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function initSelector(element){ $('.skiptocontent').removeAttr("href"); $(element).parent().before(""); window.location.hash = '#top'; $(window).scrollTop($("#top").offset().top-100); window.location.hash=''; } $('#skiptocontent').keydown(function (e) { var code; try { code = (window.event) ? window.event.keyCode : event.which; } catch(err) { code = e.keyCode || e.which; } //click Enter if(code == 13){ var mainPageId=$("#main-page-content").text(); if(mainPageId){ initSelector('#main-page-content'); }else{ var firstH1=$('h1:first').text(); if(firstH1){ initSelector('h1:first'); }else{ $('#skiptocontent').css('display','none'); } } } });

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img.loggedInArrow').hide(); //$('.myprofile-label').text("Sign In"); }; $('#portalLoginBar .sage-login-widget').attr('tabindex', '0'); $('.sage-login-widget img.user-logo').each(function(){ //console.log($(this).attr('src')); if($(this).attr('src').indexOf('templates')===-1) $(this).addClass('bannerImage'); else $(this).removeClass('bannerImage'); }); initMyProfileInfo(); initInstitutionInfo(); initSocietyInfo(); if (inPbEditorMode()) $('.sage-login-widget').attr('onclick', 'toggleLoginPopup(true);return false;'); if (isIE()) { $("img.user-logo").each(function () { let imgUrl = $(this).prop("src"); if (imgUrl) { $(this).css("backgroundImage", 'url(' + imgUrl + ')').addClass("ie-object-fit"); $(this).prop("src",""); } }); } }

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'+$user.attr('name')+'

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'; } else { appendTag+='

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'; appendTag+='

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'; } $('#inst-login-status').append(appendTag); } function setRedirectUrl() { let currentUrl =

window.location.pathname; $('.Shibboleth').attr("href", $('.Shibboleth').attr('href') + currentUrl); $('.OpenAthens').attr("href", $('.OpenAthens').attr('href') + currentUrl); } function initInstitutionInfo() { setInstitutionLoginStatus(); setRedirectUrl(); if ($('.id-institution-activated>img.user-logo').attr('title')===undefined) $('.id-institution-activated>img.user-logo').attr('title', 'You are signed in via your institution'); $('.id-institution-deactivated>img.user-logo').attr('title', 'You are not signed in via an institution'); $('#institution-info .portalInsitutionalButton').after('

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$('#ru-society').attr('href', '/action/doLogout?redirectUri='+window.location.href); function restyleJournalAd(){ if ($('#society-login-form .literatumAd').length!==0) { $('#society-login-form #society-info-text, #society-login-form .topSeparator').hide(); } } function initSocietyInfo() { if ($('.id-society-activated>img.user-logo').attr('title')===undefined) $('.id-society-activated>img.user-logo').attr('alt', 'You are signed in via your society'); $('.id-society-deactivated>img.user-logo').attr('title', 'You are not signed in via a society'); $('#society-info .portalInsitutionalButton').after('

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if ('2.2830.2360.0040.0003.97.41.0702.3600.900Google ScholarCiteScoreSCImago Journal Rank (SJR)117360616'.trim().length>0) $('.impactFactorContainer').removeClass('not-show-important'); if ($(".impact-factor-container") && $(".impact-factor-container").size()>0) $("#showAllSocietiesBtn").addClass("ifBorder"); switch ($('#showNoFoldedSocietyLogos .societyImageLink').size()) { case 2: $('#showNoFoldedSocietyLogos').addClass('two-logos'); break; case 1: $('#showNoFoldedSocietyLogos').addClass('one-logo'); break; case 0: default: break; } function resizeHeaderFont() { var headerTitleElement = document.getElementById('headerTitle'); if (headerTitleElement) { var fontsize = 32; if ("" && "FALSE"==="TRUE") fontsize=28; $('#headerTitle').css('font-size', fontsize+"px"); /*Max font size, then reduce from there*/ $('#headerTitle h1').css('font-size', fontsize+"px"); /*Max font size, then reduce from there - journal home only*/ var headerTitleSize = headerTitleElement.getBoundingClientRect(); var textHeight = headerTitleSize.height; var textWidth = headerTitleSize.width; var containerElement = document.getElementById('headerTitleContainer'); var containerSize = containerElement.getBoundingClientRect(); var containerHeight = containerSize.height; var containerWidth = containerSize.width; var fontstring = ""; while (textHeight > containerHeight) { fontsize--; fontstring = fontsize.toString(); fontstring = fontstring + "px"; $('#headerTitle').css('font-size', fontstring); $('#headerTitle h1').css('font-size', fontstring); headerTitleSize = headerTitleElement.getBoundingClientRect(); textHeight = headerTitleSize.height; textWidth = headerTitleSize.width; } } }; resizeHeaderFont(); $(window).resize(function() { resizeHeaderFont(); });

function offset(el) { let rect = el.getBoundingClientRect(), scrollLeft = window.pageXOffset || document.documentElement.scrollLeft, scrollTop = window.pageYOffset || document.documentElement.scrollTop; return { top: rect.top + scrollTop, left: rect.left + scrollLeft, bottom: rect.bottom + scrollTop , right: rect.right + scrollLeft } } window.addEventListener("scroll", function() { let y = window.pageYOffset; let $quickSearchId = $("#journalQuickSearch").parent(); if (y

>= offset(document.getElementById("portalQuickSearch")).bottom) { $quickSearchId.removeClass("doNotShow"); } else { $quickSearchId.addClass("doNotShow"); } });

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Keywords pulmonary arterial hypertension, versican, vascular smooth-muscle cell, hypoxia, mechanical strain

