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Differentiation of Cardiomyocytes from Human Embryonic Stem Cells Is Accompanied by Changes in the Extracellular Matrix Production of Versican and Hyaluronan

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ABSTRACT

Proteoglycans and hyaluronan play critical roles in heart development. In this study, human embryonic stem cells (hESC) were used as a model to quantify the synthesis of proteoglycans and hyaluronan in hESC in the early stages of differentiation, and after directed differentiation into cardiomyocytes. We demonstrated that both hESC and cardiomyocyte cultures synthesize an extracellular matrix (ECM) enriched in proteoglycans and hyaluronan. During cardiomyocyte differentiation, total proteoglycan and hyaluronan decreased and the proportion of proteoglycans bearing heparan sulfate chains was reduced. Versican, a chondroitin sulfate proteoglycan, accumulated in hESC and cardiomyocyte cultures. Furthermore, versican synthesized by hESC contained more N- and O-linked oligosaccharide than versican from cardiomyocytes. Transcripts for the versican variants, V0, V1, V2, and V3, increased in cardiomyocytes compared to hESC, with V1 most abundant. Hyaluronan in hESC had lower molecular weight than hyaluronan from cardiomyocyte cultures. These changes were accompanied by an increase in HAS-1 and HAS-2 mRNA in cardiomyocyte cultures, with HAS-2 most abundant. Interestingly, HAS-3 was absent from the cardiomyocyte cultures, but expressed by hESC. These results indicate that human cardiomyocyte differentiation is accompanied by specific changes in the expression and accumulation of ECM components and suggest a role for versican and hyaluronan in this process. J. Cell. Biochem. 111: 585–596, 2010.

KEY WORDS: VERSICAN; HYALURONAN; CARDIOMYOCYTE; DIFFERENTIATION; STEM CELLS; GLYCOSYLATION

Normal development of the heart requires synthesis, deposition, and degradation of the extracellular matrix (ECM) components versican and hyaluronan. Versican is a large chondroitin sulfate proteoglycan (CS/DSPG) which is present in most soft tissues and fills the space between cells that is not occupied by fibrous proteins such as collagens and elastic fibers [Wight et al., 1991]. Versican binds to a number of other ECM components, as well as to cellular receptors, to influence cell adhesion, proliferation, migration, and survival [Zimmermann, 2000; Wight, 2002; Wu et al., 2005]. Versican is expressed in the myocardium and myocardial basement membrane in the developing chick heart [Zanin et al., 1999] and is necessary for cardiac cushion formation, atrioventricular (AV) valve development, ventricular septation, and outflow tract development [Henderson and Copp, 1998; Mjaatvedt et al., 1998; Kern et al., 2006, 2007; Kruithof et al., 2007]. Disruption of the versican gene in heart defect (hdf) mice is embryonic lethal at day E 9.5–10.5, and the phenotype is characterized by abnormalities in endothelial-mesenchymal transition as well as defects along the anterior–posterior cardiac axis [Yamamura et al., 1997; Mjaatvedt et al., 1998]. More recently, microRNA-138 was found to target versican and to be necessary for cardiac maturation and patterning in Zebrafish heart [Morton et al., 2008]. Furthermore, knockdown in

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Zebrafish of NDRG4, a gene that appears to be required for restricting the expression of versican and bone morphogenetic protein 4 (BMP4) to the developing atrio-ventricular (AV) canal, causes a marked reduction in the proliferation of myocytes, resulting in a hypoplastic heart [Qu et al., 2008].

Molecules that have been shown to associate with versican, including hyaluronan, a high molecular weight glycosaminoglycan, which interacts with versican to form high molecular weight aggregates [LeBaron et al., 1992; Matsumoto et al., 2003] are also critical for heart development. Disruption of the gene encoding hyaluronan synthase (HAS-2), which is the major source of hyaluronan in the developing heart, leads to embryonic lethality in mice at the same stage that is affected in the hdf mouse [Mjaatvedt et al., 1998; Camenisch et al., 2000]. Furthermore, the protein that promotes and stabilizes the interaction of versican with hyaluronan, link protein, has also been shown to be important for heart development. A comparative DNA microarray analysis using tissues from the AV junction and ventricular chambers of mouse hearts at embryonic day E 10.5–11.0 revealed differential expression of cartilage link protein 1 (Crtl1) [Wirrig et al., 2007]. Mice deficient in the gene for Crtl1 [Watanabe and Yamada, 1999; Czipri et al., 2003] show a spectrum of cardiac abnormalities including AV septal and myocardial defects [Wirrig et al., 2007] and resemble those seen in the hdf mouse [Mjaatvedt et al., 1998] and Has-2$^{-/-}$ mouse [Camenisch et al., 2000]. Interestingly, a similar heart defect phenotype was recently described in *Xenopus*, in which another member of the link protein family, link protein 3, was knocked down in developing tadpoles by morpholino antisense oligos [Ito et al., 2008]. Together, these results indicate that a composite ECM requiring both hyaluronan and versican is essential for many of the events required for normal cardiac development.

Despite the known importance of versican, hyaluronan, and associated molecules in heart development, it is not known if cardiomyocytes participate in the synthesis of these molecules, nor have the relative amounts of versican and hyaluronan that compose the developing cardiac ECM been determined. Importantly, what little is known about the biochemical composition of the developing cardiac ECM was found in vertebrate animal models, but not in human tissues. Human embryonic stem cell (hESC) lines are capable of differentiating into cell types of the three germ layers, and therefore, offer a useful model to address factors associated with the differentiation of specific cell types [Thomson et al., 1998]. hESC withdraw from the cell cycle as they differentiate into a cardiomyocyte culture with spontaneously contracting areas. These cells stain for specific cardiomyocyte markers such as cardiac myosin heavy chain, α actinin, desmin, and cardiac troponin [Kehat et al., 2001; Xu et al., 2002; Snir et al., 2003]. Recent success has been achieved in efficiently promoting cardiomyocyte differentiation from hESC using exogenous factors, as well as in promoting hESC-derived cardiomyocyte proliferation and survival [McDevitt et al., 2005; Laflamme et al., 2007]. Using this system, we have determined that human cardiomyocyte cultures synthesize and secrete both versican and hyaluronan and demonstrate that significant differences exist in the amount and structure of versican and hyaluronan molecules produced by undifferentiated hESC and cardiomyocytes.

MATERIALS AND METHODS

CELL CULTURE

hESC were purchased from the WiCell Research Institute (Madison, WI) and maintained according to previously established techniques [Xu et al., 2001]. In brief, undifferentiated H7 hESC (NIH Stem Cell Registry ID# WA07 [NIHheSC-10-0061]), between passages 28 and 53, were cultured with mouse embryonic fibroblast-conditioned media (MEF-CM) on substrates coated with Matrigel (Becton Dickinson, San Diego, CA) in the absence of feeder layers, re-fed daily, and passaged weekly at a 1:6–1:12 split. The media conditioned by the MEFs consisted of 80% knockout DMEM (KO-DMEM), 2% serum replacement, 1% non-essential amino acids, 1 mM L-glutamine (all from Invitrogen, Carlsbad, CA), 0.1 mM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO), and 4 ng/ml bFGF (Peprotech, Rocky Hill, NJ).

