluronan compared with vehicle control (Fig. 7A). This agreed with previous reports that exogenous hyaluronan inhibits myogenic differentiation (12–14). However, the addition of hyaluronan to 4MU-treated cultures did not restore creatine kinase activity to normal vehicle con-

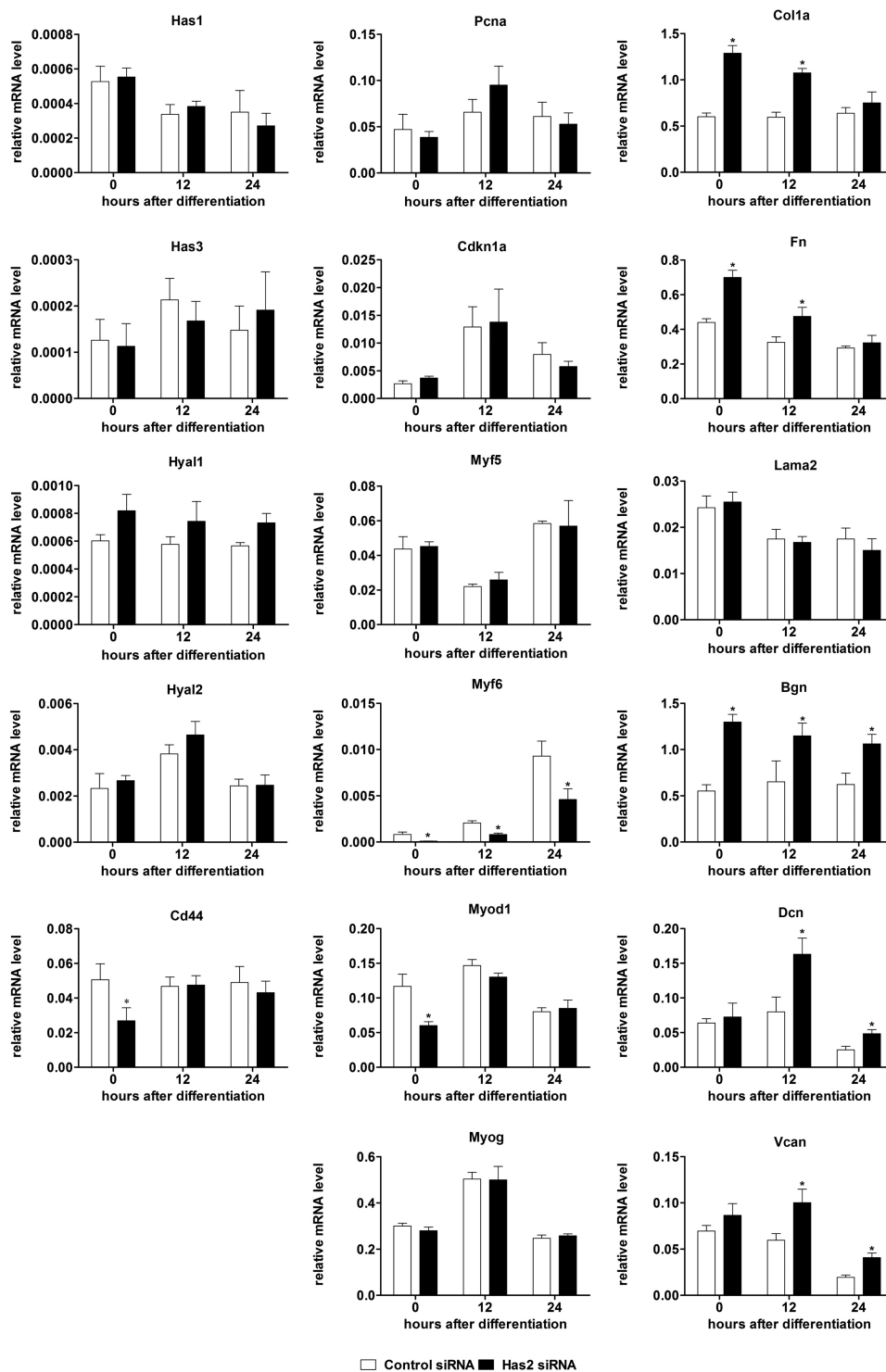


FIGURE 5. The effect of *Has2* knockdown on myogenic and extracellular protein transcripts. Relative mRNA levels of genes related to hyaluronan synthesis and degradation (*Has1*, *Has3*, *Cd44*, *Hyal1*, and *Hyal2*), cell proliferation (*Pcna*), cell cycle withdrawal (*Cdkn1a*), MRFs (*Myf5*, *Myd1*, *Myf6*, and *Myog*), and extracellular matrix (*Col1a1*, *Fn1*, *Lama2*, *Vcan*, *Bgn*, and *Dcn*) by qPCR following transfection with *Has2* and control siRNA and after the induction of differentiation in C2C12 myoblasts. *, $p < 0.05$, $n = 4$. Error bars, S.E.