Pulmonary hypertension is defined as a mean pulmonary arterial pressure Ã¢Â‰Â¥25 mmHg at rest. In the subgroup of patients with pulmonary arterial hypertension (PAH), the pathological changes behind the increased pressure are precapillary.[1] The vasculopathy in PAH is characterized by endothelial dysfunction, inflammation, and vascular remodeling, which leads to an increase in vascular resistance and eventually to right heart failure. Histopathological findings in PAH include extension of muscularization distally into previously nonmuscularized arteries, medial hypertrophy (vascular wall thickening), intimal hyperplasia (neointima), fibroproliferative changes in the adventitia, and plexiform lesions. In plexiform lesions, the hallmark of PAH, abnormal proliferation of endothelial cells and ÃŽÂ±-actinÃ¢Â€Â“positive cells and recruitment of many other cell types, including progenitor cells (e.g., $CD31^{+}$/$c\text{-}kit^{+}$ endothelial progenitors and circulating fibrocytes), contribute to obliteration of small pulmonary arteries and formation of complex capillary-like channels.[2Ã¢Â€Â“5] Pulmonary artery smooth-muscle cell (PASMC) hypertrophy, proliferation, migration, and antiapoptosis properties are thought to play key roles in the pathology of PAH.[6]

PASMCs synthesize and secrete bioactive extracellular matrix (ECM) proteins (e.g., elastin and collagens), which have been shown to play important roles in control of cell migration and proliferation during development of PAH.[7,8] Versican is a large and complex chondroitin sulfate proteoglycan produced by smooth-muscle cells (SMCs).[9] The versican core protein contains an N-terminal G1 domain containing a hyaluronan binding region, a C-terminal G3 domain, and glycosaminoglycan (GAG)Ã¢Â€Â“attachment regions between the two, the ÃŽÂ±GAG and ÃŽÂ²GAG domains.[10] Alternative splicing generates at least 4 isoforms, which vary in the GAG attachment regions. While both ÃŽÂ±GAG and ÃŽÂ²GAG are present in V0, a single GAG-

attachment domain is found in V1 (ÃŽÂ²GAG) and V2 (ÃŽÂ±GAG), and V3 lacks both ÃŽÂ±GAG and ÃŽÂ²GAG. All 4 isoforms can be present in the vascular ECM.[11]Ã¢Â€Â“[13] However, whether versican has a role in the vascular remodeling process of PAH is unclear.

Versican has a pivotal role in modulating cell behavior, including cell adhesion, migration, proliferation, and assembly of ECM.[14] Versican expression is low in most tissues but increases dramatically in a number of different pathologies, such as diseases of the lung and the cardiovascular system, cancer, and autoimmune conditions.[15]Ã¢Â€Â“[18] In vascular pathology in the systemic circulation, versican is upregulated in restenosis after vascular intervention as well as in atherosclerotic plaques, where versican mediates cell proliferation and lipid retention.[16,19,20] Experimentally, inhibition of versican synthesis in SMCs leads to reduced proliferation.[21] On the other hand, overexpression of V3, the versican variant without GAG-attachment domains, inhibits SMC growth and migration.[22] In pulmonary pathology, versican has been identified as a macrophage activator in lung cancer, and increased versican production by bronchial fibroblasts has been linked to airway hyperresponsiveness in patients with asthma.[23,24] Fibroblasts derived from distal airway from patients with chronic obstructive pulmonary disease (COPD) have also been shown to produce increased levels of versican compared with cells from control subjects.[25] Furthermore, increased immunostaining for versican in patients with moderate COPD has been shown to correlate with a decrease in elastin-binding protein.[26] This indicates that versican may be involved in elastin regulation in pulmonary pathology.

Recent studies have shown that inflammation is likely to be one of the principal factors driving vascular remodeling in PAH.[27] Aberrant $CD4^+$ T cell function and accumulation of $CD68^+$ macrophages and immature dendritic cells lead to perivascular inflammation in PAH. In addition, overproduction of cytokines and chemokines (e.g., interleukin [IL] type 1, IL-6, and macrophage inhibitory factor) has been shown to influence the proliferative response of PASMCs.[28]Ã¢Â€Â“[30] Versican may affect inflammatory responses by interactions with receptors on inflammatory cells (e.g., CD44, P-selectin glycoprotein ligand 1, and P- or L-selectins) or by interactions with other ECM components of importance for inflammation (e.g., hyaluronan and fibronectin).[31] Versican, together with hyaluronan, tumor necrosis factorÃ¢Â€Â“inducible gene 6 protein, and inter-ÃŽÂ±-trypsin inhibitor, can form organized filaments permissive for leukocyte adhesion.[32] A viscous hyaluronan- and versican-rich matrix has also been shown to impede T cell migration, which could play a role in T cell trafficking and function in inflamed tissues.[33] High levels of hyaluronan, which can bind to versican G1 domain, have been found in plasma and explanted human pulmonary artery smooth-muscle cells (hPASMCs) from patients with PAH.[34] In addition, Lauer et al.[35] recently showed that the hyaluronan that accumulates in patients' pulmonary vessels is modified by covalently bound heavy chains from plasma-derived inter-ÃŽÂ±-trypsin inhibitor. This heavy chainÃ¢Â€Â“hyaluronan complex was also shown to partially colocalize with leukocyte infiltration areas in plexiform lesions of PAH, indicating that hyaluronan may influence the inflammatory response in PAH.[35] However, the role of versican in the immuno-pathology of PAH has not been explored.

Since the pulmonary circulation is a low-resistance and low-oxygen system, the knowledge gained from studies of systemic arteries about the roles of vascular proteoglycans may not be fully applicable. In this study, we aimed to investigate the role of versican in pulmonary vascular remodeling in PAH. We examined whether versican is increased in different types of vascular lesions in PAH of different etiology in humans and explored the association between versican and inflammation. We also tested whether increased mechanical strain or hypoxia could influence versican production in hPASMCs, given the pathophysiological environment in the pulmonary

vasculature in PAH.