To promote cell differentiation [Laflamme et al., 2007], stock hESC were released from their plastic dishes with versene buffer, suspended in MEF-CM plus 4 ng/ml bFGF, re-seeded at 2×10^5 cells per well on Matrigel-coated 24-well plates, and re-fed daily with MEF-CM with 4 ng/ml bFGF for 6 days. By convention, this time point was designated "day 0" post-differentiation. Control hESC cultures were harvested for analysis at this time point. To achieve cardiomyocyte differentiation, culture medium was then replaced with 0.5 ml/well RPMI-B27 (Invitrogen) with 100 ng/ml rhu Activin A (R&D Systems, Minneapolis, MN). After 1 day (day 1), cells were switched to 1 ml/well RPMI-B27 with 10 ng/ml rhu BMP-4 (R&D Systems). There were no medium changes thereafter until day 5. On day 5, the medium was replaced with RPMI-B27 without cytokines and fed every other day. These cells were analyzed on day 14 or 15. Proteoglycan and hyaluronan synthesis in hESC early in the differentiation process (day 0 post-differentiation) was compared to cardiomyocyte cultures generated by culturing hESC under directed differentiation conditions (Activin A and BMP4), which have been reported to significantly enhance cardiomyocyte yield in monolayer cultures. Differentiated cardiomyocyte cultures were stained with an antibody against human β-myosin heavy chain (Clone A4.951, American Type Culture Collection, Manassas, VA) to determine what fraction of the cells was successfully differentiated to cardiomyocytes.

PROTEOGLYCAN ISOLATION AND ANALYSIS

To obtain radiolabeled proteoglycans, cells were metabolically labeled with 100 μCi/ml $Na_2{}^{35}SO_4$ (MP Biomedicals, Irvine, CA) or trans-methionine containing [^{35}S]-(methionine and cysteine) (40 μCi/ml) during the last 24 h of culture. The medium was then collected and combined with protease inhibitors (5 mM benzamidine, 100 mM 6-aminohexanoic acid, and 1 mM phenylmethylsulfonyl fluoride). The cell layer was rinsed with phosphate-buffered saline and solubilized in 8 M urea buffer (8 M urea, 2 mM EDTA, 0 or 0.25 M NaCl, 50 mM Tris–HCl, and 0.5% Triton X-100 detergent, pH 7.4) containing protease inhibitors [Schönherr et al., 1991, 1993]. Total [^{35}S]-sulfate incorporation into proteoglycans was determined by cetylpryidinium chloride (CPC) precipitation [Wasteson et al., 1973]. The media contained secreted proteoglycans, whereas cell layers contained cell membrane-associated, intracellular, and ECM

proteoglycans. Media and cell layer extracts were concentrated and purified by ion-exchange chromatography on diethylaminoethyl (DEAE) Sephacel in 8 M urea buffer with 0.25 M NaCl and eluted with 8 M urea buffer containing 3 M NaCl [Schönherr et al., 1991, 1993]. Alternatively, both media and cell layer extracts were bound to DEAE Sephacel in 8 M urea buffer without 0.25 M NaCl and were eluted by a gradient of 0–0.8 M NaCl [Kinsella and Wight, 1988]. To determine the molecular size of different proteoglycan populations, [^{35}S]-sulfate-proteoglycan and [^{35}S]-methionine-labeled core protein molecular sizes [Lemire et al., 2007] were characterized by SDS–PAGE (4–12% with 3.5% stacking gel) under reducing conditions according to the procedure of Laemmli [1970]. DEAE-purified radiolabeled samples were digested with either chondroitin ABC lyase (0.05 U chondroitin ABC lyase [North Star BioProducts, East Falmouth, MA] in 0.3 M Tris–HCl, pH 8.0, 0.6 mg/ml bovine serum albumin, and 18 mM sodium acetate with protease inhibitors for 3 h at 37°C [Schönherr et al., 1991]); heparinase enzyme mixture (0.5 U heparinase I, 0.25 U heparinase II, and 0.15 U heparinase III [all from Sigma–Aldrich] in 0.1 M Tris–HCl, pH 7.0, 10 mM calcium acetate, and 18 mM sodium acetate with protease inhibitors for 3 h at 37°C); or the combination of both chondroitin ABC lyase and heparinase digestion. The ^{35}S-labeled intact proteoglycans and core proteins (after various enzyme digestions) were visualized by fluorography of dried gels and exposed to Blue Basic Autorad Film (ISC Bioexpress, Kaysville, UT).

To determine the hydrodynamic size of different proteoglycan populations, equal amounts of protein as DEAE-purified [^{35}S]-sulfate-labeled proteoglycans were chromatographed on an analytical Sepharose CL-2B molecular sieve column in 4 M guanidine buffer (4 M guanidine HCl, 2.5 mM EDTA, 100 mM Na_2SO_4, 100 mM Tris, 0.5% Triton X-100 detergent, pH 7.4) [Hascall et al., 1994].

WESTERN ANALYSIS

For Western blotting, DEAE-purified proteoglycans were digested by chondroitin ABC lyase, applied to a gradient of 4–12% SDS–PAGE, and electrophoretically transferred to 0.2 μm nitrocellulose membranes (GE Healthcare, Piscataway, NJ) using a BioRad Transblot SD Semi-Dry Transfer Cell (BioRad, Hercules, CA) [Olin et al., 1999]. The transferred proteins were then detected with the primary antibody to versican, 2-B-1 (North Star Bioproducts), and enhanced chemiluminescence (Western-Light Chemiluminescent Detection System) with CSPD proprietary luminescent substrate (Applied Biosystems [AB], Foster City, CA).

ENZYMATIC DEGLYCOSYLATION

DEAE purified samples were first digested with chondroitin ABC lyase, exchanged into water using the Millipore (Bedford, MA) minicon centrifugal filtration device and then deglycosylated using the Enzymatic Deglycosylation Kit (Prozyme, San Leandro, CA) according to the manufacturer's directions. Following deglycosylation, products were then analyzed by Western analysis using 2-B-1 antibody. The Enzymatic Deglycosylation Kit utilizes *N*-glycanase, *O*-glycanase, and sialidase A to completely remove all N- and O-linked oligosaccharides from glycoproteins.

QRT-PCR (QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE PCR)

DNA-free RNA was obtained from cell culture monolayers using the Total RNA Isolation Kit from Agilent Technologies (Wilmington, DE) according to the manufacturer's directions. Complementary DNA was prepared by reverse transcription using random primers with the "High Capacity cDNA Archive Kit" from AB. PCR was carried out using an ABI 7900HT Sequence Detection System with TaqMan Fast Universal PCR Master Mix also from AB as directed by the manufacturer. TaqMan Gene Expression Assays used were as follows: HAS-1 Hs00758053_m1; HAS-2 Hs00193435_m1; HAS-3 Hs00193436_m1; Hyal1 Hs00537920_g1; Hyal2 Hs00186841_m1; versican V0 Hs01007944_m1; versican V1 Hs01007937_m1; versican V2 Hs01007943_m1; versican V3 Hs01007941_m1; ADAMTS1 Hs00199608_m1; ADAMTS4 Hs00192708_m1; ADAMTS5 Hs01095523_m1; 18S Hs99999901_s1. The ABI Gene Expression Assay forward and reverse primers are proprietary. Probe sequences for the spanning exon junctions are listed in Table S1. For each group, assays were run in triplicate on RNA samples isolated from individual dishes. Messenger RNA levels were then expressed as estimated copy numbers of mRNA for the versican isoforms and HAS-1, -2, and -3 using the Relative Standard Curve Method (ABI). Standard curves with $R^2 > 0.995$ were generated using versican PCR products greater than 500 bp spanning the isoform splice junctions and full-length cDNAs for HAS-1, -2, and -3. Target isoform QRT-PCR efficiency was not affected by the other isoforms or by background nucleic acids.