control levels (Fig. 7B). In fact the inhibition of myogenic differentiation caused by 4MU and hyaluronan were additive. *Has2* siRNA-mediated inhibition of myogenic differentiation was also not restored with exogenous hyaluronan addition (Fig. 7C). This indicates that there is an important functional difference between exogenous hyaluronan and endogenously synthesized

hyaluronan; whereas exogenous hyaluronan may inhibit myogenic differentiation, endogenous hyaluronan is absolutely critical for myogenic differentiation.

To determine if the exogenous hyaluronan addition could restore the pericellular matrix lost with 4MU treatment, we once again utilized the pericellular erythrocyte exclusion assay

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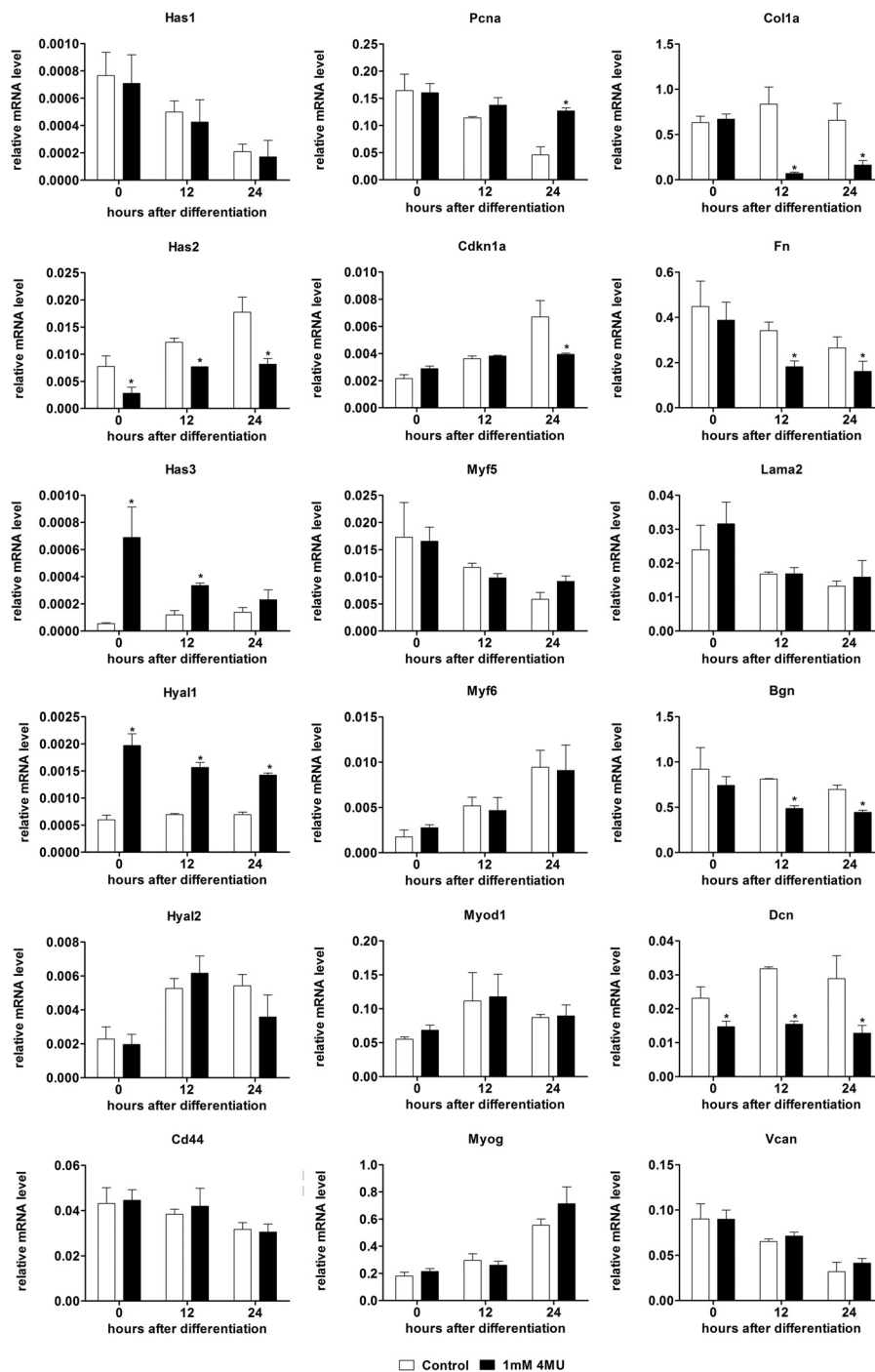


