

A preliminary proteomic evaluation of smooth muscle cells in thoracic aortic aneurysms

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Abstract: Aortic aneurysm is characterized as localized degeneration of the aorta leading to advanced weakening and widening of the vessel. While the exact mechanisms have yet to be determined, current studies indicate that the degradation of extracellular matrix (ECM) proteins and apoptosis of vascular smooth muscle cells (SMCs) may result in extensibility, dilation, and rupture of the vessel. Within the aortic wall, SMCs are implicated as key components involved in disease development, as numerous molecular changes have been reported to occur. Most current studies involve either investigation of proteins constituting a group or pathway in SMCs, or analyses of the whole aortic tissue. In order to determine which proteins are important in the development of thoracic aortic aneurysms (TAAs), we performed comparative proteomic analyses using cultured SMCs from TAAs versus controls. Label-free nano LC-MS/MS analysis of cell extracts resulted in the identification of 256 proteins, 26 of which were differentially regulated by ≥ 1.4 -fold. Both previously described and new proteins were identified that were involved in oxidative stress, ECM formation, energy metabolism, or the 14-3-3 pathway. Among these, differential expression of SerpinH1, a protease inhibitor for collagenases, was further verified via immunoblotting. Here we have attempted to shed light on the cellular mechanisms of TAAs.

Key words: Label-free proteomics, thoracic aortic aneurysm, smooth muscle cell, SerpinH1/HSP47, oxidative stress, protein expression

1. Introduction

Aortic aneurysm can be defined as a localized structural degeneration of a part of the aorta, which leads to weakening of the wall and its progressive dilation (MacSweeney et al., 1994; Boddy et al., 2008). In time, the weakened and dilated vessel tends to rupture, which is the most dangerous outcome of this disease with a considerably high mortality rate (31.7/100,000 individuals) (Booher and Eagle, 2011; <http://wonder.cdc.gov/cmfc-icd10.html>). Unfortunately, there are still many unanswered questions on how aneurysms begin and develop; hence, identifying the molecular factors involved in disease development is an active research area.

The aortic wall is a dynamic and firmly regulated structure mainly made up of 3 major types of cells [endothelial cells, fibroblasts, and smooth muscle cells (SMCs)] and the surrounding extracellular matrix (ECM) (Thompson et al., 2002). It has been reported that vessel dilation is not just a passive enlargement process, but rather a combination of multiple factors including

increased degradation, decreased buildup of ECM proteins, and loss of SMCs through apoptosis (Weintraub, 2009; Lindsay and Dietz, 2011). During the pathogenesis of aneurysms, inflammation also appears to play a role contributing to the aforementioned processes through the release of cytokines, the increase in the expression matrix metalloproteinases (MMPs), and the increase in oxidative stress resulting in the death of SMCs (Newman et al., 1994; McCormick et al., 2007). Inflammatory aneurysms are frequent in the abdominal aorta and are very rare in the thoracic aorta, where they are mostly characterized by medial degeneration (He et al., 2006). In addition, while abdominal aortic aneurysms (AAAs) present later in life, thoracic aortic aneurysms (TAAs) may be observed at younger ages, indicating the presence of genetic factors (Lindsay and Dietz, 2011). Hence, the 2 types of aneurysms are different both pathologically and mechanistically for the factors that lead to disease development.

Two cytoskeletal gene mutations specific to SMCs have been implicated in the formation of nonsyndromic

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familial TAAs: the SMC-specific beta-myosin (*MYH11*) gene (Zhu et al., 2006) and the alpha-actin (*ACTA2*) gene (Guo et al., 2007). Mutations in *FBN1*, *TGF β R2*, and *TGF β R1* have also been linked to causing TAAs, resulting in Marfan's disease in the first gene and Loeys–Dietz syndrome in the latter 2 (Milewicz et al., 1996; Pannu et al., 2005; Loeys et al., 2006). Mutations affecting SMC functioning may have serious outcomes. In addition to being a structural component populating the medial layer of the aorta, SMCs have various roles within the vessel, including contraction and secretion of ECM molecules (El-Hamamsy and Yacoub, 2009). Furthermore, SMCs link the outside (the ECM) to the inside of the cell (the cytoskeleton) via cell surface receptors (integrins, G-protein coupled receptors, and the discoidin domain receptors), regulating cell migration, proliferation, shape, or contraction (Alenghat and Ingber, 2002; Berrier and Yamada, 2007; Luo et al., 2007). Consequently, SMCs can sense mechanical stress and respond by changing the microenvironment through both intra- and extracellular arrangements, including realignment of stress fibers or cytoskeleton, or by the synthesis of new ECM components (mechanotransduction) (Wang et al., 1993; Kim et al., 1999; Alenghat and Ingber, 2002; Parker and Ingber, 2007). Thus, SMCs provide the necessary hemodynamic environment for the proper functioning of the aortic wall. Indeed, during TAA development, the loss of SMCs through apoptosis results in major changes due to their physical absence in the dilated wall as well as differentially regulated gene and protein profiles, i.e. increased MMP expression (Koullias et al., 2004; Schmoker et al., 2007; Phillippi et al., 2009), further contributing to disease development.

Development of effective strategies for early diagnosis and treatment of aneurysms depends on determining the cell-specific changes rather than those of the whole aortic section (Abdulkareem et al., 2013). Assessing expression from a mixture of cells, as in tissue samples, carries the risk of missing real differences when genes are differentially regulated in different types of cells, or over-interpretation of results when highly expressing cells exist, albeit few in number. Hence, studying a single cell type could yield different results and be more informative than studying a mixture of different cell types.

In order to have a comprehensive understanding of the molecular changes that lead to TAA development, we concentrated on SMCs and performed a label-free differential proteome analysis by comparing SMCs from TAAs to SMCs from normal nondilated aortae. We were able to identify 26 significant differentially regulated proteins, which were involved in energy metabolism, protein folding, oxidative stress response, regulatory pathways, cytoskeleton, ECM organization,

or DNA packaging. Among the proteins that are found to be significantly differentially expressed, we found that SerpinH1 (or HSP47), a human serine protease inhibitor, is downregulated in the TAA samples and may be one of the proteins that contribute to aneurysm development. To our knowledge, this is the first proteomic study focused on vascular SMCs in TAAs and may form a basis for new hypotheses on the development of TAAs.

2. Materials and methods

2.1. Materials

Acetonitrile (LC-MS grade), water (LC-MS grade), dithiothreitol, Tris, trifluoroacetic acid (TFA), formic acid (FA), iodoacetamide, and sequencing grade modified trypsin (proteomic grade) were purchased from Sigma-Aldrich. SDS and acrylamide-bis (40%) were purchased from Bio-Rad. Ammonium bicarbonate (NH_4HCO_3) was purchased from Fluka. RapiGest, an MS-compatible detergent, and the internal standard MassPREP alcohol dehydrogenase digest UniProt Accession #P00330 were purchased from Waters Corp.

2.2. Aortic tissue and cell culture

During this study, 2 control samples and 2 TAA samples were used. The study was approved by the Ethics Committee of the Turkish Ministry of Health, Kartal Koşuyolu Advanced Training and Research Hospital (Ethics Report Number 23, dated 21-03-2008, and Protocol Number 184-04). Informed consents were signed by all participants. Both samples were transported to our laboratory within 12 h after surgery, and SMC extraction procedures were started. Both control samples were obtained at the same time, and hence there is not likely to be an expression problem between controls. Similar timelines were also applied for aneurysm samples.