HYALURONAN SIZE COMPARISON

Cells were labeled with 25 μCi/ml of [^{3}H]-glucosamine during the last 24 h of culture. Media and cell layers were isolated and digested with 300 μg/ml pronase (Roche, Indianapolis, IN) in 0.5 M Tris, pH 6.5, for 18 h at 37°C. Following digestion, the pronase was inactivated by heating to 100°C for 20 min. Radiolabled samples were then passed through a Sephadex G-50 Fine (GE Healthcare) column to remove free radiolabeled glucosamine. The relative molecular size of the hyaluronan synthesized by these cells was compared by chromatography of [^{3}H]-glucosamine-labeled glycosaminoglycans on a Sephacryl S-1000 column (GE Healthcare) eluted in 0.5 M sodium acetate, 0.025% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 0.02% sodium azide at pH 7.0. An equivalent aliquot was digested with *Streptomyces* hyaluronidase (North Star Bioproducts) before chromatography to identify radiolabeled hyaluronan [Wilkinson et al., 2004].

HYALURONAN ELSA (ENZYME-LINKED SORBENT ASSAY)

Media and cell layers were digested with 300 μg/ml pronase for 18 h at 37°C. To isolate hyaluronan from the cell layer, tissue culture dishes were rinsed with PBS and incubated in pronase in 0.5 M Tris, pH 6.5, for 18 h, scraped, and removed to Eppendorf tubes for storage. Following digestion, the pronase was inactivated by heating to 100°C for 20 min. We used a modification [Wilkinson et al., 2004] of a previously described [Underhill et al., 1993] competitive ELSA in which the samples to be assayed were first mixed with bPG (the N-terminal hyaluronan binding region of aggrecan which has been biotinylated) and then added to hyaluronan-coated microtiter

plates; therefore, the final signal is inversely proportional to the amount of hyaluronan in the sample (hyaluronan in the sample binds to bPG and competes with its binding to the microtiter plate). Specifically, Nunc Maxisorp 96-well plates were coated with an excess of hyaluronan (Sigma), which we have covalently bound to BSA to enhance its retention by the plastic, and blocked with PBS containing serum. In tubes, different amounts of hyaluronan (standard or unknown) were mixed with a single quantity of bPG, which was limiting. After incubation, the mixtures were added to the wells and the remaining free bPG bound to the hyaluronan in the wells. bPG already bound to hyaluronan was washed away. Thus, increasing amounts of hyaluronan resulted in decreasing amounts of bPG free to be retained in the wells. After the bPG had bound to the wells, a series of reagents were added to produce a colored product. Specifically, the wells were incubated with peroxidase-labeled streptavidin, which binds to biotin, followed by incubation with a peroxidase substrate consisting of peroxide, and 2,2 azinobis (3-ethylbenzthiazoline sulfonic acid) in sodium citrate buffer, pH 4.2. This gave a green colored product which absorbs at OD_{405}. This procedure results in a standard curve where the colored signal, which is proportional to the amount of bPG retained, is inversely related to the amount of hyaluronan in the sample.

STATISTICAL ANALYSIS

The Student's *t*-test was used and results are given as means ± SEM. Differences with *P*-values <0.05 were considered statistically significant.

RESULTS

CHANGES IN PROTEOGLYCAN SYNTHESIS AND ACCUMULATION IN hESC AND hESC-DERIVED CARDIOMYOCYTE CULTURES

Treatment of high-density hESC monolayer cultures with Activin A and BMP4 yielded clusters of beating cells that were prevalent throughout the culture wells as has previously been found [Laflamme et al., 2007]. In parallel experiments, 59 ± 6% of equivalently prepared differentiated cells were positive for the cardiomyocyte marker β-myosin heavy chain by immunocytochemistry while hESC cultures contained no β-myosin-positive cells (data not shown). A representative image is provided in Figure 1A. In contrast, the hESC cultures at day 0 post-differentiation consisted of dense monolayers on non-beating, fibroblast-like cells. Total proteoglycan accumulation was significantly decreased in cardiomyocyte cultures compared to hESC ($P<0.01$; Fig. 1B). [^{35}S]-sulfate-labeled extracts from media and cell layers were then analyzed by ion-exchange and molecular sieve analysis, revealing a mix of proteoglycans of different types. [^{35}S]-sulfate-labeled extracts from media and cell layers subjected to DEAE-Sephacel ion-exchange chromatography showed that proteoglycans from hESC and cardiomyocyte cultures eluted at similar positions (Fig. 2). Radiolabeled media from both hESC (Fig. 2A) and cardiomyocyte (Fig. 2B) cultures yielded a single major peak that eluted at 0.52–0.55 M NaCl, while cell layer extracts from both cultures produced a broad peak at 0.61 M NaCl with a shoulder at about 0.48–0.54 M NaCl (Fig. 2C,D). hESC cultures also contained a small peak, which was absent from the cardiomyocyte cultures, eluting at 0.10 M NaCl in both medium (Fig. 2A) and cell layer (Fig. 2C). This peak, which may contain sulfated glycoproteins, was not further analyzed.

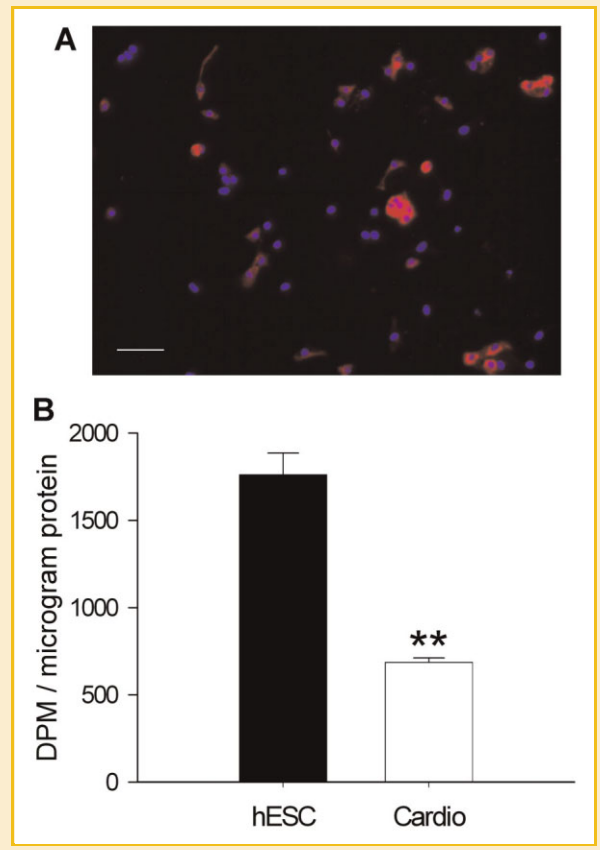

Fig. 1. Directed differentiation of cardiomyocyte cultures results in a reduction of total proteoglycan synthesis. A: hESC-CM preparations express the cardiac marker β-myosin heavy chain. This confocal image shows a representative directly differentiated hESC culture that has been immunostained with an antibody against the human striated muscle marker β-myosin heavy chain. Note that, as was typical for the preparations used elsewhere in this study, approximately 50% of the cells were immunoreactive for this marker. Scale bar = 50 μm; red: β-myosin heavy chain; blue: Hoechst dye nuclear stain. B: Proteoglycan accumulation by hESC and cardiomyocyte cultures. After 24 h incubation, total accumulated [^{35}S]-sulfate-labeled proteoglycans (cell plus medium extracts, normalized to total protein) were reduced in differentiated cells. This experiment was repeated three times with similar results. $^{**}P<0.01$ compared to hESC.