METHODS

Section:
Choose

Lung tissues from patients with pulmonary hypertension and failed donor lungs

Paraffin-embedded lung sections and frozen lung tissue from patients with PAH who had undergone lung transplantation and control samples from unused donor lungs were provided by Marlene Rabinovitch under the Pulmonary Hypertension Breakthrough Initiative and obtained from the pathology biobank at SkÃƒÂ¥ne University Hospital, Lund, Sweden (approved by the regional ethical review board, 534/2005 and 248/2010). Samples used for this project had been completely deidentified. In total, tissue from 15 patients with idiopathic PAH (IPAH), 3 patients with scleroderma, 4 patients with PAH associated with congenital heart disease, and 15 control subjects were used for this study.

Immunohistochemistry

After deparaffinization and rehydration, endogenous peroxidase activity was quenched using 0.7% hydrogen peroxide in 100% methanol for 20 minutes, and then the sections were pretreated with chondroitinase ABC (0.2 units/mL) in 18 mM Tris-acetate buffer pH 8.0 containing 0.1% bovine serum albumin (BSA) for 1 hour at 37Ã‚Â°C, followed by 1 hour of blocking with 3% nonfat dry milk and 2% BSA in phosphate-buffered saline (PBS) buffer. Primary antibody (mouse monoclonal antiversican, clone 2B1, Seikagaku) was applied for overnight incubation at 4Ã‚Â°C. PBS buffer was used for all washing steps. The sections were then incubated with a biotinylated goat anti-mouse secondary antibody (1 : 300, Zymed) for 1 hour at room temperature. After detection with Vectastain Elite ABC kit and DAB HRP substrates (Vector Labs), the sections were counterstained with hematoxylin.

For double staining of versican and leukocytes, a mouse monoclonal antihuman CD45 antibody was used (1 : 100, clones 2B11 + PD7/26, Dako) and heat-induced antigen retrieval with citrate buffer for 20 minutes was added to the protocol above. The double staining was performed using a Leica Bond-Max automated immunostainer. The incubation with primary antibodies was done for 1 hour at room temperature, and DAB HRP was used as substrate for versican and Vector Red for CD45.

Double immunofluorescence

After deparaffinization and heat-induced antigen retrieval with citrate buffer, the lung sections were treated with chondroitinase ABC (0.2 units/mL) in 18 mM Tris-acetate buffer pH 8.0 containing 0.1% BSA for 1 hour at 37Ã‚Â°C, followed by 20 minutes of blocking with BlockAid blocking solution (Thermo Fisher Scientific). The sections were then incubated with rabbit monoclonal antiversican G3 domain antibody (1 : 100, ab177480, Abcam) overnight at 4Ã‚Â°C. After washing with PBS buffer, cyanine Cy3 goat antirabbit immunoglobulin G (IgG; 1 : 200, Jackson ImmunoResearch) and fluorescein isothiocyanateÃ¢Â€Â“conjugated mouse monoclonal antiÃ¢Â€Â“smooth-muscle ÃŽÂ±-actin antibody (1 : 500, ab8211, Abcam) were applied simultaneously for 1 hour at room temperature. Following washing with PBS buffer, the slides were mounted with ProLong Diamond Antifade Mountant with 4Ã¢Â€Â²,6-diamidino-2-phenylindole (Thermo Fisher Scientific).

Proteoglycan extraction and Western blot

Proteoglycan extraction from cells. Cells were solubilized in 8 M urea buffer (8 M urea, 50 mM Tris-base, 0.5% Triton X-100, 0.25 M NaCl, 2 mM EDTA, pH 7.4) with protease inhibitors (5 mM benzamidine, 100 mM 6-aminohexanoic acid, and 1 mM phenyl-methylsulfonyl fluoride). After measuring the protein concentration by Coomassie (Bradford) protein assay (Pierce), equal protein aliquots from each sample were used for purification of proteoglycans by ion-exchange chromatography on diethylaminoethyl (DEAE) Sephacel in 8 M urea buffer. The columns were washed with 8 M urea buffer and eluted with 8 M urea buffer containing 2 M NaCl. Isolated proteoglycans were purified by ethanol precipitation as described elsewhere.[36] After chondroitinase ABC digestion (1.67 units/mL) for 3 hours at 37Ã‚Â°C, the samples were reduced and electrophoresed on a 4%Ã¢Â€Â“12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were then transferred to nitrocellulose membranes using a Bio-Rad Transblot SD Semi-Dry Transfer Cell. After incubation with the blocking buffer (10% Aqua Block in Tris-buffered saline [TBS] containing 0.1% Tween-20) for 2 hours at room temperature, membranes were incubated with a goat polyclonal antibody against the human versican ÃŽÂ²GAG domain (1 : 2000, AF 3054, R&D systems) overnight at 4Ã‚Â°C in blocking buffer. TBS containing 0.1% Tween-20 was used for all washing steps. After incubation with fluorescence-conjugated secondary antibody for 1 hour and washing, membranes were scanned by Odyssey CLx infrared imaging system (LI-COR). Protein band signal intensities were measured by Image Studio Lite software (LI-COR).

