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# Proteomic evidence for the plasticity of cultured vascular smooth muscle cells

**Ahmet Tarık BAYKAL[1], Betül BAYKAL[1], Müge SERHATLI[1], Zelal ADIGÜZEL[1], Mehmet Altuğ TUNÇER[2], Ömer KAÇAR[1], Kemal BAYSAL[1,3], Ceyda AÇILAN AYHAN[1,*]**

[1]Genetic Engineering and Biotechnology Institute, TÜBİTAK Marmara Research Center, Gebze, Kocaeli, Turkey
[2]Kartal Koşuyolu Education and Research Hospital, Republic of Turkey Ministry of Health, İstanbul, Turkey
[3]Department of Biochemistry, Medical Faculty, Dokuz Eylül University, İnciraltı, İzmir, Turkey

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**Abstract:** Morphological, functional, and gene expression studies have established the phenotypic plasticity of smooth muscle cells (SMCs). These cells have been shown to respond to environmental stimulants such as extracellular matrix and growth factors. Cellular changes can vary between extremes defined as the contractile and synthetic states. Various growth factors have been shown to have profound effects on the phenotype of these cells. In this study, we intended to investigate the effects of growth factor-rich medium on the protein expression of vascular SMCs in culture. Interestingly, transiently changing the type of medium did not result in any apparent morphological differences, yet we hypothesized that some cellular factors might still be altered. In order to understand what kind of intracellular molecular changes should be expected to occur during the medium change, we analyzed global protein expression changes using nano-LC-MS/MS in smooth muscle cell cultures that were isolated and grown in one medium formulation and temporarily switched to the other. Our data indicate that proteins playing a role in energy metabolism (glycolysis), translation, and folding of proteins are affected, along with regulatory molecules and cytoskeletal proteins. The individual proteins and their significance are discussed within the scope of this paper.

**Key words**: Thoracic aortic aneurysm, label-free LC-MS, smooth muscle cell, pathway analysis, smooth muscle cell growth medium, DMEM/F12 growth medium

## 1. Introduction

Cell culture has been one of the most popular methods for researchers studying mammalian systems since its establishment in the mid-1900s (1). It is a complicated task, and cells must be maintained under certain growth conditions such as temperature, humidity, $CO_2$ content, well-adjusted salt concentrations, optimum pH, and the presence of growth factors (2). Today, many of these factors have been well established and it has become routine to keep most cell contents under these controlled settings. However, one of the critical issues regarding cell culture maintenance that constantly evolve is the formulation of media, whose requirements vary greatly for different cell types. This is especially difficult with primary cells (2).

Mature vascular smooth muscle cells (SMCs) are able to undergo transition from a quiescent, contractile state to a synthetic, proliferative form (3). Differentiated SMCs express a group of cytosolic and membrane proteins that are necessary for contractile function (4). Various factors and diseases such as atherosclerosis, restenosis, and hypertension cause the cells to dedifferentiate into a highly synthetic phenotype (5). Such phenotypic modulation has recently been reported in aortic aneurysms (6). Furthermore, many studies report that there is a continuum from the contractile stage to the more proliferative stage, and cells quickly adapt the synthetic phenotype in cell culture (7). A wide range of signaling factors have been implicated in the transition between these 2 stages, including basic fibroblast growth factor (bFGF), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), angiotensin-II, α-thrombin, factor Xa, endothelin-1, unsaturated lysophosphatidic acids, and fetal bovine serum (FBS) (8–13). Hence, a phenotypic change can be expected when cells are cultured in specific medium formulations and then switched to another.

There have been several reports studying vascular SMCs that were grown in different types of media ranging from regular media such as M199 (14,15), DMEM (16–18), or DMEM/F12 (19) supplemented with FBS, with or without the addition of the above mentioned growth factors, or commercially available media specially optimized for the

* Correspondence: ceyda.acilan@tubitak.gov.tr

growth of SMCs (20–23). While the optimized media appear valuable in terms of being successful in establishing new primary cell lines and being able to passage longer, they are not cost-effective. Given the variability for the choice of media, it is also hard to cross-compare results from different laboratories. Furthermore, switching from one formulation to another between experiments (14,19) for several purposes can also lead to changes that may interfere with the problem under study.

In order to understand what kind of changes should be anticipated by shifting growth conditions, we used SMCs isolated from human aorta and grown in enriched or poor medium formulations (medium with or without growth factors) from the day of isolation and then switched to the other medium for 72 h. We observed the effect of changing medium conditions in both of these samples by analysis of global proteomic changes via nano-liquid chromatography–tandem mass spectrometry (LC-MS/MS). The hypothesis was that cells grown in identical media would exhibit similar proteomes. As expected, we found that switching media resulted in changes in the global proteome. Among the identified proteins, the expression of more than 100 proteins was significantly altered after the medium shift. Within these, 43 proteins were common in the 2 study groups. To our surprise, both the direction of the change (upregulation or downregulation) and the fold change were parallel regardless of whether the change was to the enriched or to the poor medium conditions. We concluded that cells adapt to environmental changes by changing the proteome and similar proteins are effectuated during this adaptation, yet the effect appears to be a result of the environmental change itself, rather than a response to the particular medium used.