To examine the hydrodynamic sizes of proteoglycans synthesized by cultures of cardiomyocytes and hESC, [^{35}S]-sulfate-labeled extracts of the media and cell layer were subjected to Sepharose CL-2B molecular sieve chromatography which resolved the mixture into two broad peaks containing heparan sulfate and CS/DSPGs. There was generally more CS/DSPG in the larger size class than in the smaller peak. For hESC media, peak 1 eluted at $K_{av} \sim 0.12$ and peak 2 at $K_{av} \sim 0.62$, whereas the cardiomyocyte radiolabeled medium contained slightly smaller proteoglycans with peak 1 at $K_{av} \sim 0.20$ and peak 2 at $K_{av} \sim 0.65$ (Fig. 3A). Cell layer samples also showed similar elution positions with cardiomyocyte culture

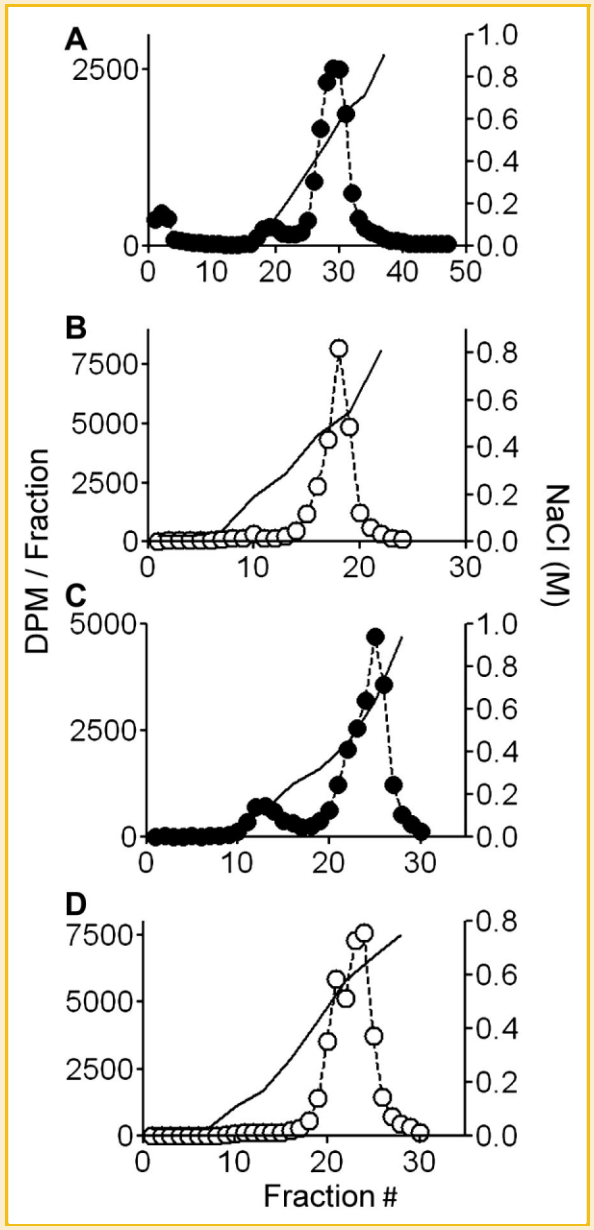

Fig. 2. Ion-exchange analysis of radiolabeled proteoglycans in hESC and cardiomyocyte cultures. [^{35}S]-sulfate-labeled proteoglycans in 8 M urea buffer were applied to DEAE sepharose and eluted with a 0–0.8 M NaCl gradient in 8 M urea buffer. A: hESC medium; (B) cardiomyocyte medium; (C) hESC cell layer; and (D) cardiomyocyte cell layer. This experiment was repeated two times with similar results.

samples shifted to a lower K_{av} relative to that of proteoglycans synthesized by hESC (Fig. 3B). Peaks 1 and 2 in hESC cell layers had K_{av} values of 0.12 and 0.62, respectively, while for cardiomyocyte cell layers, the K_{av} for peak 1 was 0.22 and peak 2 existed as a shoulder on peak 1, thereby preventing determination of its K_{av} value. Consistent with the results in Figure 1, hESC contained more proteoglycan than cardiomyocyte cultures on a per-protein basis.

To analyze the composition of the proteoglycans loaded on Sepharose CL-2B, subsequent columns were presented on the basis of equal counts. Chondroitin ABC lyase digestion (Fig. 3C–F) revealed that peak 1 contained predominantly CS/DSPGs (90% in hESC and 98% in cardiomyocyte culture media; Fig. 3C,E). Analysis of peak 1 from the hESC cell layer extracts (Fig. 3D) revealed a much lower percentage of CS/DSPGs (60%) compared to cardiomyocyte extracts (85%; Fig. 3F). Peak 2 in media contained less CS/DSPG than peak 1 with approximately 10% of incorporated [^{35}S]-sulfate in hESC (Fig. 3C) and 45% in cardiomyocyte cultures (Fig. 3E). Only a small fraction of peak 2 contained CS/DSPG in the cell layer extracts (15% in hESC and 2% in cardiomyocyte cultures; Fig. 3D,F). The elution position of chondroitin ABC lyase-resistant [^{35}S]-sulfate-labeled materials (heparan sulfate or keratan sulfate) ranges from $K_{av} \sim 0.20$ to 0.85 for all samples except differentiated cardiomyocyte media samples which eluted as a single peak at $K_{av} \sim 0.62$ (Fig. 3E). These results indicate that hESC synthesize a larger proportion of proteoglycans that do not contain chondroitin sulfate compared to cardiomyocytes.