SMCs were prepared from thoracic aortic tissue samples or aortae from organ-donor cadavers without aneurysms (1 donor and 1 acceptor individual were used as control samples). TAA specimens removed during surgery were transferred to the laboratory in cold phosphate-buffered solution (PBS) solution with penicillin (10,000 U/mL) and streptomycin (10 mg/mL) (Biol. Industries, Cat. #03-031), and all samples were processed within a few hours of operation. SMCs were isolated using the explant method (Leik et al., 2004). Cells were isolated on the same day the surgery was performed. After the adventitia and endothelial layer of the vessel were removed, the tissue was cut into small pieces (~2 mm²), which were kept at 5% CO₂ and 37 °C for 0.5–1 h on gelatin-coated plates for attachment before the addition of culture medium. The medium was changed every 2–3 days, and cells were passaged once approximately 70%–80% confluency was reached. For initial experiments, nonenriched medium formulations (DMEM/F12, GIBCO Cat. #32500-035 +

10% fetal bovine serum, Biochrom AG Cat. #S0115) were used. For the proteomic analyses, only cells grown in commercially purchased enriched growth medium (Cell Applications, Smooth Muscle Cell Growth Medium) were used from the first day of the isolation process and were always kept in the enriched medium. We routinely ran trypan blue exclusion assays while harvesting cells, and the cultures used in this study were performed with >95% viable cells. Cells were harvested when they were 90% confluent.

2.3. Immunofluorescence staining

Cells were fixed at approximately 80% confluency using $-20\text{ }^{\circ}\text{C}$ methanol for 10 min at $-20\text{ }^{\circ}\text{C}$. Briefly, after washing with 1X PBS, cells were blocked using 1% bovine serum albumin (BSA) and stained using antialpha smooth muscle actin (Abcam, ab5694, 1/100, $25\text{ }^{\circ}\text{C}$, 2 h) and antirabbit (Invitrogen, Alexa Fluor 546, 1/50, $25\text{ }^{\circ}\text{C}$, 1 h). Cells were visualized under fluorescence microscope, scored for smooth muscle actin positivity, and imaged using the same camera settings.

2.4. Immunoblotting

First, 20 μg of total cell lysate (prepared as described for the sample preparation) was loaded into each well, separated by 10% SDS-PAGE, and transferred to 0.22- μm PVDF membranes (Millipore, PSQ #ISEQ00010), which were then blocked for 1 h at $25\text{ }^{\circ}\text{C}$ in TBS (Tris-HCl, 20 mM, pH 7.4; NaCl, 150 mM) containing 3% BSA (Amresco, #0332-100G). The membrane was incubated overnight at $4\text{ }^{\circ}\text{C}$ with anti-SerpinH1 antibodies (1/1000, Abcam, #ab109117) and antiactin (1/200, pan Ab-5, Labvision, #MS1295-P1) diluted in TBS/0.1% Tween 20 (v/v), and for 1 h at $25\text{ }^{\circ}\text{C}$ with secondary antibodies (1:5000, Lab Vision #TR-001-HR, and 1/1250, Pierce, Cat. #32400). The proteins were detected using the SuperSignal chemiluminescent substrate (Pierce #34080).

2.5. Sample preparation for analysis

Approximately 250,000 SMCs were scraped from 25- cm^2 cell culture flasks when they were between 80% and 90% confluency, washed twice with 50 mM cold ammonium bicarbonate, and lysed with an ultrasonic homogenizer (5 s on, 5 s off; 3 cycles). The mixture was centrifuged at 15,000 rpm and the protein concentration measurement was performed for the supernatant based on the Bradford method. Fifty micrograms of total protein extract was transferred to a 1.5-mL Eppendorf vial. Proteins were reduced with 5 mM dithiothreitol at $60\text{ }^{\circ}\text{C}$ for 15 min, alkylated with 10 mM iodoacetamide in the dark at room temperature for 30 min, and digested with trypsin (50 μL , 20 ng/ μL ; Sigma Proteomics Grade) overnight at $37\text{ }^{\circ}\text{C}$. The hydrolysis of the acid-labile MS-compatible detergent, RapiGest (Waters Corp.), was done by the addition of TFA and ACN to a 1% final volume and incubation at $60\text{ }^{\circ}\text{C}$ (600 rpm shaker, 2 h). During RapiGest removal,

standard internal calibrant digest (alcohol dehydrogenase, UniProt #P00330, Waters Corp.) was added to the samples (25 fmol/ μL final concentration). The resulting mixtures had 250 ng/ μL tryptic peptide mixture. After RapiGest hydrolysis, the mixture was centrifuged (15,000 rpm, 15 min) and an aliquot was taken into an LC vial for analysis; the rest of the tryptic peptides were stored at $-80\text{ }^{\circ}\text{C}$.

2.6. LC-MS/MS analysis

Each sample was analyzed in triplicate to eliminate technical errors. A 2- μL volume of sample (containing 500 ng of tryptic peptide mixture) was loaded onto the LC-MS/MS system (nanoACQUITY UPLC coupled to SYNAPT high-definition mass spectrometer with NanoLockSpray ion source; Waters Corp.). Prior to the injection, the columns were equilibrated with 97% mobile phase A (water with 0.1% FA) and 3% mobile phase B (acetonitrile containing 0.1% FA). The column temperature was set to $35\text{ }^{\circ}\text{C}$. First, peptides were trapped on a nanoACQUITY UPLC Symmetry C18 trap column (5 μm particle size, 180 μm i.d. \times 20 mm length) at 5 $\mu\text{L}/\text{min}$ flow rate for 5 min. Peptides were eluted from the trap column by gradient elution onto the analytical column (nanoACQUITY UPLC BEH C18 Column, 1.7 μm particle size, 75 μm i.d. \times 250 mm length), at 300 nL/min flow rate with a linear gradient from 5% to 40% acetonitrile over 90 min. Data independent acquisition mode (MS^E) was used by operating the instrument at positive ion V mode, applying the MS and MS/MS functions over 1.5-s intervals with 6 V of low collision energy and 15–40 V of high collision energy ramp to collect the peptide mass-to-charge ratio (m/z) and the product ion information to deduce the amino acid sequence. To correct for mass drift, the internal mass calibrant Glu-fibrinopeptide (500 pmol/ μL) was infused every 45 s through the NanoLockSpray ion source at 300 nL/min flow rate. Peptide signal data between 50 and 1600 m/z values were collected.

2.7. LC-MS/MS data processing

Tandem mass spectra extraction, charge state deconvolution, and deisotoping steps were processed with the ProteinLynx Global Server v. 2.4 (Waters Corp.) and searched with the IDENTITY^E algorithm against the *Homo sapiens* reviewed protein database from UniProt (1 December 2010; 25,690 entries). IDENTITY^E was set up to search null assuming the digestion enzyme trypsin and searched with a fragment ion mass tolerance of 20 ppm and a parent ion tolerance of 10 ppm. The amino acid sequence of the internal standard (yeast alcohol dehydrogenase, UniProt accession #P00330) was included in the FASTA file of the database. The Apex3D data preparation parameters were set to 0.2 min chromatographic peak width, 10,000 MS TOF resolution, 150 counts for low energy threshold, 50 counts for elevated energy threshold, and 1200 counts for the intensity threshold. The databank search query was

set to a minimum of 3 fragment ion matches per peptide, minimum 7 fragment ion matches per protein, minimum 1 peptide match per protein, and 1 missed cleavage. Carbamidomethyl-cysteine fixed modification and acetyl N-TERM, deamidation of asparagine and glutamine, and oxidation of methionine variable modifications were set. Absolute quantification of the peptides was calculated with the Hi3 functionality of the IDENTITY^E system using the spiked known amount of the internal standard. The false positive rate of the IDENTITY^E algorithm is around 3%–4% with a randomized database (D'Aguzzo et al., 2010). The quantitative analysis was based on the identified proteins, which were detected in 2 out of the 3 technical replicate injections. Normalization of the proteins was achieved against the digest of the internal calibrant P00330. The acquired protein fold changes were used in the IPA analysis (IPA v. 8.5). The canonical pathways used to construct the protein–protein interaction map were generated with protein identifications having a P-value of <0.05 and greater than or equal to 40% (≥ 1.4 -fold) expression change. The peptide m/z values and sequence information related to the identified proteins are provided in Table 1.