## 2. Materials and methods

### 2.1. Cell culture

SMCs were either thoracic aortic tissue samples from patients undergoing aneurysm surgery or coronary bypass surgery, as described previously (24,25), or were purchased commercially (Cell Applications, Inc., 354-05a). The tissue samples were collected and SMCs were isolated on the same day the surgery was performed. The tissues were kept in phosphate buffered saline (PBS) solution with penicillin (10,000 U/mL, streptomycin 10 mg/mL) until isolation of cells. After the adventitia and endothelial layer of the vessel were removed, the tissue (~2 $mm^2$) was kept at 5% $CO_2$, 37 °C, for 0.5–1 h on gelatin-coated plates for attachment before the addition of culture medium. The medium (DMEM/F12 (Sigma, D0547) + 10% FBS (Biochrome, S0115)) was changed every 2 to 3 days, and cells were passaged once approximately 70%–80% confluency was reached. Cells were maintained in this formulation of medium and were switched to enriched human aortic smooth muscle cell growth medium (SMC-GM, Cell Applications, Inc., 311K) for 72 h, and were then lysed as described below. Cells that were purchased commercially were isolated by the company, grown in an enriched medium, and switched to poor growth conditions (DMEM/F12 + 10% FBS) for 72 h before lysates were prepared. The complete formulation for DMEM/F12 can be found on the Sigma company website, but the formulation for SMC-GM is proprietary. Cell Applications has kindly agreed to partially disclose the ingredients within the growth medium supplement as FBS, rhEGF, rhbFGF/heparin, and insulin. The growth medium also contained 1 g/L glucose and no oxalates.

### 2.2. Immunohistochemistry staining

SMCs were characterized with α-SMC-actin. Cells were seeded on sterile 18-mm cover slips and grown in culture for 48 h. For fixation, cells were incubated in ice-cold methanol for 10 min at –20 °C. Cells were then washed with PBS and stored until staining at 4 °C in PBS. Staining was performed similarly to the protocol used by Akkoyunlu et al. (26). A ready-to-use detection system was used for immunohistochemistry (UltraVision Anti-Polyvalent, HRP, Thermo Scientific). Initially, the cells were incubated with $H_2O_2$ at room temperature (RT) for 10–15 min to block endogenous peroxidase activity. This was followed by incubation in blocking solution for 5 min at RT to block nonspecific binding (Thermo Scientific Ultra V Block). The polyclonal primary antibodies (α-actin, Abcam, ab5694) were subsequently added and the slides were incubated for 30 min (1:100 in PBS). Color formation was achieved by adding diaminobenzidine (DAB) chromogen and DAB substrate mixture (RT, 5–10 min) and the cells' nuclei were visualized by staining with Mayer's hematoxylin.

### 2.3. Sample preparation

Sample preparation was done according to an earlier published protocol (27). Cultured cells were collected with HyQtase (Hyclone, SV3003001) treatment and later washed twice with 50 mM ice-cold ammonium bicarbonate. Cells were pelleted by centrifugation at 1200 rpm and frozen at –80 °C until LC-MS analysis. Next, 100 µL of 0.1% RapiGest (Waters Corp.), an MS-compatible detergent (in 50 mM AmBic), was added to the frozen cells containing a protease inhibitor cocktail and lysed by ultrasonication (10 s on, 10 s off, for 3 cycles). Cell debris was removed via centrifugation at 14000 × *g* and the supernatant was collected. Sample desalting was done by washing the protein mixture with 50 mM Ambic in 5-kDa cut-off spin columns (Vivaspin, Sartorius). Next, 50 µg of protein from each sample measured with the Bradford method was transferred to an Eppendorf vial and alkylated with iodoacetamide (10 mM, 30 min of incubation in the dark at RT) following disulfite bond reduction with dithiothreitol (5 mM, 15 min of incubation at 60 °C). Tryptic peptides were generated with incubation of the

protein mixture with proteomics-grade trypsin (1:100 protein-to-trypsin ratio) at 37 °C overnight.