FIGURE 6. **The effect of 4MU on myogenic and extracellular protein transcripts.** Relative mRNA levels of genes related to hyaluronan synthesis and degradation (*Has1*, *Has3*, *Cd44*, *Hyal1*, and *Hyal2*), cell proliferation (*Pcna*), cell cycle withdrawal (*Cdkn1a*), MRFs (*Myf5*, *Myd1*, *Myf6*, and *Myog*), and extracellular matrix (*Col1a1*, *Fn1*, *Lama2*, *Vcan*, *Bgn*, and *Dcn*) by qPCR following treatment with 4MU and control (DMSO vehicle) after the induction of differentiation in C2C12 myoblasts. *, $p < 0.05$, $n = 4$. Error bars, S.E.

(Fig. 7E). We found that the addition of hyaluronan by itself did not restore the pericellular matrix. Previous studies have demonstrated that recapitulation of a pericellular matrix requires both a hyaluronan-binding proteoglycan, such as aggrecan or versican, in conjunction with hyaluronan (38). Therefore, we examined the addition of hyaluronan to C2C12 cells transfected with either a control (GFP) or versican (V1 variant) construct following 4MU inhibition of hyaluronan synthesis. C2C12 myoblasts were transfected with either the versican

construct or a GFP reporter control construct and allowed to grow for 3 days, after which time they were incubated with 0.25 mg/ml hyaluronan in growth medium or control growth medium for 24 h with or without 1 mM 4MU. Individually, hyaluronan and versican did not restore the pericellular matrix; however, when combined, large pericellular matrices could be seen even in cells that were treated with 4MU.