***Proteoglycan extraction from tissues*.** Frozen lung tissue from each subject was homogenized with 4 M guanidine buffer (4 M guanidine HCl, 100 mM sodium sulfate, 100 mM Tris-base, 2.5 mM Na_2 EDTA, 0.5% Triton X-100, pH 7.0) and then dialyzed extensively against 7 M urea buffer (7 M urea, 50 mM Tris-base, 2 mM EDTA, 0.5% Triton X-100, pH 7.5). Protein concentration was determined by Coomassie (Bradford) protein assay. Equal amounts of total proteins of each sample were subjected to ion exchange chromatography as described above. Equal volumes of isolated proteoglycans were ethanol precipitated, digested with chondroitin ABC lyase, and electrophoresed on 4%Ã¢Â€Â“12% SDS-PAGE gels with 3.5% stacking. The samples were then subjected to Western blot analysis as described above. Two different primary antibodies were used to detect versican, a goat polyclonal antibody against ÃŽÂ²GAG domain (1 : 3000, AF 3054, R&D systems) and a mouse monoclonal antibody against C-terminal G3 domain (2B1, 1 : 3000, Seikagaku).

Cell culture

The hPASMCs and aortic SMCs were obtained from Life Technologies. Medium 231 and smooth-muscle growth supplement were used as growth medium for cell culture propagation. Final concentration of fetal bovine serum in the growth medium was 5%. Cells with passage 5Ã¢Â€Â“9 were used for in vitro experiments.

Cyclic stretch

Silicon chambers (self-made; inner dimensions: 4 cm ÃƒÂ— 4 cm ÃƒÂ— 4 cm) with newly attached bottoms were sterilized and coated with bovine fibronectin (10 ÃŽÂ¼g/cm^2) as described elsewhere.[37] The hPASMCs were plated on the coated chambers with the cell density of 8,000 cells/cm^2. After 72 hours of culture with growth medium, the medium was changed to DMEM containing 0.4% fetal bovine serum, and 10% of biaxial cyclic stretch was applied to the silicone chambers with the frequency of 0.5 cycles per second. After 6 hours of cyclic stretch, the medium was collected, and the cell layer was lysed for additional studies. For metabolic labeling experiments, 50 ÃŽÂ¼Ci/mL of [^{35}S]sulfate was added during 6 hours of cyclic stretch. To ensure that our biaxial mechanical stretch device had the same effect on versican production in aortic SMCs as previously described with a different device and lower degree of mechanical stretch, human aortic SMCs from the same donor as hPASMCs were used as positive controls.[38]

One percent hypoxia

The hPASMCs were plated on 100-mm Petri dishes (8,000 cells/cm^2) and cultured in growth medium for 72 hours. At this time point, the cultures were subconfluent. The medium was then changed to DMEM with 0.4% fetal bovine serum. Cells in the hypoxic group were incubated in an Invivo$_2$ 300 hypoxia workstation (Ruskinn) with 1% O_2 and 5% CO_2 for 24 hours at 37Ã‚Â°C. Normoxic control cells were incubated in a regular cell incubator (5% CO_2). After 24 hours, the medium was collected, and the cell layer was lysed for RNA purification or proteoglycan extraction.

Metabolic labeling and gel filtration chromatography

A total of 1 ÃƒÂ— 10^6 SMCs were metabolically labeled with 50 ÃŽÂ¼Ci/mL of [^{35}S]sulfate (PerkinElmer) in DMEM with 0.4% fetal bovine serum for 6 hours. After labeling, the medium was collected. The cells were washed with PBS, incubated in solubilization buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.15 M NaCl) for 30 minutes at 4Ã‚Â°C, and then centrifuged at 800 *g* for 10 minutes. The supernatants from the cell lysates and the medium samples were applied on 0.2-mL columns of DEAE-Sephacel (GE Healthcare), equilibrated with 50 mM Tris-HCl pH 8, 0.1% Triton X-100, 0.15 M NaCl. Following washing of the DEAE-Sephacel column with equilibration buffer and a second washing with sodium acetate buffer (50 mM NaAc pH 4, 0.1% Triton X-100, 0.15 M NaCl), the [^{35}S] sulfate-labeled proteoglycans were eluted with 50 mM NaAc pH 4, 0.1% Triton X-100, 2 M NaCl. Protease inhibitor cocktail (Roche) was used during solubilization and the whole process of ion exchange chromatography.

To release the [^{35}S]sulfate-labeled GAG chains from their core proteins, a portion of the elute was

treated with 0.5 M NaOH overnight at 4Ã‚Â°C. [39] After neutralization to pH 7.4, desalting in ultra-pure water on an NAP-10 column, followed by lyophilization, half volume of the samples were treated with chondroitinase ABC digestion (0.01 units/ÃŽÂ¼L) overnight at 37Ã‚Â°C. The treated and untreated samples were subsequently analyzed by gel filtration on Sephadex G50 eluted with 0.2 M NaCl. For analysis of [^{35}S]sulfate-labeled proteoglycans, the DEAE-Sephacel purified samples were applied to a Sepharose CL-2B column (10 mm Ãƒ Â— 90 cm) in the buffer containing 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 1 M NaCl; 0.5 mL-fractions were collected and analyzed for [^{35}S]sulfate-radioactivity.

RNA isolation and quantitative polymerase chain reaction (qPCR)

RNA extraction was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Deoxyribonuclease was applied to remove genomic DNA. Total RNA integrity, RNA purity, and concentration were measured by a 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). To make complementary DNA (cDNA), 1 ÃŽÂ¼g of each RNA sample with RNA integrity number 10 was used for reverse transcription by a high-capacity RNA-to-cDNA kit (Applied Biosystems). For each 20-ÃŽÂ¼L amplification reaction, 50 ng of cDNA was used. Real-time PCR was done using Applied Biosystems 7900HT. The following TaqMan gene expression assays were applied: total versican, Hs00171642_m1; versican V0, Hs01007944_m1; versican V1, Hs01007937_m1; versican V2, Hs01007943_m1; and versican V3, Hs01007941_m1. ÃŽÂ²2 microglobulin (Hs99999907_m1) was used as reference gene. Fold changes in gene expression were calculated using the relative quantification (ÃŽÂ”ÃŽÂ”C_t) method. Individual sample values were normalized to ÃŽÂ²2 microglobulin levels. Gene expression data from 3 independent batches of cells in quadruplicates were combined before statistical analyses.