3. Results

3.1. Sample characterization

SMCs were isolated from aortic tissue via the explant technique (Leik et al., 2004) and maintained under cell culture conditions until growth was observed (Figure 1A). During initial experiments, cells were explanted in DMEM/F12 supplemented with 10% fetal bovine serum. However, our analyses showed that medium formulations that were not enriched (no additional growth factors other than serum) affect the proteome of the cells (Baykal et al., 2013). Therefore, cells grown in commercially purchased medium that is enriched for growth factors (Cell Applications, Smooth Muscle Cell Growth Medium) were used. Using this medium formulation, we were able to isolate 2 control and 2 patient cells; the SMCs were always maintained in the enriched medium from the first day of the isolation process.

The mean age of the control subjects was lower than that of the TAA patients (Table 2). The controls did not have any known cardiovascular diseases and there were no pathological signs of atherosclerosis. The aortic diameter for control individuals was not measured but was reported to be less than 3 cm. The diameters for patients were 4.75 and 5.7 cm at the time of operation, respectively. All subjects were males.

The cells were passaged until enough material for the experiment was collected (passage 2 or 3; see Section 2 for details). There was no apparent morphological difference in cultured SMCs between control and patient samples

(Figure 1B). SMCs were tested for smooth muscle α -actin positivity (>95%) to confirm smooth muscle origin (Figure 2).

The population doubling times for control cells were calculated to be 26 h and 39 h, while aneurysmal cells divided drastically more slowly, with 95 h and 124 h doubling-time measures. This finding is consistent with a recent paper, where the authors showed that TAA SMCs proliferate slowly compared to controls regardless of the age of the subject (Blunder et al., 2011).

3.2. Proteomic analysis by label-free LC-MS/MS

Differential proteome analysis of smooth muscle cells extracted from normal and aneurysmal aortae was performed by nLC-MS^E. Extracted proteins were trypsinized and loaded onto a nanoACQUITY UPLC system (Waters Corp.) coupled to a SYNAPT high-definition mass spectrometer (Waters Corp.). Technical replicates of the control samples were compared and calculated so that the mass error across all identified peptides was below 14 ppm and the average mass error was around 4 ppm. The chromatographic retention time coefficient of variation calculated as %CV RT was around 4% with the average being below 0.4%, meaning little deviation in the elution times of the identified peptides. The intensity coefficient of variation, expressed as %CV Int, averaged below 15% across all the identified peptides.

3.3. Smooth muscle cell proteome

Exact mass and retention time (EMRT) and protein tables were generated with ProteinLynx Global SERVER (PLGS v. 2.4, Waters Corp.), and 256 proteins were qualitatively identified from 36,415 EMRTs. Normalization of the absolute peptide intensities was based on the 3 most intense internal calibrant peptides. Quantitative calculation was processed only for the identified proteins that were detected in 2 out of 3 injections. Among the 256 proteins, actin, vimentin, and desmin were determined as the most abundant proteins in SMCs, constituting almost 50% of the whole cell extract, and were not depleted from the samples. Sixty-two proteins out of 256 were found to be significantly differentially regulated using PLGS. Among the 62 identified proteins, 9 had a PLGS identification score of below 100 and were eliminated after manual reevaluation of their protein spectra. Furthermore, the intensity cut-off was set to 40%, and only the proteins that were up- or downregulated more than the 40% cut-off are reported. Following the limiting criteria above, we identified 26 differentially regulated proteins that were statistically significant (Table 1).

3.4. Differentially regulated proteins in TAAs

We classified the identified differentially regulated proteins that were detected in all the samples into 8 groups: regulatory proteins, skeletal proteins, extracellular matrix-related proteins, histones, proteins that are involved in

Table 1. Proteins identified to be differentially expressed in this study. Ratio represents the absolute intensities of the aneurysmal samples divided by the control samples. Values below 1 indicate downregulation and values above 1 indicate upregulation in the aneurysmal samples. Control protein was only detected in control samples; aneurysm protein was only detected in aneurysm samples. Log(e) ratio and Log(e) StdDev are natural logarithm values and standard deviations for the ratio of a given protein. pI is the isoelectric point and MW is the molecular weight of proteins. The functions may include statements from proteins databases such as UniProt.

| Accession | Description | Score | Ratio | Log(e) ratio | Log(e) StdDev | pI | MW (kDa) | Molecular function |
|----------------------------|--|-------|---------|--------------|---------------|-----|----------|---|
| Protein folding | | | | | | | | |
| P48741 | HSP77 Putative heat shock 70 kDa protein 7, HSPA7 | 235 | 0.41 | -0.88 | 0.68 | 7.9 | 40.2 | A member of the Hsp70 family of heat shock proteins |
| P30101 | PDIA3 Protein disulfide isomerase A3, PDIA3 | 574 | 0.5 | -0.7 | 0.08 | 5.9 | 56.7 | ECM-related, catalyzes protein folding, prolyl 4-hydroxylase, a highly abundant isomerase multifunctional enzyme that belongs to the protein precursor disulfide isomerase family |
| P14625 | ENPL Endoplasmic, HSP90B1/GRP94 | 572 | 0.47 | -0.76 | 0.08 | 4.6 | 92.4 | A heat shock 90-kDa protein |
| O43852 | CALU Calumenin CALU | 122 | Control | Control | Control | 4.5 | 37.1 | Involved in ER functions such as protein folding and sorting |
| P27797 | CALR Calreticulin CALR | 387 | 0.51 | -0.68 | 0.1 | 4.1 | 48.1 | Binds to misfolded proteins, prevents export from the endoplasmic reticulum to the Golgi apparatus |
| P11021 | GRP78 78 kDa glucose regulated protein HSPA5 | 1479 | 0.51 | -0.67 | 0.06 | 4.9 | 72.3 | Heat shock 70-kDa protein 5 |
| Regulatory proteins | | | | | | | | |
| P27348 | 1433T 14 3 3 protein theta YWHAQ | 341 | 2.34 | 0.85 | 0.31 | 4.5 | 27.8 | Regulatory protein, mediates signal transduction |
| P31946 | 1433B 14 3 3 protein beta alpha YWHAB | 472 | 0.61 | -0.49 | 0.15 | 4.6 | 28.0 | Regulatory protein, mediates signal transduction |
| P09382 | LEG1 Galectin 1 LGALS1 | 1506 | 0.58 | -0.54 | 0.06 | 5.1 | 14.7 | Beta-galactoside-binding protein, modulates cell-cell and cell-matrix interactions |
| Energy metabolism | | | | | | | | |
| P04406 | G3P Glyceraldehyde 3 phosphate dehydrogenase GAPDH | 6442 | 0.55 | -0.6 | 0.04 | 8.7 | 36.0 | Catalyzes sixth step of glycolysis |
| P04075 | ALDOA Fructose biphosphate aldolase A, ALDOA | 2195 | 0.55 | -0.59 | 0.07 | 8.1 | 39.4 | Catalyzes a reverse aldol reaction |
| P25705 | ATPA ATP synthase subunit alpha mitochondrial ATP5A1 | 182 | 0.6 | -0.51 | 0.3 | 9.4 | 59.7 | Mitochondrial membrane ATP synthase |
| Skeletal protein | | | | | | | | |
| P41219 | PER1 Peripherin, PRPH | 482 | 1.57 | 0.45 | 0.32 | 5.2 | 53.6 | Type III intermediate filament |
| P60709 | ACTB Actin cytoplasmic 1, ACTB | 18483 | 0.55 | -0.6 | 0.09 | 5.1 | 41.7 | One of 6 different actin isoforms, one of the 2 nonmuscle cytoskeletal actins |
| Q9BVA1 | TBB2B Tubulin beta 2B chain, TUBB2B | 1629 | TAA | TAA | TAA | | 49.9 | Tubulin binds 2 moles of GTP, 1 at an exchangeable site on the beta chain |