To further characterize the proteoglycans synthesized by hESC and cardiomyocyte cultures, [^{35}S]-sulfate-labeled extracts of the media and cell layer were subjected to 4–12% gradient SDS–PAGE under reducing conditions (Fig. 4A). Four different broad bands were identified. Proteoglycans from cardiomyocyte cultures (media and cell layer) remained in the stacking gel or at the resolving gel interface (bands A and B) with a light band (C) in the cell layer. Proteoglycans from hESC also contained high molecular weight proteoglycans (bands A and B) and, in addition, smaller proteoglycans; one larger than 250 kDa (C) and one at about 200 kDa (D). Medium and cell layer extracts were digested with chondroitin ABC lyase, or heparinase I, II, and III, or a combination of all four enzymes, prior to electrophoresis on SDS–PAGE (Fig. 4B). Radiolabeled proteoglycans from cardiomyocyte cultures were completely digested by the chondroitinase plus heparinase mixture and most of the material was sensitive to chondroitin ABC lyase alone. This indicates that the small amount of material in bands B and C which is insensitive to chondroitin ABC lyase is heparan sulfate proteoglycan (HSPG). Bands A and B from hESC cultures were mostly degraded by chondroitin ABC lyase, while bands C and D were degraded by heparinase. A small amount of high molecular weight material remained after both chondroitin ABC lyase and heparinase digestion of the hESC material and thus may be keratan sulfate proteoglycan. The results from SDS–PAGE shown in Figure 4 are generally consistent with the results of molecular sieve chromatography in Figure 3. For example, the presence of a large amount of chondroitin ABC lyase-insensitive material in bands C and D from hESC, but not cardiomyocyte cell layers in gels is consistent with the results in Figure 3B where the second peak from hESC cell layers greatly exceeds the peak in the cardiomyocyte cultures and this peak is shown to be chondroitin ABC lyase resistant in Figure 3D.

VERSICAN IS SYNTHESIZED BY BOTH hESC AND CARDIOMYOCYTE CULTURES

Results from molecular sieve and SDS–PAGE analysis suggest that there are one or more large CS/DSPGs in both the hESC and

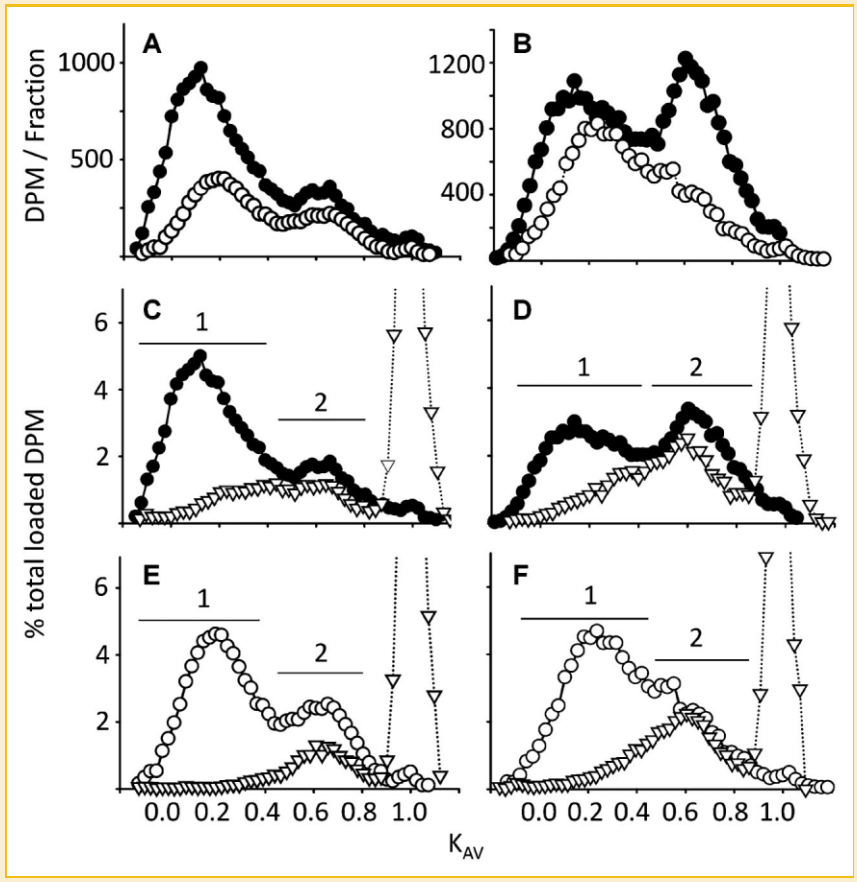

Fig. 3. Molecular sieve chromatography of radiolabeled proteoglycans synthesized by hESC and cardiomyocyte cultures. [^{35}S]-sulfate-labeled proteoglycans were characterized by size exclusion chromatography using Sepharose CL-2B. A: hESC medium (closed circles) and cardiomyocyte medium (open circles). B: hESC cell layer (closed circles) and cardiomyocyte cell layer (open circles). Samples were applied on the basis of equal protein in (A) and (B). Panels C–F depict column profiles before and after chondroitin ABC lyase digestion; results are expressed as percent of total DPM per column. C: hESC medium; (D) hESC cell layer; (E) cardiomyocyte medium; and (F) cardiomyocyte cell layer. Triangles indicate enzyme digestion. Bars indicate area used to calculate percent composition. This experiment was repeated three times with similar results.

cardiomyocyte cultures (Peak 1, Fig. 3), and bands A and B (Fig. 4). To probe for versican, core proteins were revealed by Western analysis and radiolabeling (Fig. 5). [^{35}S]-methionine-labeled proteoglycans were treated with chondroitin ABC lyase, or heparinase I, II, III, or the combination of all four enzymes prior to 4–12% gradient SDS–PAGE analysis. Following chondroitin ABC lyase digestion, two strong bands from the media and cell layers of cardiomyocytes appeared in the region above ~250 kDa, consistent with the location of V0 and V1 isoforms of versican [Sandy et al., 2001; Kenagy et al., 2006]. Although those bands are absent from the hESC cultures, there are two large bands that appeared after chondroitin ABC lyase digestion at higher molecular weights than the two bands identified in the cardiomyocyte culture extracts and just lower than the stacking–resolving gel interface in the undifferentiated cultures (Fig. 5A). To identify these bands, Western analysis was performed using an antibody against human versican (2-B-1). Surprisingly, the antibody identified the two bands from cardiomyocyte culture samples as versican and revealed that the two higher molecular weight bands from the hESC extracts were also versican (Fig. 5B).

The presence of an unusually large versican core protein in the hESC cultures could be due to differences in glycosylation. To examine this possibility, Western analysis for versican was performed on conditioned media from hESC and cardiomyocyte culture extracts after chondroitin ABC lyase digestion alone or with the addition of complete enzymatic N- and O-linked deglycosylation. After complete deglycosylation, staining with the antibody to versican revealed that the higher molecular weight bands have shifted to a lower molecular weight position which is approximately the same for both hESC and cardiomyocyte cultures. These bands are located in a position in the gel which is consistent with the molecular weights of versican splice variants, V0 and V1 (372 and 265 kDa), when calculated for core proteins with no post-translational modifications. Complete deglycosylation appears to have caused the formation of an insoluble aggregate of versican, possibly with other proteins, which fails to enter the resolving gel (Fig. 5C).