Statistical analysis

IBM SPSS statistics version 21 software was used for statistical analyses. Student t test was used for comparisons between two groups. Data were presented as mean Ã‚Â± SEM. A *P* value <0.05 was considered significant.

RESULTS

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Versican accumulates in vascular lesions of patients with PAH

The presence of versican in vascular lesions of PAH was demonstrated by immunohistochemistry (Fig. 1). In PAH of patients with IPAH, in PAH secondary to scleroderma, and in PAH secondary to congenital heart disease, strong staining for versican was seen in neointima, in hypertrophic media, and in plexiform lesions (Fig. 1*A*, 1*B*). In contrast, little versican was found in the vessels of control subjects (Fig. 1*B*). The increased deposition of versican in PAH was confirmed by Western blotting of lung tissue homogenates using 2 different antibodies, one for the G3 domain (same as for immunohistochemistry) and one for the ÃŽÂ²GAG domain (Fig. 2). The Western blots revealed multiple bands in lung homogenates from patients with IPAH, which may represent breakdown products of versican. Similar results have been reported in differentiating embryoid bodies accompanied with increased expression levels of versican degradation enzymes.[36] Notably, versican levels were consistently lower in one of the patients with IPAH, possibly due to fewer vascular lesions in the specimen.

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Figure 1. Versican accumulates in the vascular lesions of individuals with pulmonary arterial hypertension (PAH). A, Immunohistochemistry for versican (in brown) in different vascular lesions. *B*, Representative images of versican staining in lungs from patients with PAH of different etiologies. A monoclonal antiversican antibody (clone 2B1) against the C-terminal G3 domain was used for immunostaining. Images were captured with a x20 objective. One section per subject was analyzed (n = 7 for idiopathic PAH [IPAH], n = 7 for failed donor lung as control, n = 3 for atrial septal defect with PAH [ASD], and n = 3 for scleroderma with PAH [Scl]). Donor: healthy donor lungs unused for transplantation as controls; Neg: mouse immunoglobulin G used as negative control.

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Figure 2. Western blots confirm increased levels of versican in the lungs of patients with pulmonary arterial hypertension (PAH). Proteoglycan extracts from lung tissue homogenates treated with the chondroitinase ABC were used. For each sample, 1.5 mg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, and immunoblotting for versican was performed using two different antibodies, mAb 2B1 for the C-terminal G3 domain (*A*) and pAb 3054 for the ÃŽÂ²GAG domain (*B*). Samples from 4 patients with idiopathic PAH (IPAH)

were compared with age-matched unused donor lungs (control). Increased versican fragments (lanes 5, 6, and 8) were observed in subjects with IPAH compared with age-matched control subjects. *C*, Schematic drawing of versican splice variants and recognition sites for mAb 2B1 and pAb 3054. Versican has two globular domains, the G1 domain at the amino terminus and the G3 domain at the carboxy terminus. The splice variants vary in the GAG-attachment domains (ÃŽÂ±GAG and ÃŽÂ²GAG). C: complement regulatory region; E: epidermal growth factorÃ¢Â€Â“like domain; HABR: hyaluronan-binding region; Ig: immunoglobulin-like domain; L: lectin-binding domain.

Only sporadic colocalization of versican immunostaining with leukocyte infiltration in PAH

To examine the relationship between inflammation and versican in PAH, double staining for CD45 (leukocyte common antigen) and versican was performed. However, there was no clear correlation between versican staining in the vasculature and the inflammatory status of the lungs. In both patients and controls, vessels with clear accumulation of $CD45^+$ cells in the adventitia often showed little versican staining (Fig. 3*A*Ã¢Â€Â“3*C*), and most of the versican deposition did not colocalize with CD45 in plexiform lesions (Fig. 3*D*Ã¢Â€Â“3*F*). In neointima, the majority of areas positive for versican were not positive for CD45 (Fig. 3*G*, 3*H*), although a few regions with positive staining for both antibodies were observed (Fig. 3*I*Ã¢Â€Â“3*K*).

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Figure 3. Versican and inflammation in pulmonary arterial hypertension (PAH). Representative images of double immunostaining for versican (in brown) and CD45 (leukocyte common antigen, in red). *A* and *B*, Pronounced perivascular inflammation with little versican staining. *C*, Infiltration of inflammatory cells in the adventitia without colocalization with versican. *D*Ã¢Â€Â“*F*, Versican and CD45 are rarely colocalized in plexiform lesions. *G* and *H*, Versican accumulates in the neointima, but there is almost no colocalization with inflammatory cells. *I*Ã¢Â€Â“*K*, Some degree of colocalization in neointima, but it is not consistent. Arrows indicate the colocalization of versican and CD45. The letter L indicates the lumen of pulmonary vessels. Mouse immunoglobulin G (IgG) was used as a negative control. The images were captured with a x4 objective for *K* and a x20 objective for *A*Ã¢Â€Â“*J*. There were 4Ã¢Â€Â“6 sections per slide, and 1 slide per subject was analyzed (*n* = 13 for PAH group; *n* = 7 for control group).