Table 1. (continued).

| | | | | | | | | |
|------------------|---|------|---------|---------|---------|------|-------|--|
| Q13885 | TBB2A Tubulin beta 2A chain, TUBB2A | 1629 | Control | Control | Control | 4.6 | 49.9 | Tubulin binds 2 moles of GTP, 1 at a nonexchangeable site on the alpha-chain |
| Oxidative stress | | | | | | | | |
| Q13162 | PRDX4 Peroxiredoxin 4, PRDX4 | 117 | Control | Control | Control | 30.5 | 30.5 | An antioxidant enzyme |
| P04732 | MT1E Metallothionein 1E, MT1E | 1396 | 0.59 | -0.53 | 0.15 | 7.8 | 6.0 | Regulation of physiological metals (Zn and Cu), provides protection against oxidative stress |
| Q9H299 | SH3L3 SH3 domain binding glutamic acid rich like protein 3, SH3BGR3 | 334 | 1.46 | 0.38 | 0.16 | 4.6 | 10.4 | Could act as a modulator of glutaredoxin that acts in antioxidant defense |
| P26641 | EF1G Elongation factor 1 gamma, EEF1G | 146 | Control | Control | Control | 6.2 | 50.1 | Responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome, contains an N-terminal glutathione transferase domain |
| ECM-related | | | | | | | | |
| P08123 | CO1A2 Collagen alpha 2 I chain, COL1A2 | 116 | Control | Control | Control | 9.2 | 129.3 | One of the chains for type I collagen, an extracellular matrix protein |
| P50454 | SERP1 Serpin H1, SERPINH1 | 469 | 0.31 | -1.16 | 0.35 | 9.0 | 46.4 | Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, human chaperone protein for collagen |
| Others | | | | | | | | |
| P06454 | PTMA Prothymosin alpha, PTMA | 120 | Control | Control | Control | 3.4 | 12.2 | Remodeling of chromatin fibers through its interaction with histone H1, abundant in nucleus, expression is related to cell proliferation |
| P80723 | BASP1 Brain acid soluble protein 1, BASP1 | 229 | Control | Control | Control | 4.4 | 22.7 | A membrane-bound protein with several transient phosphorylation sites and PEST motifs |
| Histones | | | | | | | | |
| Q96KK5 | H2A1H Histone H2A type 1 H, HIST1H2AH | 2964 | Control | Control | Control | 11.3 | 13.9 | A member of the histone H2A family |
| P62805 | H4 Histone H4, HIST1H4A | 9489 | 0.3 | -1.2 | 0.04 | 11.8 | 11.3 | One of the main histone proteins |

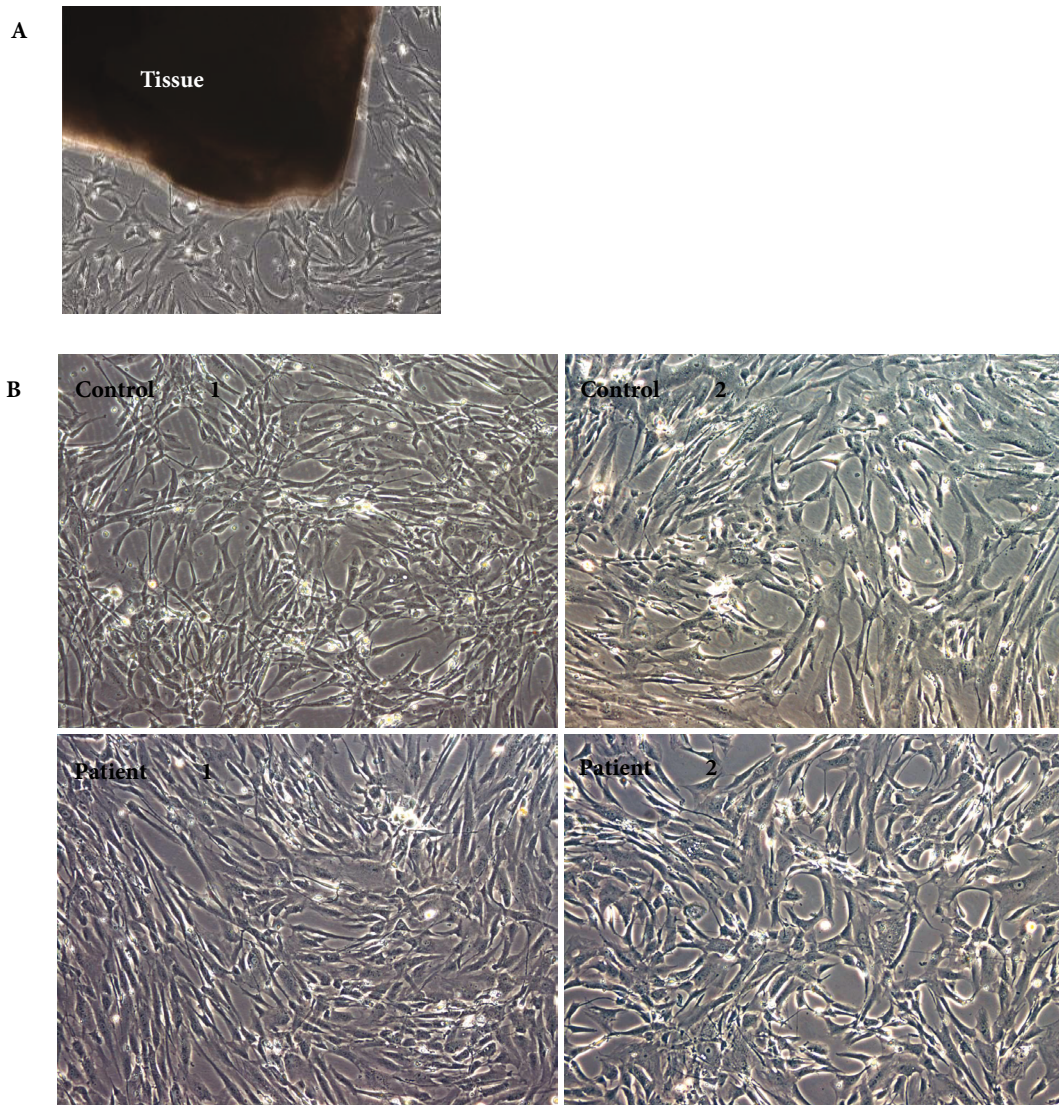


Figure 1. A) Smooth muscle cells were isolated using the explant technique: a representative phase contrast image is shown in the picture with the tissue seen as dark and the cells growing around it (10× magnification). B) Morphological appearance of SMCs used in this study: top panel shows images from control samples, bottom panel shows images from TAA samples; the identities are labeled on the images. Phase contrast microscopy, 10× magnification.

protein folding, oxidative stress, energy metabolism, and 2 proteins classified as “others” that did not fall into any of these categories (Table 1). Out of the 26 differentially regulated proteins, 9 were abundant in 1 group and below our detection limit in the other. Furthermore, while there was a general downregulation of proteins (15/26), only 3 were upregulated in aneurysm samples. A brief summary of all expression changes is given in Figure 3.