Messenger RNAs for all of the versican splice variants, V0, V1, V2, and V3, were significantly increased in the cardiomyocyte cultures compared to the hESC cultures. V0 and V1 were the most abundant mRNAs, and they both increased two- or threefold as cardiomyo-

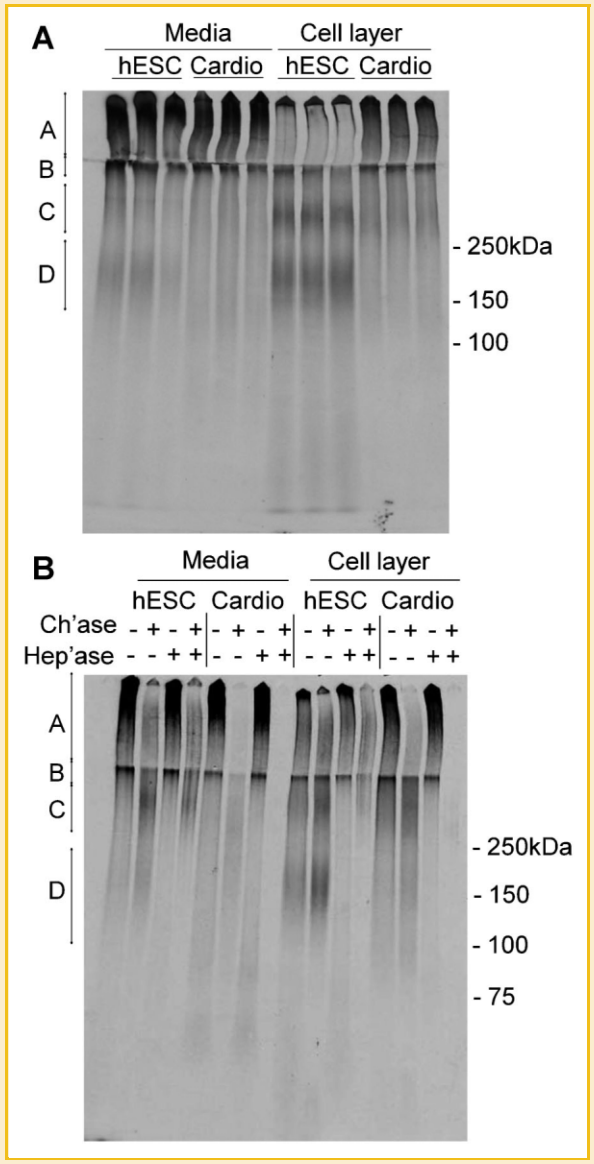

Fig. 4. Characterization of radiolabeled proteoglycans by SDS–PAGE [^{35}S]-sulfate-labeled proteoglycans were subjected to SDS–PAGE. A: Intact proteoglycans. B: Proteoglycans were digested with chondroitin ABC lyase (Ch'ase), heparinase I, II, and III (Hep'ase), or the combination of all four enzymes. This experiment was repeated three times with similar results. Data shown are from a representative experiment. Each lane contains material from a separate well.

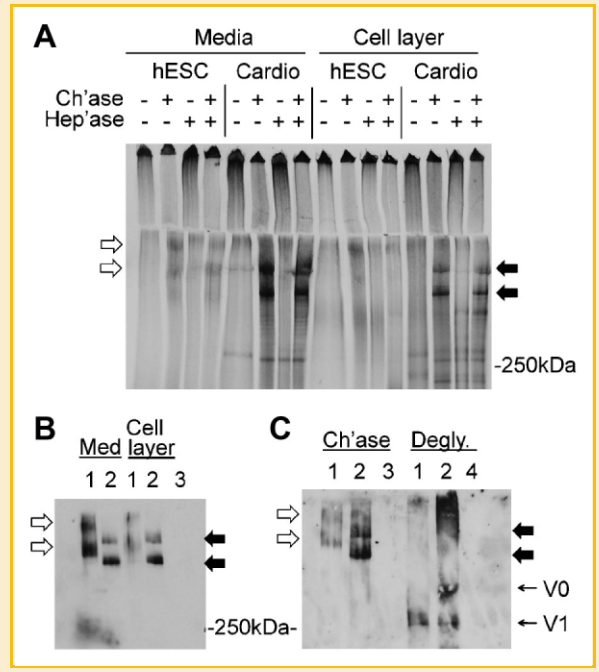

Fig. 5. Identification of proteoglycan core proteins. A: [^{35}S]-methionine-labeled proteoglycans were digested with chondroitin ABC lyase (Ch'ase), heparinase I, II, and III (Hep'ase), or the combination of all four enzymes and applied using an equal amount of radioactivity to 4–12% gradient SDS–PAGE. B: Western analysis for versican after Ch'ase digestion. Gels were loaded with equal amounts of protein. C: Western analysis of media from hESC or cardiomyocytes incubated with Ch'ase, or Ch'ase plus the total deglycosylation mixture to completely remove all N- and O-linked carbohydrates from glycoproteins. The protein content of lanes in (C) was not normalized. Lanes: 1: hESC; 2: cardiomyocyte cultures; 3: Ch'ase plus buffer; 4: Ch'ase mixture, exchanged into water, plus the deglycosylation enzymes and buffer. Arrows indicate versican after Ch'ase: solid arrows: cardiomyocyte versican; open arrows: hESC versican; fine arrows: cardiomyocyte or hESC versican after Ch'ase digestion and total deglycosylation. Experiments shown in (A) and (B) were repeated three times with similar results. The experiment shown in (C) was repeated three times, but with a single batch of conditioned medium.

cytes differentiated from hESC. Versican V2 and V3 mRNA transcripts were orders of magnitude less abundant but showed similar increases when compared to hESC (Fig. 6A). The expression of the versicanases [Apte, 2009], ADAMTS1 and 5, were increased in the cardiomyocyte cultures in comparison to the hESC cultures. No significant change was seen in ADAMTS4 mRNA. The copy number of ADAMTS1 was an order of magnitude greater than for the others (Fig. 6B). This increase in versicanase expression level may help to explain the decreased amount of CS/DSPG found in cardiomyocyte cultures in spite of the elevated levels of versican transcripts.

DIFFERENCES EXIST IN THE SYNTHESIS AND SECRETION OF HYALURONAN BETWEEN hESC AND CARDIOMYOCYTE CULTURES

Hyaluronan is frequently co-localized with versican in the ECM and binds to versican in a link-stabilized manner [Matsumoto et al., 2003]. Media and cell layer fractions were subjected to ELSA to determine the amount of hyaluronan accumulation before and after hESC differentiation. Total hyaluronan accumulation was significantly lower in cardiomyocyte cultures compared to hESC cultures (Fig. 7A). To examine the hydrodynamic size of hyaluronan, cells were radiolabeled with [^{3}H]-glucosamine and subjected to chromatography on Sephacryl S-1000 columns. All of the [^{3}H] dpm eluted above $K_{av} \sim 0.5$ were susceptible to hyaluronidase. In media samples, cardiomyocyte cultures accumulated a higher proportion of higher molecular weight hyaluronan compared to hESC cultures (Fig. 7B). Cell layers from both cell types contained similar size profiles of hyaluronan (Fig. 7C). To determine if a specific HAS isoform was involved in the differentiation of cardiomyocytes, transcripts for HAS-1, -2, and -3 were determined by QRT-PCR.