Versican accumulates in ECM surrounding smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells

The abundance of versican in the neointima and medial layer of pulmonary vessels implies that hPASMCs may play a key role in versican production. Immunofluorescent double staining for versican and smooth-muscle ÃŽÂ±-actin revealed that the majority of versican was in the extracellular space in close proximity to smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells, both in neointimal areas (Fig. 4*A*Ã¢Â€Â“4*C*) and in plexiform lesions (Fig. 4*D*Ã¢Â€Â“4*F*). In the healthy control subjects (Fig. 4*G*, 4*H*), little versican was observed in areas with smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells.

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Figure 4. Versican and smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells. Representative images of double immunofluorescence staining for smooth-muscle ÃŽÂ±-actin (in green) and versican (in red). Nuclear staining was shown in blue. A, Smooth-muscle ÃŽÂ±-actin staining of neointimal lesion (idiopathic pulmonary arterial hypertension [IPAH]). *B*, Strong versican immunostaining in the same neointima. *C*, Merged image (*A* and *B*), which shows versican accumulation in areas with smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells within the neointima. D, Smooth-muscle ÃŽÂ±-actin staining of plexiform lesion (IPAH). E, Prominent versican staining in the same plexiform lesion. *F*, Merged image (*D* and *E*). *G* and *H*, Double immunofluorescence staining of vessels from healthy control subjects, with very low levels of versican in areas with smooth-muscle ÃŽÂ±-actinÃ¢Â€Â“positive cells. I, Rabbit immunoglobulin GÃ¢Â€Â“negative control. The images were captured with a Ã*f*Â—20 objective. One section per subject was analyzed (*n* = 4 for IPAH; *n* = 3 for healthy control subjects).

Majority of GAGs secreted by hPASMCs are chondroitin sulfate and dermatan sulfate

Since little is known regarding the composition of proteoglycans secreted by hPASMC, proteoglycan profiles were examined by metabolic labeling with [^{35}S]sulfate. Sephadex G50 chromatography after digestion of isolated GAG chains with chondroitinase ABC showed that almost all GAGs secreted by hPASMC were susceptible to the treatment, indicating that the proteoglycans carried chondroitin sulfate and dermatan sulfate and not heparan sulfate side chains (Fig. 5*A*). In the cell-layer samples, but not in medium samples, there was a small peak resistant to digestion, representing heparan

sulfate. Sepharose CL-2B chromatography showed that, compared with aortic SMCs from the same healthy donor, proteoglycans produced by hPASMCs were of smaller hydrodynamic size, suggesting that the composition of proteoglycans may differ between the two cell types (Fig. 5*B*).

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Figure 5. Characterization of proteoglycans produced by human pulmonary artery smooth-muscle cells (hPASMCs). A, Gel filtration chromatography on Sephadex G50 of [^{35}S]sulfate-labeled glycosaminoglycan chains. [^{35}S]sulfate-labeled glycosaminoglycans were released from their core proteins by alkaline treatment. The graph represents profiles before and after chondroitinase ABC digestion. *B*, [^{35}S]sulfate-labeled intact proteoglycans synthesized by hPASMCs and aortic smooth-muscle cells (AoSMCs) from the same donor were characterized by gel filtration chromatography on Sepharose CL-2B. Samples were applied on the basis of equal amount of radioactivity (measured in counts per minute [CPM]). Similar results were seen in 3 independent experiments.

Mechanical strain has no immediate effect on versican production in hPASMCs in vitro

Since we observed that versican was increased in patients with PAH independent of whether the PAH was idiopathic or associated with scleroderma or congenital heart disease, we hypothesized that an initial increase in pressure, possibly mainly caused by vasoconstriction, may induce versican deposition and further vascular remodeling. To test our hypothesis, hPASMCs were subjected to 10% biaxial cyclic stretch for 6 hours. Under these conditions, no obvious cell death was found as examined by Trypan blue staining. As a positive control, aortic SMCs from the same donor were used.[38]

During the 6 hours of 10% cyclic stretch, SMCs were labeled with [^{35}S]sulfate, and [^{35}S]sulfate-labeled proteoglycans were analyzed by Sepharose CL-2B chromatography. The results showed that mechanical strain did not affect the hydrodynamic size of proteoglycans. However, the peak representing small proteoglycans clearly disappeared after stretch (Fig. 6*A*). The results for hPASMCs were similar to control aortic SMCs from the same donor (Fig. 6*B*).

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Figure 6. The hydrodynamic size of proteoglycans after mechanical strain. Sepharose CL-2B chromatography of [^{35}S]sulfate-labeled proteoglycans. *A*, Results for stretched human aortic smooth-muscle cells (hAoSMCs) compared with nonstretched controls. *B*, Stretched human pulmonary artery smooth-muscle cells (hPASMCs) compared with nonstretched controls. CPM: counts per minute.

For the aortic control SMCs, qPCR and Western blot revealed that stretch significantly increased both versican mRNA and protein levels after 6 hours of cyclic stretch, compared with static conditions (Fig. 7*A*, 7*B*). However, for hPASMCs, the same stretch conditions did not affect versican mRNA levels immediately after 6 hours of cyclic stretch (not shown). Following prolonged mechanical strain up to 12 hours, a trend toward increase in versican protein and mRNA was seen in hPASMCs compared with static conditions, but the differences did not reach statistical significance with the exception of an increase in V0 mRNA (Fig. 7*C*, 7*D*).