Restoration of the pericellular matrix in this fashion, however, did not restore differentiation in 4MU-treated cells, as

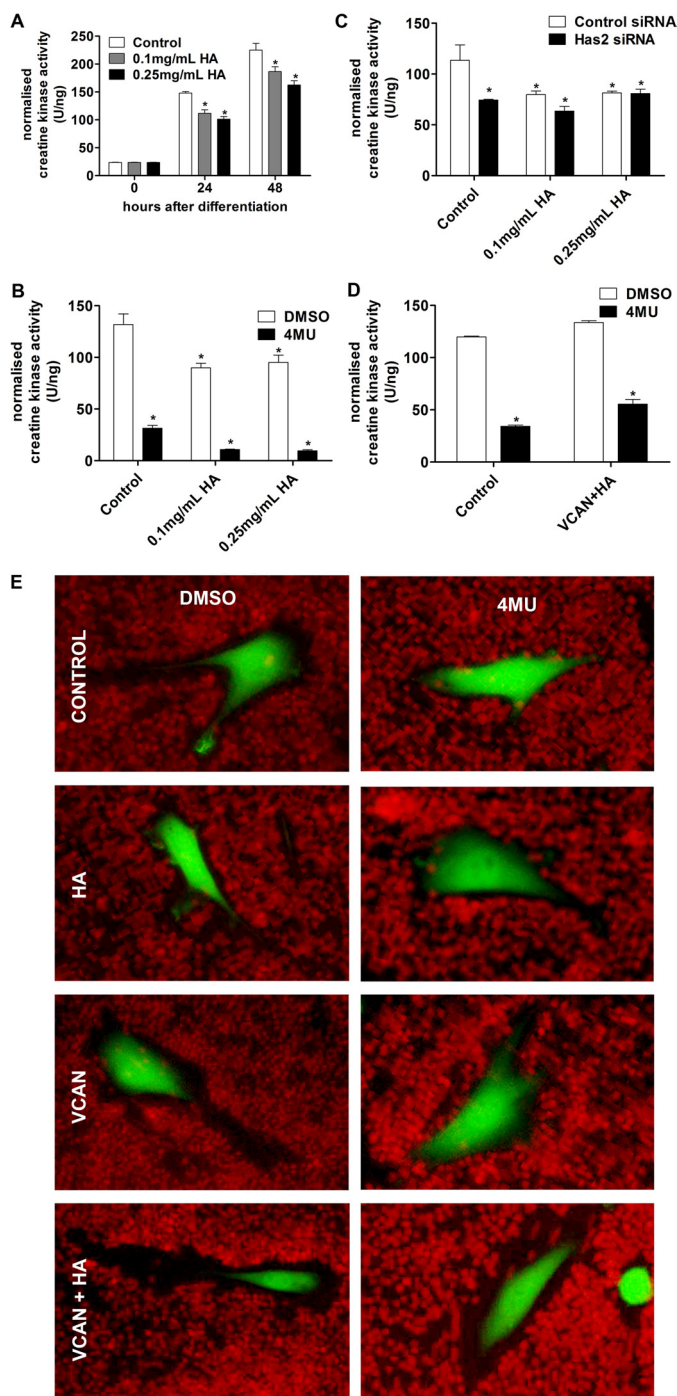


FIGURE 7. Inhibition of differentiation by blocking hyaluronan synthesis cannot be rescued by restoration of the pericellular matrix. *A*, creatine kinase activity in C2C12 cultures that were incubated with hyaluronan-containing medium prior to and during differentiation show a minor reduction in differentiation. *B*, the addition of hyaluronan does not increase creatine kinase activity in 4MU-treated cultures 24 h after differentiation. *C*, the addition of hyaluronan does not increase creatine kinase activity in *Has2* siRNA-treated cultures 24 h after differentiation. *D*, hyaluronan addition in conjunction with versican expression does not restore creatine kinase levels to control 24 h after differentiation. *E*, hyaluronan addition in conjunction with versican expression restores the erythrocyte-excluding pericellular matrix to 4MU hyaluronan synthesis-blocked cells, but neither hyaluronan nor versican could achieve this independently. *, $p < 0.05$, $n = 4$. Error bars, S.E.