3.5. Ingenuity Pathway Analysis

In order to understand the signal transduction pathways and associated proteins with these differentially expressed proteins, the data were further analyzed using Ingenuity

Pathway Analysis (IPA v. 8.5) software, which is a knowledge database relying on published literature assessing protein function, localization, relevant interactions, and biological mechanisms. Interestingly, most of these proteins (18 out of 26) were merged strongly in a single network (Figure 4A; Table 3) with a score of 49. A score of ≥ 2 is significant; the score indicates the log of the probability of network-eligible proteins appearing in a network by random chance. The higher the score, the lower the probability of randomness. The second network had a score of 8, and 4 out of 26 molecules were included in this signaling pathway (Figure 4B; Table 3). Figure 4C shows merged networks 1 and 2.

Table 2. Patients' demographic and clinical properties. NA: Not available.

| | Sex | Age | Hypertension | Diabetes mellitus | Smoking |
|-----------|-----|-----|--------------|-------------------|---------|
| Patient 1 | M | 40 | 1 | 0 | 1 |
| Patient 2 | M | 63 | 1 | 1 | 1 |
| Control 1 | M | 28 | NA | NA | NA |
| Control 2 | M | 30 | NA | NA | NA |

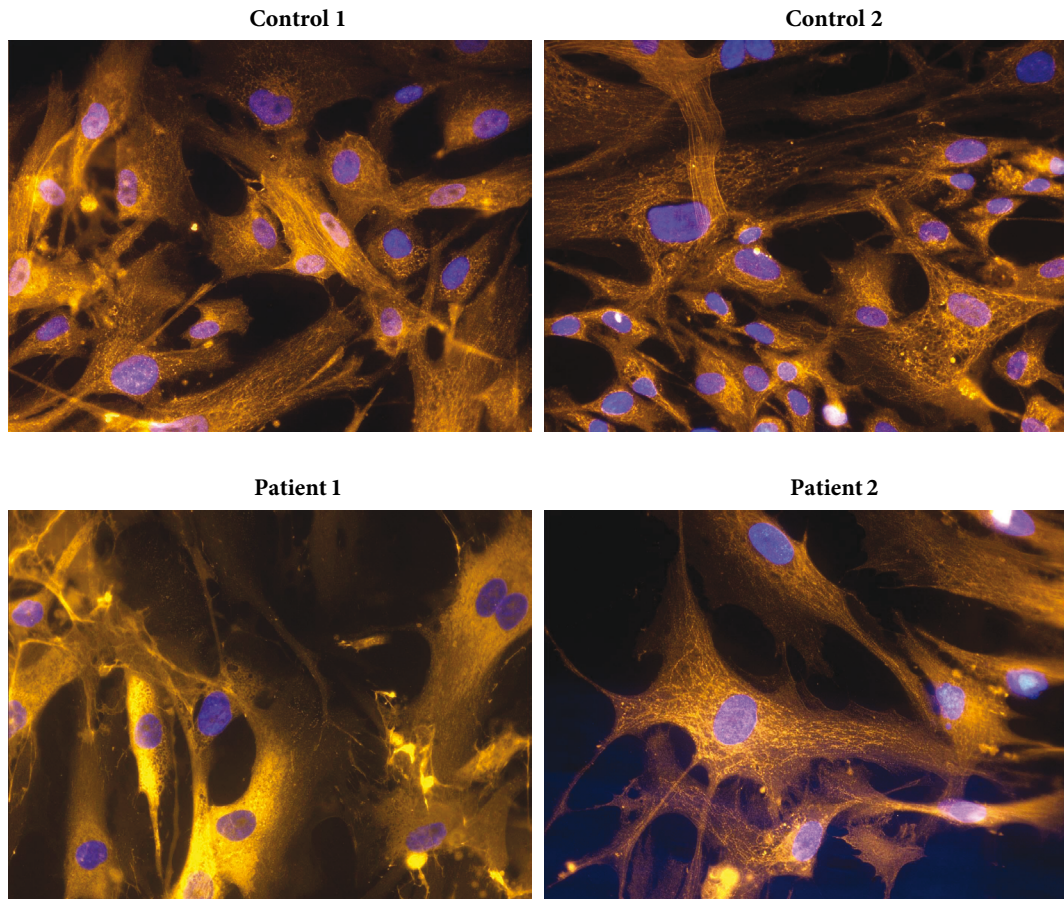


Figure 2. α -Actin staining of smooth muscle cells used in this study: cells were characterized by α -actin positivity for smooth muscle origin; >95% positivity was observed. Fluorescent images were taken with a Leica DMI 6000 microscope and a Hitachi 3-CCD HV-D20P color camera using the same settings and exposure times (40 \times magnification).

While the top canonical pathway appeared as the 14-3-3 signaling pathway including proteins such as protein disulfide isomerase A3, tubulin beta 2A and 2B chains, 14-3-3 theta, and 14-3-3 beta ($p: 4.68 \times 10^{-7}$), 15 molecules were identified that are implicated in playing a role in skeletal and muscular disorders (actin cytoplasmic 1, fructose biphosphate aldolase A, brain acid soluble protein 1, calreticulin, collagen alpha 2 I chain, human elongation factor 1 gamma, glyceraldehyde 3 phosphate dehydrogenase, endoplasmic, 78-kDa glucose

regulated protein, galectin, tubulin beta 2A and 2B chains, metallothionein, protein disulfide isomerase A3, prothymosin alpha) ($p: 1.39 \times 10^{-5}$). The top 10 canonical pathways and biological functions are given in Figure 5.

3.6. Implication for SerpinH1 downregulation in TAAs
Our analyses showed that one of the downregulated proteins was SerpinH1/HSP47, which belongs to a serine protease inhibitor family and is involved in the correct folding of collagens (Nagai et al., 2000). In accordance, the expression of one of the collagens, collagen A1 (Col1A1),

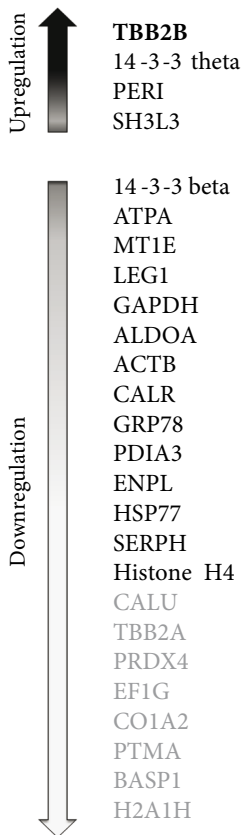


Figure 3. A summary of the proteins that were identified to be significantly differentially regulated in this study: upregulated proteins are represented alongside the upwards arrow, and downregulated proteins alongside the downwards arrow. The order is based on the fold change from the most upregulated to the most downregulated except for the proteins in bold, which were identified only in control or aneurysm samples.

was also reduced in our study (Table 1). Due to its potential role in disease development as a previously unidentified molecule in aneurysms, we focused on SerpinH1. Immunoblotting for SerpinH1 confirmed that this protein was downregulated in aneurysm samples (Figure 6).