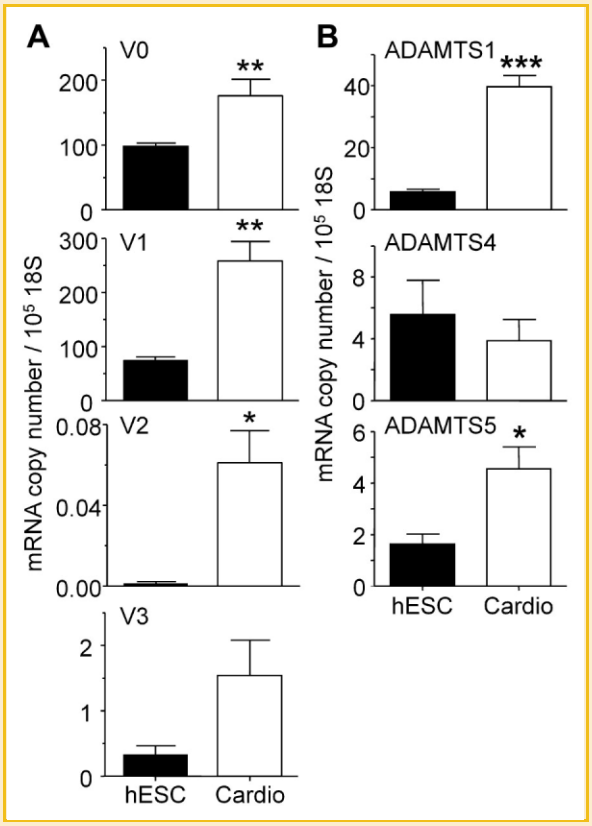

Fig. 6. Versican splice variant and ADAMTS mRNA expression by QRT-PCR. Relative copy numbers of (A) versican splice variants, V0, V1, V2, V3, and (B) versicanases, ADAMTS1, 4, and 5 were determined by QRT-PCR and normalized to the mRNA copy number for 18S ribosomal protein. This experiment was repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to hESC.

HAS-1 and -2 were the major HAS isoforms expressed in cardiomyocyte cultures and their expression levels were significantly higher than in hESC cultures. hESC expressed a high level of the HAS-3 isoform, while it was reduced 200-fold in the cardiomyocyte cultures (Fig. 8). This is consistent with the production of a large amount of lower molecular weight hyaluronan by the hESC since HAS-3 enzymes produce shorter hyaluronan molecules [Wilkinson et al., 2006]. Since hyaluronan degrading enzymes are also responsible for the existence of low molecular weight hyaluronan, we examined the expression levels of HYALs 1 and 2 [Stern, 2004]. Both are elevated in cardiomyocyte cultures in comparison to hESC cultures (Fig. 8B). So, it does not seem likely that alterations in the expression of the HYALs is responsible for the larger size of hyaluronan found in cardiomyocyte cultures. The possibility remains that the activity level of existing HYALs has been altered in some way.

DISCUSSION

We have shown that both hESC and hESC-derived cardiomyocyte cultures synthesize an ECM enriched in versican and hyaluronan.

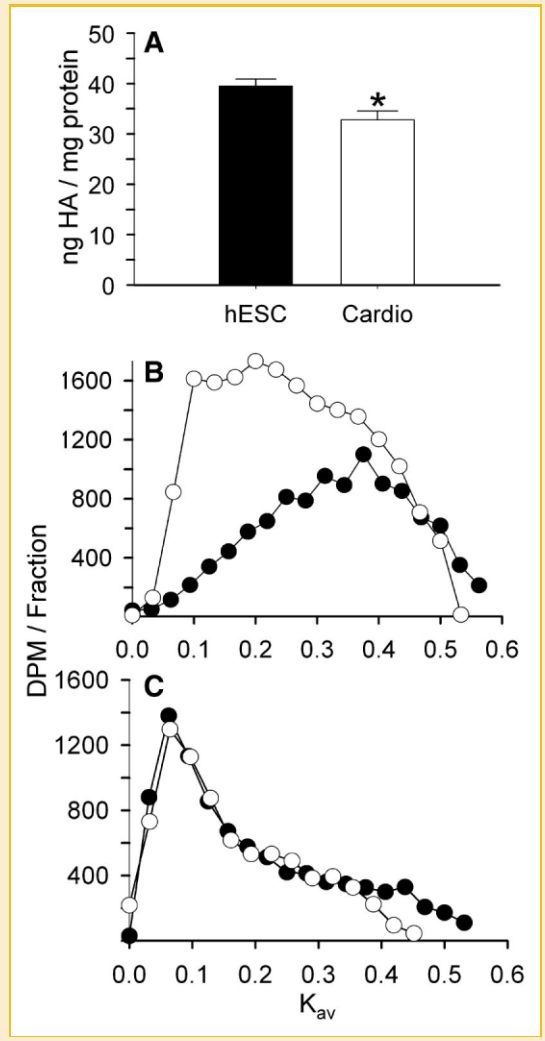

Fig. 7. Alterations in hyaluronan production which accompany differentiation of cardiomyocytes. A: Hyaluronan accumulation by hESC and cardiomyocytes. After 24 h incubation with fresh medium, hyaluronan content was determined in the total cultures and normalized to total cell layer protein. This experiment was repeated four times with similar results. $^{*}P < 0.05$ compared to hESC. B–C: Molecular sieve analysis of hyaluronan synthesized by hESC and cardiomyocyte cultures. Cultures were incubated for 24 h in medium containing [^{3}H]-glucosamine after which, cell layers and media were applied to Sepharose-S-1000 molecular sieve columns. Results show the distribution of hyaluronidase sensitive material. Open circles: cardiomyocytes; closed circles: hESC. This experiment was repeated two times with similar results. B: medium; C: cell layer.

While total proteoglycan and hyaluronan accumulation decreases as cardiomyocytes differentiate from hESC, there are both quantitative and qualitative differences in the proteoglycans and hyaluronan synthesized by the two sets of cells. While the proteoglycans synthesized and secreted into the media by both by hESC and cardiomyocyte cultures contain chondroitin sulfate, hESC deposit considerably less CS/DSPG and more HSPG in their ECM than cardiomyocyte cultures. HSPGs have been linked to hESC self-renewal and pluripotency, and reduction in HSPGs in hESC promotes their differentiation [Sasaki et al., 2008]. The importance

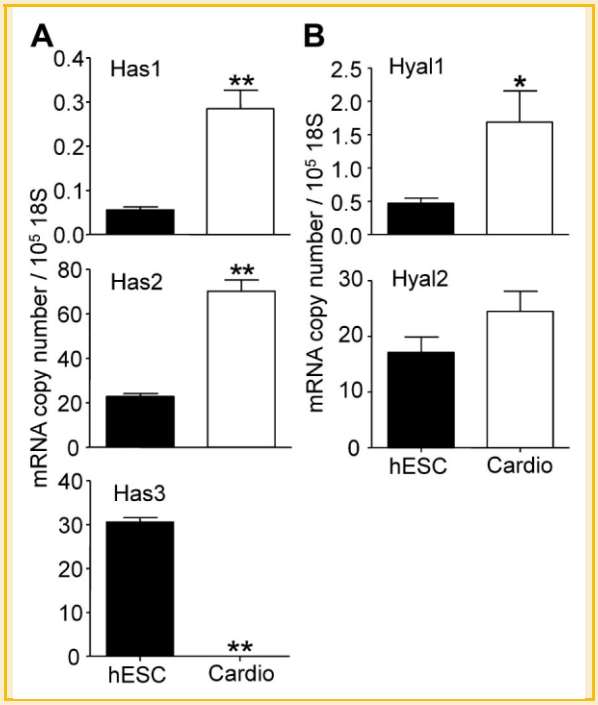

Fig. 8. HAS and HYAL mRNA expression by hESC and cardiomyocyte cultures. Differentiated and undifferentiated cultures were fed fresh medium and then harvested after 24 h for mRNA analysis. Levels of mRNA expression of (A) HAS-1, -2, or- 3 or (B) HYALS 1 or 2 were determined by QRT-PCR and expressed as mRNA copy number per 10^5 18S. This experiment was repeated three times with similar results. $^{**}P < 0.01$ compared to control.