measured by creatine kinase activity, to levels similar to that without 4MU treatment (Fig. 7D). In this experiment, C2C12 myoblasts were transfected with either the versican construct or a GFP reporter control construct and allowed to grow for 3 days, after which time they were incubated with 0.25 mg/ml hyaluronan in growth medium or control growth medium for 24 h with or without 1 mM 4MU; following this, they were then induced to differentiate for 24 h with differentiation medium either with or without hyaluronan and 4MU. No appreciable myotube formation could be seen in 4MU-treated cultures that also received hyaluronan and versican treatments. This suggested that although hyaluronan is essential to myogenic differentiation, it is not the hyaluronan- and proteoglycan-dependent pericellular matrix that is necessary to allow myoblast differentiation and fusion.

DISCUSSION

Hyaluronan and *Has* genes have been studied extensively biochemically and in certain contexts, such as the embryo, developing heart, metastases, and adult articular cartilage, yet few studies have focused specifically on hyaluronan and synthase gene functions in skeletal muscle, a tissue rich in hyaluronan. Recent reports have indicated almost ubiquitous expression of all three HAS enzymes on myoblasts (M -cadherin⁺) and fibroblasts ($FSP1$ ⁺) as well as synthase-dependent expression in other cell types, such as endothelial ($CD31$ ⁺) and monocytic ($F4/80$ ⁺) cells, in the context of compensatory skeletal muscle hypertrophy (25). We present herein the first findings that describe changes in *Has* gene expression in skeletal muscle during differentiation, development, and skeletal muscle remodeling in response to notexin-induced injury and dystrophic pathology. We discovered gene expression profiles of *Has1* and *Has3*, which temporally associated with muscle necrosis and inflammation, and we established a clear link between *Has2* and myogenic differentiation *in vivo* and *in vitro*. Importantly, we demonstrate for the first time that hyaluronan synthesis is essential for myogenic differentiation and that *Has2* mediates myogenic differentiation, muscle cell hyaluronan synthesis, and maintenance of a pericellular matrix around myoblasts.

Has1 and *Has3* Expression Is Correlated with Inflammation—The pattern of *Has1* expression *in vivo* and *in vitro* was not in keeping with a role for *Has1* in myogenesis. *Has1* expression in injured muscle did, however, coincide well with the acute inflammatory response induced by notexin that we have described previously (39); moreover, *Has1* expression was significantly up-regulated in *mdx* compared with wild type diaphragms at 3 weeks of age, which is typically considered the time of onset of pathology, when necrosis and inflammation first become visible histologically. These observations strongly suggest that *Has1* may be involved in the initial inflammatory response to necrosis in skeletal muscle.

As for *Has1*, changes in *Has3* transcript expression were not particularly indicative of a role in myogenesis. *Has3* expression was not altered during C2C12 myoblast differentiation or by pathology in the *mdx* diaphragm; however, there was an increase in expression neonatally compared with embryonic and postnatal time points. Most striking, however, was the sim-

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ilarity in expression patterns between *Has3* and *Has1* following notexin injection. Both genes showed a very similar trend with a peak at 1 day after notexin injection associated with the acute inflammatory response.

It was therefore very interesting to observe a large accumulation of hyaluronan in necrotic fibers being invaded by inflammatory cells not long after injection of notexin. This strongly suggests that hyaluronan could have a role in phagocytosis of necrotic myofibers by leukocytes and could promote the migration and adhesion of leukocytes to necrotic fibers. Numerous studies have suggested that hyaluronan is linked with inflammation, including the ability of small hyaluronan fragments to induce chemokine expression (40). Studies by Calve *et al.* (25) have indicated that hyaluronan synthesis was increased in a model of compensatory skeletal muscle hypertrophy induced by tenectomy. Indeed Calve *et al.* (25) demonstrated increased hyaluronan synthase gene expression associated with inflammation and expression of *Has1*, *Has2*, and *Has3* co-localized with non-resident inflammatory macrophages. In addition, very recent studies have indicated that overexpression of *Has1* promotes dermal regeneration by decreasing the number of inflammatory cells in the healing excision wound (41). Combined with our studies presented herein, these findings suggest that hyaluronan synthases may regulate inflammation associated with remodeling in skeletal muscle.