4. Discussion

In this study, we aimed to conduct a global comparison of protein expression profiles of SMCs in TAAs to cells of aortae from transplant tissue without aneurysm. Hence, a label-free differential proteome analysis of the whole-cell extracts was run on a nano LC-MS/MS setup. Proteomic analysis resulted in identifying 26 differentially expressed proteins greater than 1.4-fold with significance. Importantly, to our knowledge, this is the first report focusing on SMCs in TAAs analyzing the global proteome.

Although this study was performed in a cell-culture model with a limited number of samples, we think that the data reveal important results that might be relevant

to *in vivo* alterations. In accordance, proteins involved in the main TAA pathological mechanisms (i.e. extracellular matrix, oxidative stress) were identified by LC-MS/MS, which is consistent with previous reports. Furthermore, several differentially expressed proteins are in accordance with the literature, as 10 of the identified proteins were implicated in connective tissue disorders and 15 in skeletal and muscle disorders (IPA analysis), implying that even a small number of samples can reveal disease-related changes.

Alterations in the ECM components are the key factors in medial degeneration in aortic aneurysms. Collagen, as one of the main components that make up the ECM that is crucial for maintaining the arterial wall structure and stiffness, determines the mechanical properties of the aorta. Our data present evidence of a reduction in collagen protein levels (Col1A2, Table 1) that is accompanied by a reduction in SerpinH1/HSP47 levels, a protein involved in the correct folding of the collagen triple helix (Nagai et al., 2000). Although complete knockout of this gene is embryonically lethal (Nagai et al., 2000), mutations in SerpinH1 have been associated with other connective tissue disorders (Drogemuller et al., 2009; Christiansen et al., 2010). The proposed mechanism of action for SerpinH1 is via its interactions with triple-helical procollagen molecules that are distinct from protein disulfide isomerase (PDIA3), another molecule observed to be diminished in our study. We confirmed the downregulation of SerpinH1 through western blotting. Indeed, a larger sample size is necessary to extrapolate SerpinH1 as a biomarker, yet it appears that downregulation of SerpinH1 could be one of the factors leading to TAA development.

Interestingly, apart from SerpinH1, despite the increased stress within the aorta, SMCs isolated from TAAs expressed fewer cytoprotective molecules involved in protein folding (Table 1), suggesting that correct folding of cellular proteins might be perturbed, perhaps leading to an accumulation of misfolded proteins that are essential for aortic wall integrity. It has been previously shown that artificial elevation of molecular chaperones can exhibit cardioprotection (Marber et al., 1995; Shamaei-Tousi et al., 2007). Thus, the opposite could be true for the reduction in the protein quality-control mechanism by downregulation of chaperones, which might directly or indirectly contribute to disease development and aneurysm formation.

To further elucidate how these proteins are regulated and which molecular networks are in play, IPA analysis was performed. Our IPA analyses suggested that one of the pathways that did not previously receive much attention in relation to aneurysm could be the 14-3-3 pathway. This regulatory pathway has a large plethora of targets that play a role in the regulation of cell cycle, mitogenic signaling,

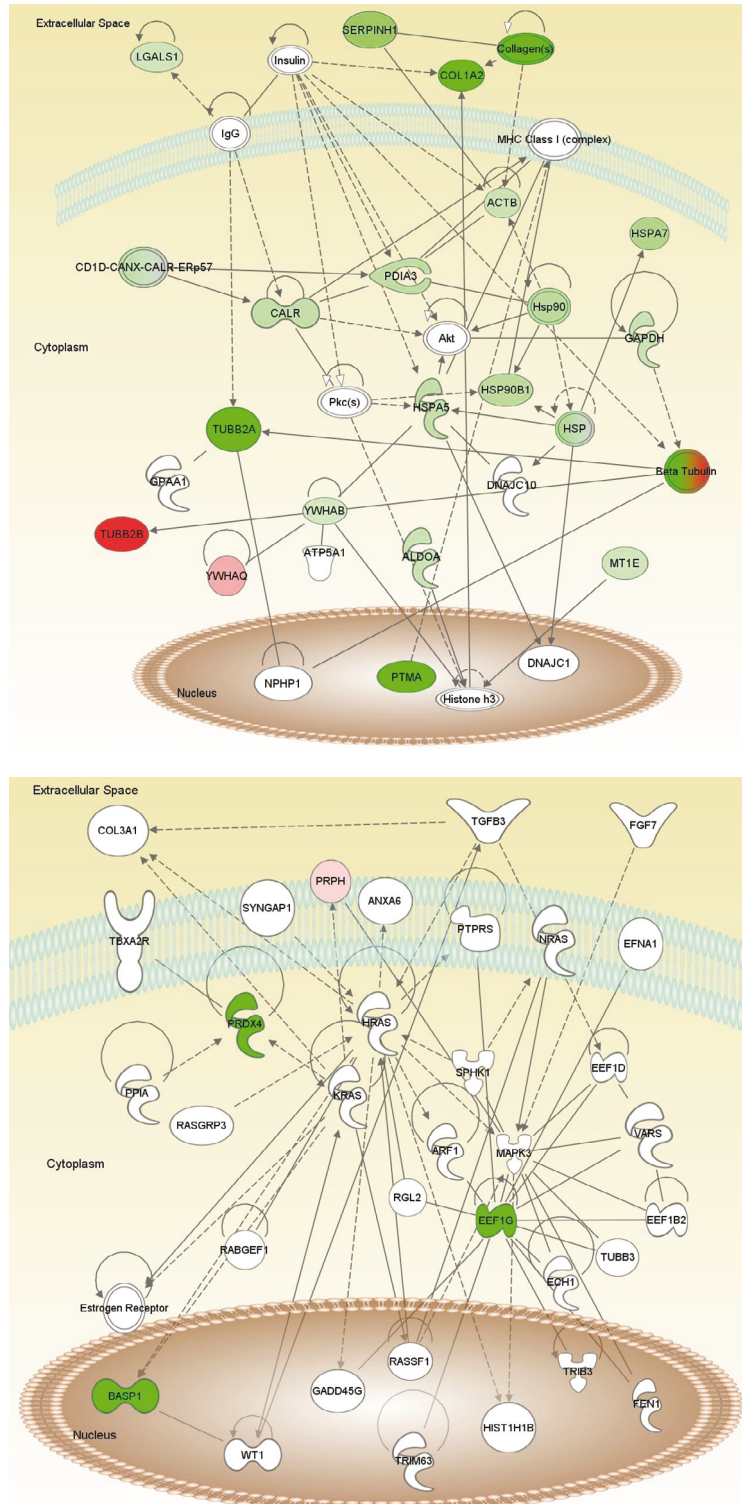


Figure 4. Most high-scored networks: pathway generated by Ingenuity Pathway Analysis (IPA) with proteins identified as specific target antigens. Proteins with no color were not detected by LC-MS/MS, yet were added to the network by the IPA algorithm. Green indicates downregulation, red indicates upregulation. The intensity of the color correlates with the degree of fold change in expression. Proteins are located based on their subcellular location. A) Most high-scored network 1; B) most high-scored network 2; C) the merge of most high-scored networks 1 and 2; D) IPA legends for path designer shapes and edge types.