Figure 7. Upregulation of versican messenger RNA and protein by mechanical strain in human aortic smooth-muscle cells (SMCs) but not in human pulmonary artery smooth-muscle cells (hPASMCs). Results of quantitative polymerase chain reaction (qPCR) for versican isoforms (V0, V1, V2, and V3; *A*) and a representative Western blot (*B*) for versican from cell cultures of stretched aortic SMCs and nonstretched controls. Samples were collected after 6 hours of cyclic stretch. Results of qPCR (*C*) for versican isoforms and a representative Western blot (*D*) for versican from cell cultures of stretched hPASMCs and nonstretched controls. Samples were collected for another 6 hours after 6 hours of cyclic stretch. For qPCR (*A* and *C*), fold changes were calculated using ÃŽÂ”ÃŽÂ”C_t method compared with nonstretched controls. ÃŽÂ²$_2$-microglobulin was used as reference gene (n = 3; asterisk indicates $P < 0.05$ by Student t test). For Western blots (*B* and *D*), equal amount of total proteins from conditioned medium and cell lysates, respectively, were applied to ion-exchange chromatography for proteoglycan purification. Chondroitinase ABC-treated samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing condition and probed with goat antihuman versican ÃŽÂ² glycosaminoglycan domain antibody for versican isoforms V0 and V1. N: nonstretched control group; S: stretched group; (+): positive control extracted from human aortic SMCÃ¢Â€Â“conditioned medium.

Hypoxia influences versican expression in hPASMCs in vitro

Hypoxia is a known contributing factor for vascular remodeling in PAH.[40] To determine whether versican expression can be regulated by hypoxia in hPASMCs, cells were cultured in an incubator with 1% O_2 and 5% CO_2. After 24 hours of 1% O_2, versican isoforms V0 and V1 were both significantly increased at the mRNA level, whereas V2 transcripts were significantly decreased compared with the normoxic group of PASMCs (Fig. 8*A*). Western blot analysis confirmed the increase of versican protein following hypoxia exposure (Fig. 8*B*). The results suggest that hypoxia influences versican production in hPASMCs.

Figure 8. Versican is regulated by hypoxia. A, quantitative polymerase chain reaction (qPCR) for versican isoforms (V0, V1, V2, and V3) from cell lysates of hypoxic human pulmonary artery smooth-muscle cells (hPASMCs). Data were analyzed using ÃŽÂ”ÃŽÂ”C_t method compared with normoxic controls. ÃŽÂ²$_2$-microglobulin was used as reference gene (n = 3; asterisk indicates $P < 0.05$ by Student t test). B, Representative Western blot for versican from cell cultures of hypoxic hPASMCs and normoxic controls. Purified, chondroitinase-treated samples were probed with goat antihuman versican ÃŽÂ² glycosaminoglycan domain antibody for versican isoforms V0 and V1. H: hypoxia; N: normoxia; (+): positive control extracted from human aortic smooth-muscle cellÃ¢Â€Â“conditioned medium.

DISCUSSION

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In this study, we show versican accumulation in neointima, in medial hypertrophy, and in plexiform lesions of patients with IPAH, as well as in PAH secondary to connective tissue disease or congenital heart disease. In addition, our data show that hypoxia, but not mechanical strain, induces versican expression in hPASMCs in vitro. Hypoxia may be an important factor for versican deposition during vascular remodeling of PAH.

SMCs have previously been demonstrated to be the main source of versican in blood vessels.[41] Here, versican was mainly found in areas with positive staining for smooth-muscle ÃŽÂ±-actin. Therefore, hPASMCs are likely to be a major source of versican during vascular remodeling in PAH. In plexiform lesions, there were often some strongly stained areas and some almost negative areas, possibly indicating different cell populations, because the origin of cells in plexiform lesions has been shown to be very diverse.[3]

Earlier studies of vascular development and remodeling support the idea that increased vessel wall thickness is an adaptation to higher blood pressure, which may also apply to vascular remodeling in PAH.[42,43] Our hypothesis was that versican, secreted by vascular cells exposed to increased strain, contributes to vascular wall thickening. Mechanical strain may have a direct impact on versican production, which may facilitate vascular remodeling. Lee and coworkers have previously shown that versican protein is increased in aortic SMCs after 36 hours of 4% cyclic stretch.[38] In our study, we increased the mechanical strain to 10% stretch, and an upregulation of versican protein was observed in aortic SMCs already after 6 hours of stretch exposure. However, no significant increase in versican protein was found at this time point in hPASMCs. We also observed that hPASMCs (but not aortic SMCs) began to detach from the fibronectin-coated silicone chambers after 12 hours of 10% cyclic stretch. We were therefore unable to examine long-term effects of mechanical strain on hPASMCs using our system. It should be noted that the cells used for this study are from a healthy donor, and there is a possibility that hPASMCs from patients with pulmonary hypertension would respond differently to mechanical strain.

Hypoxia may partly explain versican accumulation in pulmonary vessels. Hypoxia-inducible factors (HIFs) are transcriptional factors that respond to cellular oxygen deficiency to maintain cell survival. HIF-1ÃŽÂ± has been shown to be an important factor in PAH.[44] Asplund et al.[45] reported that the versican gene, *VCAN*, has a hypoxia response element, DNA sequence 5Ã¢Â€Â²-[AG]CGTG-3Ã¢Â€Â², in the promoter region and that HIF-1ÃŽÂ± and HIF-2ÃŽÂ± can mediate versican transcription. Here, we show that hypoxia induces increases in versican V0 and V1 mRNA levels but a decrease in V2 mRNA, indicating that selective splicing between exons encoding ÃŽÂ±GAG and ÃŽÂ²GAG domains exists under hypoxia. Whether HIFs have a direct impact on alternative RNA splicing awaits additional investigations.