Has2 Expression Is Correlated with Myogenesis—A strong positive correlation between *Has2* mRNA expression and myogenesis was observed in all four models examined, suggesting that *Has2* is involved in the formation and regeneration of muscle fibers.

The formation of skeletal muscle myofibers during mouse development occurs in several phases. Following the formation of the muscle anlage, primary myogenesis (when embryonic myoblasts begin to differentiate and fuse into the primary syncytial muscle fibers) occurs around E11–E14 and is followed subsequently by fetal myoblast fusion in the secondary wave of myogenesis beginning around E14–E16 (42, 43). The data presented here show that in developing skeletal muscle, *Has2* expression peaks at E14.5, a time that is associated with the onset of fetal myoblast fusion.

Has2 transcript expression was also up-regulated following notexin injection into the tibialis anterior. The expression profile of *Has2* associated best with macrophage accumulation or myoblast differentiation, myotube formation, and maturation (39).

Increased expression of *Has2* at 6 and 12 weeks of age in *mdx* compared with wild type diaphragms was during a phase that also corresponds with both myogenic differentiation and inflammation. Taken together with the observed up-regulation of *Has2* during C2C12 myoblast differentiation, these observations constitute a substantial body of evidence implicating *Has2* in fetal myoblast differentiation and also potentially in postnatal myoblast differentiation and myotube formation. These expression profiles suggested a function for *Has2* in muscle growth and regeneration potentially relating to myogenesis. Therefore, we examined the involvement of *Has2* in the process of myogenesis in culture by siRNA-mediated knockdown.

Has2 Knockdown and Loss of Hyaluronan Inhibit Myogenesis—*Has2* transcript was successfully knocked down in C2C12 myoblasts induced to differentiate, and no change in *Has1*, *Has3*, *Hyal1*, or *Hyal2* expression was observed, which, in conjunction with HABP reactivity, suggested that hyaluronan levels were greatly reduced as a consequence of knockdown without compensation toward hyaluronan anabolism or inhibition of catabolism. The reduction in HABP reactivity caused by *Has2* knockdown, however, was not as complete as that achieved with 4MU, suggesting residual hyaluronan synthesis even with *Has2* knockdown. Indeed, 4MU and *Has2* siRNA caused distinct changes in transcriptional profiles, indicating that although these treatments can cause similar but different decreases in hyaluronan production and the pericellular matrix, which is useful for comparison of the effects of differentiation, these treatments should not be viewed as the same.

The hypothesis for this series of experiments was that the knockdown of *Has2* would inhibit differentiation due to the positive correlation between *Has2* expression and myogenesis. Myogenic differentiation was indeed inhibited by *Has2* knockdown, as assessed by changes in morphology, creatine kinase activity, fusion index, and MRF gene expression. This inhibition of differentiation was mimicked by the hyaluronan synthesis inhibitor 4MU. The prevention of differentiation with 4MU was so pervasive that it suggests that hyaluronan synthesis is critical to myogenic differentiation. Because of the importance of hyaluronan in epithelial-mesenchymal transitions (44), we cannot exclude a more generalized cellular effect of 4MU, whereby loss of hyaluronan shifts C2C12 cells to a more epithelial rather than mesenchymal-like phenotype incapable of differentiating toward the myogenic lineage. There are many possible reasons for why and how loss of *Has2* and hyaluronan could inhibit differentiation, and those investigated in this study are discussed.