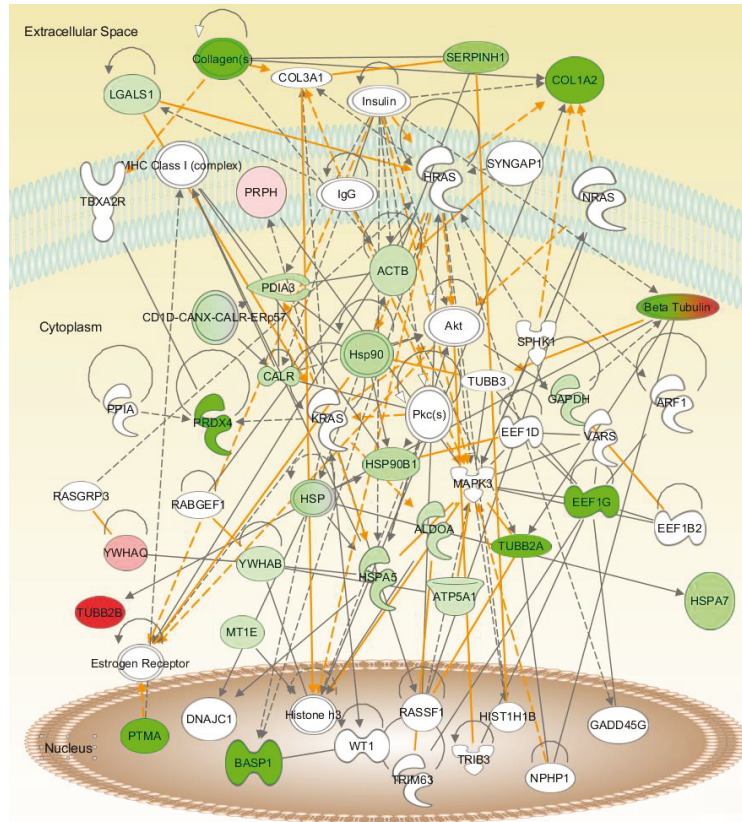


Figure 4. (continued).

Table 3. The high-scored biological networks in human aortic SMCs based on Ingenuity Pathway Analysis: proteins in bold were identified to be significantly differentially regulated in this study, and others were added by IPA for network analysis. A total of 5 networks were found with a score of >2, which is considered to be significant. The top functions, number of focus molecules, and the score number for each network are given. Networks 1 and 2, and their merge, are drawn in Figure 4.

| Network ID | Molecules in network | Top functions | Score | Focus molecules |
|------------|---|---|-------|-----------------|
| 1 | ACTB , Akt, ALDOA , ATP5A1 , Beta Tubulin, C7orf25, CALR , CD1D-CANX-CALR-ERp57, COL1A2 , collagen(s), DNAJC1, DNAJC10, GAPDH , GPAA1, histone 3, Hsp90, HSP, HSP90B1 , HSPA5 , HSPA7 , IgG, insulin, LGALS1 , MHC Class I (complex), MT1E , PHP1, PDI3 , Pkc(s), PTMA , SAMD4B, SERPINH1 , TUBB2A , TUBB2B , YWHAB , YWHAQ | Cellular compromise, cellular function and maintenance, posttranslational modification | 49 | 18 |
| 2 | ANXA6, ARF1, BASP1 , COL3A1, ECH1, EEF1B2, EEF1D, EEF1G , EFNA1, estrogen receptor, FEN1, FGF7, GADD45G, HIST1H1B, HRAS, KRAS, MAPK3, NRAS, PPIA, PRDX4 , PRPH , PTPRS, RABGEF1, RASGRP3, RASSF1, RGL2, SPHK1, SYNGAP1, TBXA2R, TGFB3, TRIB3, TRIM63, TUBB3, VARS, WT1 | Cell signaling, cellular development, tissue development | 8 | 4 |
| 3 | HIST1H2AH , HNF4A | Cellular development, tissue development, gene expression | 3 | 1 |
| 4 | ERBB2, miR-1/miR-206/miR-1a, SH3BGR13 | Cell-to-cell signaling and interaction, cellular assembly and organization, nervous system development and function | 3 | 1 |
| 5 | APCS, AURKA, Ca2+, CALU , CEP192, GGCX, IGF1R, PLCB2, RHOJ, TERT, TPX2 | Cancer, tissue development, cellular growth and proliferation | 2 | 1 |