In addition to mechanical strain and hypoxia, several growth factors and transcription factors are likely to be involved in the regulation of versican expression. Transforming growth factor ÃŽÂ² and platelet-derived growth factor subunit B have, for example, previously been demonstrated to increase versican secretion.[46,47] In Wnt signaling, the ÃŽÂ²-catenin/T cell factor complex has been identified as a transcriptional activator of versican in SMCs.[48] These factors are also known to contribute to the proliferative vasculopathy of PAH.[49Ã¢Â€Â“51] So far, we can only speculate about the function of versican in vascular remodeling in PAH. Increased versican accumulation makes the vascular ECM enriched in chondroitin sulfate. The negatively charged GAG chains may attract proteoglycan-binding mitogens and act as indirect regulators of growth factor signaling. The GAG chains also absorb water and provide a water-rich pericellular matrix, which can facilitate cell proliferation and migration by creating space for cell movement.[52]

In this study, we saw little colocalization of versican with CD45 staining for inflammatory cells. However, this does not exclude a role for versican in inflammation in PAH, because vascular remodeling is a dynamic process, and our methodology did not allow us to analyze more than a single time point for each patient (i.e., the time of transplantation). An association with inflammation may be more evident earlier in the remodeling process. A close association between versican and

hyaluronan has been shown in inflammation, as mentioned above, and it has also been reported in several other pathological processes, such as intimal hyperplasia in the systemic arteries.[53] However, in the case of PAH, versican staining is mainly localized in the tunica media and in neointima and is less pronounced in the adventitia, where hyaluronan deposits have been reported.[34] Hyaluronan-versican complexes are therefore not likely to be present in PAH, except possibly in plexiform lesions, where staining for both molecules is seen. However, as with inflammation, it is also possible that the two molecules may be more closely associated during certain stages of the remodeling, such as during rapid neointima expansion. This would be interesting to explore in animal models where lesions from different time points can be studied.

It is not easy to answer the question of whether versican plays an important functional role in PAH or whether it is just a marker for the disease and part of the normal response of the vessels when there is a pathological insult. However, in a recent study by de Jesus Perez et al.,[54] point mutations in the *VCAN* gene were described in patients with IPAH. Versican was one of the top 3 mutated genes detected in samples from patients with IPAH by whole-exome sequencing, indicating that versican could be a major player in PAH. It is possible that point mutations in the *VCAN* gene can explain why some people are more susceptible to developing pulmonary hypertension when exposed to other factors, such as high altitude and certain drugs. It is also known that a majority of family members who carry *BMPR2* mutations, the most common type of mutations in hereditary PAH, will never develop PAH, so other factors are clearly necessary for penetrance and disease progression.[55]

In summary, we show that versican accumulates in vascular lesions of PAH and that it can be regulated by hypoxia. Additional studies on the expression and localization of different versican splice variants, as well as studies of cell-type-specific or inducible-versican mutant mice, will be needed to clarify the diverse roles of this large chondroitin sulfate proteoglycan in the pathobiology of PAH. More detailed studies of the molecular mechanisms behind the regulation by hypoxia may also be helpful to elucidate the control of versican metabolism, a potential antiremodeling target for treatment of PAH.

ACKNOWLEDGMENTS

Tissue samples were provided by Marlene Rabinovitch under the Pulmonary Hypertension Breakthrough Initiative and were obtained from the pathology biobank at SkÃƒÂ¥ne University Hospital in Lund. We thank the patients and everyone involved in the tissue collection. We also especially thank Nancy Ferreira Tojais, Michal Roof, Lingli Wang, Hans BrunnstrÃƒÂ¶m, and Lena Thiman for excellent technical assistance and Marlene Rabinovitch and Leif Eriksson for facilitating the study.

Source of Support: Funding for the Pulmonary Hypertension Breakthrough Initiative is provided by the Cardiovascular Medical Research and Education Fund. This project was also funded by the National Institutes of Health (P01 HL098067 to TNW), the Swedish Heart-Lung Foundation, Thelma ZoÃƒÂ©ga's Foundation for Medical Research, Greta and Johan Kock's Foundation, Thure Carlsson Memorial Foundation, Fanny Ekdahl Foundation for Pediatric Research, and Karolinska Institutet.

Conflict of Interest: None declared.

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return $(this).text().replace(/-/g, ' '); }); $('.contentItemVol').text(function() { return $(this).text().replace('Vol 0,', '').replace('Vol.', 'Vol ').replace(/\s*,/, ','); }); $('.issueFormat').text(function() { return $(this).text().replace('issue', 'Issue').replace('vol.', 'Vol').replace(/\s*,/, ','); }); //Remove trailing dot from a.deleteAccountLink $('a.deleteAccountLink').text('Delete your account'); //Remove trailing dot from a.deleteAccountLink $('a#copyToClipBoard').text('Copy to Clipboard'); // Rename "Views" to "Views and downloads" $('.view-count').text(function() { if (inJournalScope()) return $(this).text().replace('Views:', 'Views & downloads:'); else return $(this).text(); }); // Keep only anchor element if already in citedBy page if ($('.view-all-citedBy a').attr('href') === window.location.pathname) $('.view-all-citedBy a').attr('href', ''); // Add #top-content-scroll on 'View All' citedBy link $('.view-all-citedBy a').attr('href', $('.view-all-citedBy a').attr('href') + '#top-content-scroll'); // Change MR/MC panel text $('#mostReadCitedPage .online-pub-date').text(function() { return $(this).text().replace("Online publication date", "First published"); }); //Wait for images to load, before deciding whteher to move the related journals $('.journalHomeFourRight').imagesLoaded().always(function(){ moveRelatedJournals(); //console.log('Ad(right) image is loaded'); }); // Fix for 'more...' label falling into 2nd line if ($('.authors .more-than').length && $('.authors .more-than').offset().left 1) $('input[name=AllField]').autocomplete('close'); } catch(e) {} }); //console.log('Journal: Pulmonary Circulation, Issue: , Article: Versican Accumulates in Vascular Lesions in Pulmonary Arterial Hypertension');

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