CD44, the extracellular receptor for hyaluronan, has previously been reported as a protein necessary for the differentiation of myoblasts (45). However, these studies do not indicate whether the loss of CD44 may prevent differentiation through hyaluronan-dependent mechanisms. *Has2* and *Cd44* expression frequently appear to be linked, where higher expression of *Has2* typically yields higher expression of *Cd44* and *vice versa* (46–50). Thus, the decrease in *Cd44* expression with *Has2* knockdown could potentially be mediating inhibition of differentiation or reflect a dysregulation of CD44 and hyaluronan interactions, which could even more broadly lead to a dysregulation of cell and extracellular matrix interactions.

Loss of the extracellular matrix has previously been demonstrated to inhibit differentiation of myoblasts in culture. Inhibition of collagen synthesis reduced MRF expression and prevented myogenic differentiation in a manner that could be reversed with exogenous collagen in Matrigel (51, 52). Similarly, the loss of proteoglycans in the extracellular matrix by synthesis inhibitors inhibited differentiation and could be rescued by exogenous extracellular matrix administration (53–55). Hyaluronan is an important constituent of the extracellular matrix and therefore could be equally

important for differentiation as collagens and proteoglycans and explain why *Has2* knockdown and 4MU inhibit myogenic differentiation. 4MU treatment produced conspicuous decreases in transcripts for several matrix components, including collagen-I, fibronectin, and the proteoglycans biglycan and decorin, suggesting that 4MU could limit production of the extracellular matrix.

Loss of the pericellular matrix was considered due to the importance of cell-cell adhesion in mediating myoblast fusion and the role of hyaluronan in the initial contact between cells prior to integrin-mediated adhesion (56). It has been speculated that it could be necessary to remove the hyaluronan-rich pericellular coat to allow myoblast fusion (16), presumably through allowing closer contact between cell membranes and associated proteins. This mechanism has been suggested recently as a method by which proteoglycan cleavage can prevent retention of an overwhelmingly large pericellular matrix and thus allow cell-cell contact and differentiation/fusion (57). Both *Has2* knockdown and 4MU abolished the hyaluronidase-sensitive pericellular matrix and also inhibited myogenic differentiation. Despite both treatments abolishing the pericellular matrix, 4MU caused a considerably greater inhibition of differentiation relative to *Has2* knockdown. Consideration of the above findings and the inability of hyaluronan synthesis-inhibited cells to differentiate even after restoration of the pericellular matrix would suggest that the inhibition of differentiation caused by loss of hyaluronan occurs independently of pericellular matrix ablation. In support of this, we found that although both *Has2* knockdown and 4MU treatment abolished pericellular matrix formation, hyaluronan could still be detected in or around *Has2* knockdown cells but not with 4MU treatment by HABP. This indicated a difference in total hyaluronan production between *Has2* knockdown and 4MU treatments, where 4MU appears to completely prevent hyaluronan synthesis, whereas *Has2* knockdown reduces hyaluronan synthesis sufficiently to prevent pericellular matrix formation but not to completely prevent hyaluronan production. Therefore, hyaluronan functions independent from extracellular/pericellular functions may also be regulating myogenic differentiation.

Hyaluronan has recently come to be appreciated as a factor that can also be present and act intracellularly, mediating cell homeostasis by associating with intracellular hyaluronan-binding proteins that regulate RNA transcription and cell cycle progression, such as Cdc37, IHABP4, and p32 (58–60). Intracellular hyaluronan functions, such as binding Cdc37, could provide an alternative explanation for hyaluronan-mediated differentiation, because Cdc37 interacts with the myogenic transcription factor MyoD (61).

Altogether, this work demonstrates that hyaluronan synthesis regulation occurs during skeletal muscle growth, injury, and regeneration. These changes in transcriptional regulation can be associated with different phases involved in regeneration, which can differ, depending on the synthase considered; Has1 and, to a lesser extent, Has3 associate well with an acute inflammatory response, and Has2 associates best with myoblast differentiation and myotube formation. Furthermore, this work demonstrates the requirement for Has2 and for hyaluronan synthesis in general for myogenic differentiation. This work has

important implications for the field of skeletal muscle and potentially tissue engineering.

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