A

Top Canonical Pathways

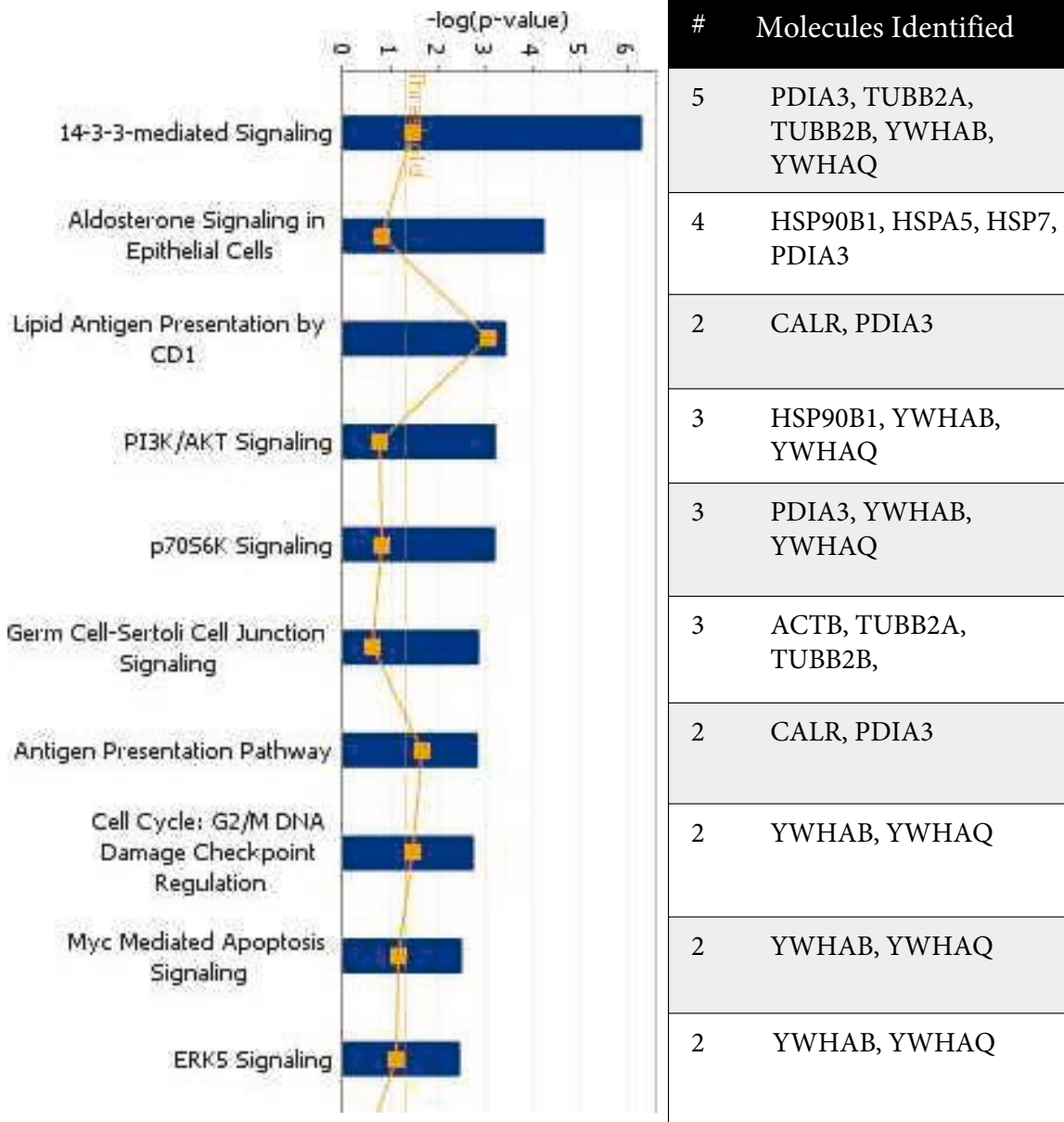


Figure 5. Top canonical pathways and biological functions based on the Ingenuity Pathway Analysis (IPA) of differentially regulated genes in TAAs. The 26 significant genes identified by LC-MS/MS were used for the analysis of canonical pathways (A) and functions (B). The bars represent the various pathways or functions described on the left of the graphs. The heights of the bars indicate the p values. The yellow boxes indicate # of genes in the list/total # of genes in the pathway. The yellow line shows the threshold of significance. The number of classified genes and the list of these identified molecules are given in the table on the right.

and cell death (Gardino and Yaffe, 2011). Supportively, our analyses showed greatly increased population-doubling times for TAA SMCs, and expression changes in the 14-3-3 pathway proteins could be partially responsible for the observed difference in the cell cycle. Abnormal expression

of 14-3-3 proteins and/or dysregulation of 14-3-3/target interactions have been previously reported and associated with various human diseases (Freeman and Morrison, 2011; Steinacker et al., 2011; Zhao et al., 2011), making 14-3-3 proteins a potential target for therapeutic agents (Zhao

Top Functions

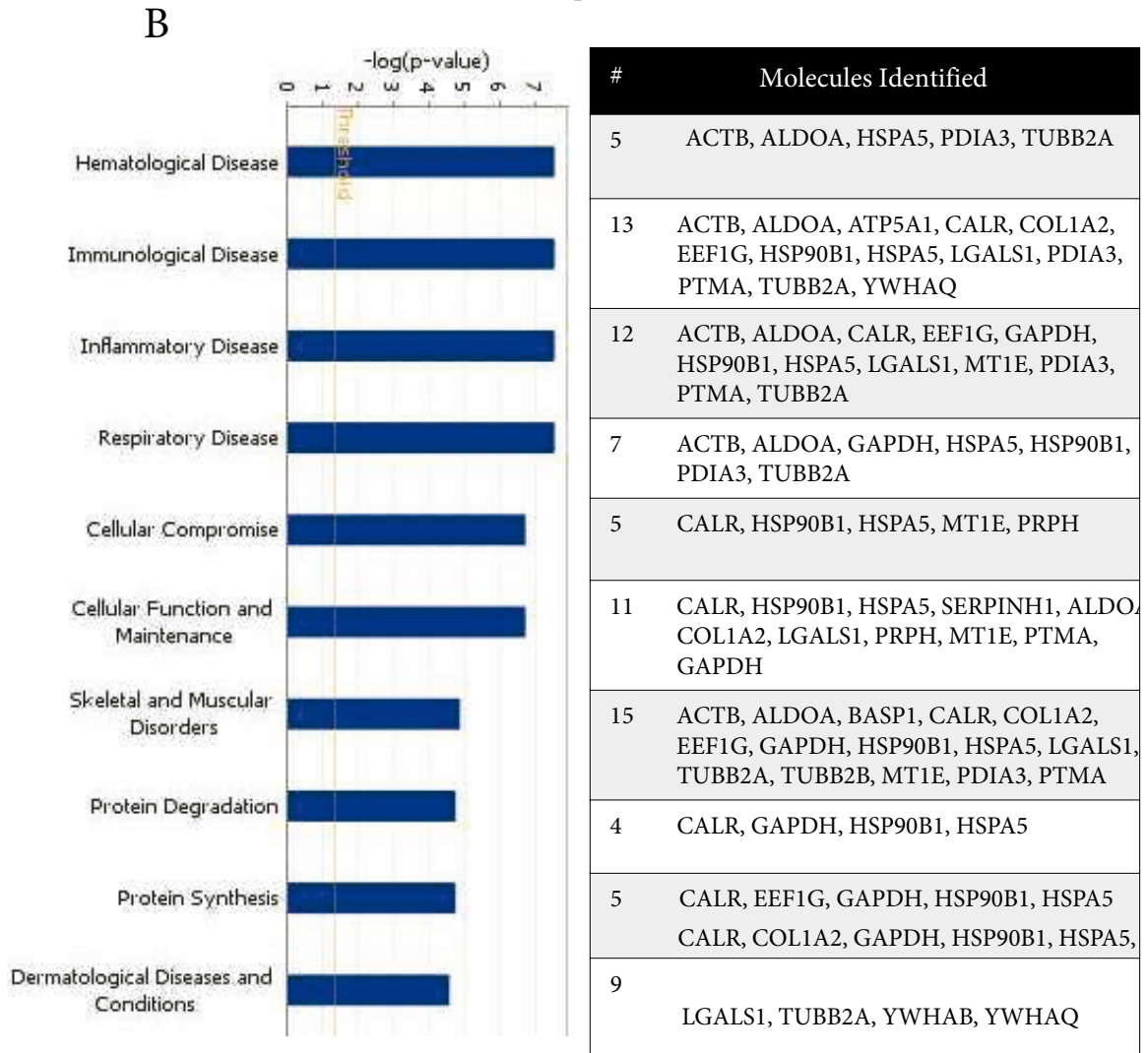


Figure 5. (continued).

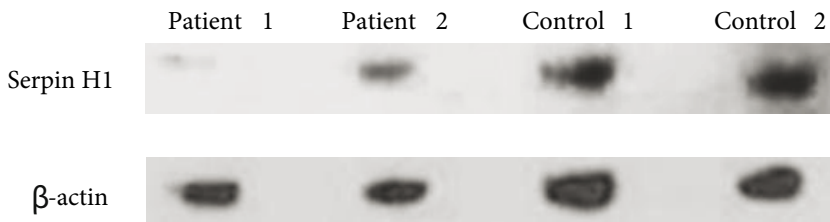


Figure 6. SerpinH1/HSP47 expression: the sample identity in each lane is indicated above the gel picture. The reduction in SerpinH1/HSP47 expression in patient SMCs was confirmed via western blotting. α -Actin was used as the loading control.

et al., 2011). Interestingly, similar to TAAs, as a result of a proteomic study in AAAs, many 14-3-3 subunits have been proposed as potential biomarkers in many biological samples and were patented for possible uses in diagnosis (Smalley et al., 2009).

The limitations of this study include the small sample size, the containment of only one sex (males), and the difference in age between the groups at the time of surgery. The low proliferative capacity of SMCs and the availability of control tissue were the major restrictive factors for the small sample

size. Despite these limitations, we were able to identify previously reported pathways, implying that this study can provide relevant information. However, these results should be confirmed using larger study groups and validated by the immunoblotting of each protein. Accordingly, this study should be considered a supportive base for new hypotheses.

In conclusion, SMCs isolated from TAAs differentially express proteins that appear to be potentially important for disease development and have not been previously reported for TAAs. In the future, it will also be interesting to see whether these results are valid *in vivo*, where SMCs are

isolated via laser capture microdissection microscopy and further examined. Indeed, studies like this one and others will serve as a database before large clinical studies take place for evaluation of the value of such biomarkers.

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