**2.4. LC-MS/MS analysis**

The parameters for the LC-MS analysis were optimized previously (27). A tryptic peptide mixture was spiked with 50 fmol MassPREP Enolase Digestion Standard (Waters Corp.). Three technical replicate analyses were performed for each biological sample, and a 2-µL volume of sample containing 500 ng of tryptic peptide was loaded onto the LC-ESI-qTOF system (nanoACQUITY ultrapressure liquid chromatography (UPLC) and SYNAPT high-definition mass spectrometer with NanoLockSpray ion source, Waters)). Columns were equilibrated with 97% mobile phase A ($H_2O$, 0.1% FA) and 3% mobile phase B (ACN, 0.1% FA). The column temperature was set to 35 °C. Peptides were trapped on a nanoACQUITY UPLC Symmetry C18 trap column (5 µm particle size, 180 µm i.d. × 20 mm length) at a flow rate of 5 µL/min for 5 min. Peptides were then eluted from the trap column by gradient elution onto an analytical column (nanoACQUITY UPLC BEH C18 Column, 1.7 µm particle size, 75 µm i.d. × 250 mm length, Waters) at a flow rate of 300 nL/min with a linear gradient from 5% to 40% acetonitrile over 90 min. Parallel collusion-induced dissociation ($MS^E$) was done at positive ion V mode, applying the MS and MS/MS functions over 1.5-s intervals with 6-V low energy and high-energy collusions of 15–40 V. Mass drift was corrected by infusing glu-fibrinopeptide (500 pmol/µL) every 45 s through the NanoLockSpray ion source at a flow rate of 300 nL/min. Peptide signal data between 50 and 1600 m/z were collected.

**2.5. LC-MS/MS data processing**

Data processing parameters were the default values for the SYNAPT system (27). Peptide mass measurement was performed at low collusion energy and peptide sequence data collection at higher collusion energies. Tandem mass spectra extraction, charge state deconvolution, and deisotoping were processed with ProteinLynx Global Server v 2.3 software (PLGS) (Waters Corp.). The protein sequence database from UniProt was used. The amino acid sequence of the internal standard (yeast alcohol dehydrogenase, UniProt accession no. P00330) was included in 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-terminal, 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 (28–30). The false positive rate of the IDENTITY$^E$ algorithm is around 3%–4% (31) with a randomized database and is 5 times larger than the original one (32). The quantitative analysis is based on the identified proteins, which are 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 version 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 40% expression change.

## 3. Results

**3.1. Sample characterization and morphology**

In order to understand the effect of switching medium formulations on the SMC proteome, we extracted SMCs from the thoracic region of the aorta and maintained the cells in the same medium formulation until the day of the experiment. A total of 4 SMC cultures were used in this study: while 2 of the cultures were kept in DMEM Nutrient Mixture F-12 (DMEM/F-12) medium supplemented with 10% FBS from the first day of isolation, 2 others were established and maintained in an optimized smooth muscle cell growth medium (SMC-GM, Cell Applications, Inc.). Due to the small size of the sample obtained, we could not divide the tissue sample into 2 to obtain cell cultures from the same origin grown in different medium formulations from the beginning. However, the differences due to different cell origins are less likely to be significant as each sample (lysate from cells after medium change) is compared to its own origin (lysate from cells always kept in the original medium).

The cells were tested for smooth muscle α-actin positivity (>95%) to confirm smooth muscle origin (Figure 1) and were used for proteomic studies between passages 3–8 (3 and 7 versus 4 and 8). The mean age of all individuals from which the cells were isolated was similar (53 and 58 versus 48 and 54 years). The subjects were all males except for one.

Interestingly, cells did not adapt to a different morphology even though they were switched to a completely different formulation for 72 h. Hence, transient medium changes may not alter basic cellular characteristics such as size, shape, or structure (Figure 2).

**3.2. Label-free LC-MS/MS and data quality**

Once cells reached a sufficient number to continue with proteomic analysis (approximately 500,000 cells) and

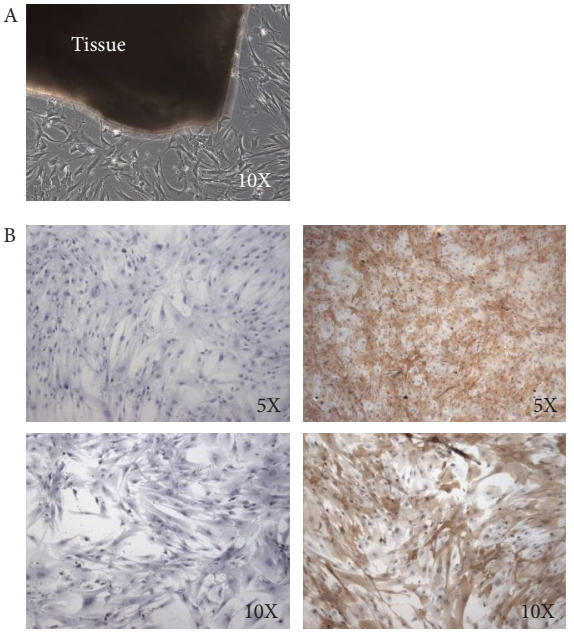


**Figure 1.** A) Images of smooth muscle cells extracted from aortic tissue. Image was taken with a phase contrast filter (10× magnification). B) Cells were characterized by α-actin positivity for smooth muscle origin. Images were taken with a bright field filter (5× and 10× magnification as indicated).