of HSPGs appears to be related to their abilities to control extrinsic signaling pathways contributing to hESC self-renewal and pluripotency. The loss of HSPGs from undifferentiated hESCs as they differentiate into cardiomyocytes suggests that they play a key role in controlling cardiomyocyte differentiation. Although total proteoglycan synthesis and accumulation decreased as cardiomyocytes underwent differentiation, the expression of versican increased. Analyses of the mRNA transcripts for the different versican variants, V0, V1, V2, and V3, reveal that all isoforms increased in the cardiomyocyte cultures in comparison to the hESC, and that the V1 variant was the most abundant in cardiomyocyte cultures. The significance of the individual versican variants in cardiomyocyte differentiation is not known. Versican expression is controlled by signaling molecules such as Wnt/β catenin and PI3K/Akt [Rahmani et al., 2005, 2006], which have also been shown to regulate cardiomyocyte differentiation [Ueno et al., 2007]. A causal link between the signals that regulate the expression of these ECM molecules and cardiomyocyte differentiation awaits further study. Versican expression has been shown to be associated with cell proliferation [Zimmermann et al., 1994; Bode-Lesniewska et al., 1996; Wight, 2002], but the role of the individual splice variants in cell proliferation, and in cardiomyocytes specifically, has not been systematically investigated. Our previous studies have shown that overexpressing the V3 variant form of versican in ASMCs slowed down their growth, while the larger forms of versican are associated with proliferating cells [Lemire et al., 2002]. Such results suggest that V3 expression is more conducive to the differentiation phenotype. In addition, versican that is synthesized by hESC contains a higher proportion of N- and O-linked oligosaccharides attached to the protein core than versican synthesized by cardiomyocyte cultures. The significance of the loss of N- and O-linked glycosylation from versican as cardiomyocytes differentiate is uncertain at the present time. However, it is of interest that specific glycan structures attached to proteins have been used to define undifferentiated stem cells [Kannagi et al., 1983; Badcock et al., 1999; Muramatsu and Muramatsu, 2004]. While the pattern of N-linked glycans is known to differ between undifferentiated and differentiated hESC [Satomaa et al., 2009], the functional consequences of these differences are not understood. Such differences may play a role in the ability of versican to interact either with growth factors, other ECM components, or other molecules; thereby affecting its processing and turnover; or otherwise influencing its biological activity. Evaluation of the biological significance of this finding awaits further investigation.

The hyaluronan that is synthesized and secreted by hESC is of lower molecular weight than the hyaluronan synthesized and secreted by cardiomyocyte cultures. This increase in molecular weight is accompanied by an increase in the expression of HAS-1 and HAS-2 mRNA, in the cardiomyocyte cultures, with HAS-2 being the most abundant. Interestingly, HAS-3 was greatly reduced or absent from the cardiomyocyte cultures, but expressed by the hESC. mRNA levels for the hyaluronidases, HYAL1 and 2, were unchanged or increased in the differentiated cultures. Hyaluronan is a predominant ECM component in the embryonic environment [Toole, 2001] and thought to be important in the self-renewal and differentiation of hESCs [Gerecht et al., 2007]. Thus, it may be that the size, quantity, and source of hyaluronan help to regulate differentiation of hESC. HAS-3 has been shown to synthesize lower molecular weight hyaluronan [Wilkinson et al., 2006]. Therefore, its absence may be partially responsible for the larger molecular weight hyaluronan found in cardiomyocyte cultures. Although we have found elevated levels of hyaluronidase mRNA in the cardiomyocyte cultures, it has not resulted in smaller hyaluronan. Increased hyaluronan turnover, mediated by the HYALS, may be required for changes in its location on the cell surface or ECM. In other systems, hyaluronan fragments regulate angiogenesis, differentiation, embryogenesis, wound repair, and inflammation using different signaling mechanisms which are dependent upon fragment size [Stern et al., 2006]. In addition, we recently observed that hyaluronan is present in developing embryoid bodies (EB) and co-localizes with versican in the vicinity of cells undergoing epithelial to mesenchymal transitions during EB differentiation [Shukla et al., 2010].

PARTICIPATION OF VERSICAN AND HYALURONAN IN CARDIOGENESIS

Versican expression has been observed in areas of the developing myocardium involved in differentiation, rather than in zones associated with active and rapid proliferation. Versican may play a role in cardiomyocyte differentiation by directing a switch from

rapid proliferation to differentiation. Cardiomyocytes undergo terminal differentiation in the ventricle during the neonatal period when versican is completely switched off. This suggests that versican must be down-regulated before terminal differentiation can take place [Henderson and Copp, 1998]. It is of interest that recent studies show that microRNA-138 represses versican expression in the heart ventricles but allows versican expression in the AV canal. Knockdown of this microRNA leads to ventricular expansion and abnormal ventricular cardiomyocyte development [Morton et al., 2008]. Such studies highlight the need to fine tune the expression of versican during heart development within specific regions of the heart.

The different splice variants of versican may control different aspects of cardiomyocyte differentiation and heart development. For example, Kern et al. [2007] recently transduced mouse embryonic cardiomyocytes with V3 and noticed a marked reduction in proliferation of the cardiomyocytes and a significant increase in myocardial cell–cell association. Furthermore, injection of the adenovirus-containing V3 into the heart field of a developing mouse heart led to an increase in the outflow track myocardium and at least a twofold increase in the compact layer of the ventricular myocardium. Such findings indicate that the non-cleavable, V3 form of versican, may lead to increased myocardial cell survival and stabilization of the myocardial cell layer. On the other hand, the other splice variants of versican, V0, V1, and V2, can be cleaved by matrix metalloproteinases (MMPs) such as several members of the disintegrin and metalloproteinase with a thrombospondin type-1 motif (ADAMTS) family [Kenagy et al., 2006] and may promote the early proliferative phases, but interfere with myocardial cell–cell aggregation and the growth and thickening of the myocardium as it develops [Kern et al., 2007]. Our finding of elevated ADAMTS1 and 5 in the differentiating cardiomyocyte cultures may indicate a need, not only for new synthesis, but also an involvement of versican turnover with cardiomyocyte differentiation.

In conclusion, we have defined changes in the production of specific components of the ECM that occur as cardiomyocytes differentiate from hESC. It is well known that stem cells lie in specialized ECM environments that help maintain and promote differentiation and survival [Scadden, 2006]. The involvement of hyaluronan and versican as part of the cardiomyocyte niche suggests that these molecules play a role in regulating cardiomyocyte differentiation; perhaps through controlling the mechanical properties of the microenvironment around the differentiating stem cells [Engler et al., 2006; Kurpinski et al., 2006], or as a depot to attract and store growth factors and other cytokines necessary for cardiomyocyte differentiation [Haylock and Nilsson, 2005; Li and Xie, 2005; Moore and Lemischka, 2006]. A causative role for these specific ECM components in cardiomyocyte differentiation awaits further study.

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