a confluence of 70%–80%, the medium was switched to DMEM/F12 if cells were regularly kept in SMC-GM, or to SMC-GM if cells were regularly kept in DMEM/F12. Cells were maintained in the new medium for 72 h and were lysed and used in LC-MS/MS analyses as described in Section 2. Tryptic peptides generated from SMCs were qualitatively and quantitatively analyzed with label-free nano-LC-MS/MS methodology. Peptide separation was performed on nanoACQUITY UPLC and peptide m/z measurement and sequencing were performed on a SYNAPT HDMS mass spectrometer. The quantitative rigor of the peptide measurement was evaluated based on 3 technical replicates. A triplicate injection of the control sample followed by the calculation of the standard deviations of the quantitative values for each protein provided the variation in the measurements. The intensity coefficient of variation (CV), expressed as %CV intensity, averaged below 16% across all the identified peptides. The accuracy of the peptide mass-to-charge ratio of the identified peptides was evaluated and the calculated average mass error for the precursors was shown to be below 5 ppm. Label-free analysis was based on the reproducibility of peptide elution times on nanochromatography instruments. The chromatographic retention time coefficient of variation, calculated as %CV RT, averaged below 0.5%, providing very reproducible peptide elutions for comparative analysis.

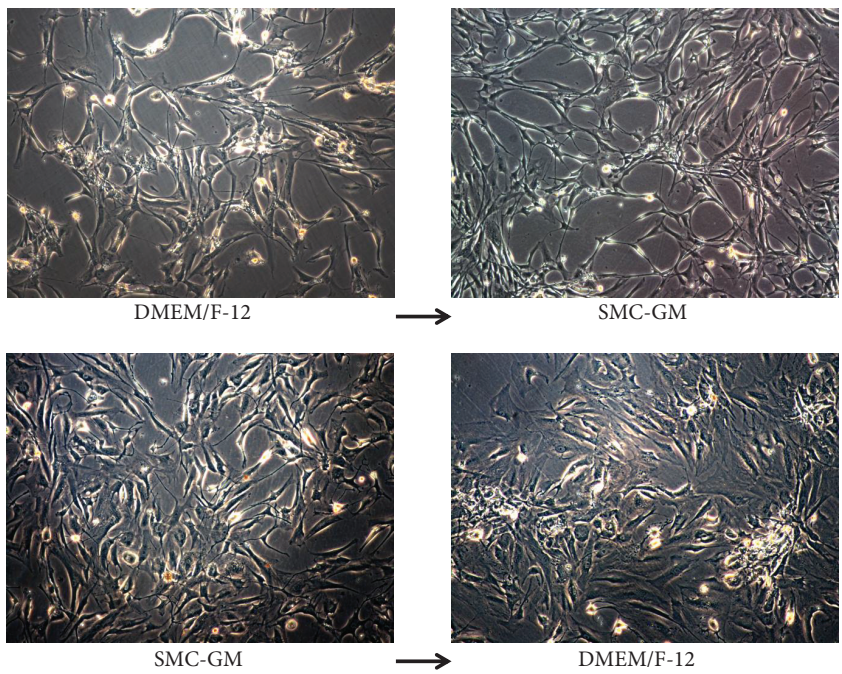


**Figure 2.** Cell morphology before and after change of medium: left column indicates the morphology in medium in which cells were extracted and never prone to medium change. The right column indicates the morphology after medium change. DMEM/F-12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; SMC-GM: Smooth Muscle Cell Growth Medium. Phase contrast microscopy, 10× magnification.

### 3.3. The effect of medium change on the smooth muscle cell proteome

Applying the identification criteria discussed in Section 2, a total of 379 proteins were identified when the medium was shifted from SMC-GM to DMEM/F12, and 294 proteins were identified when the medium was changed from DMEM/F12 to SMC-GM. Utilizing PLGS, the expressions of more than 100 proteins were significantly altered in each experiment. Two additional elimination criteria were applied: first, based on PLGS identification score (a score below 100 was considered unreliable after a manual reevaluation of their protein spectra), and second, based on fold change (intensity cut-off was set to 40% and only the proteins that were up- or downregulated by more than the 40% cut-off were reported). When these limiting criteria were followed, 8 (SMC-GM → DMEM/F12) and 29 (DMEM/F12 → SMC-GM) of the differentially expressed proteins were further eliminated, still leaving about 100 proteins that were significantly differentially expressed. A summary is given in Table 1.

Interestingly, 43 of the approximately 100 proteins that were found to be significant were the same on both lists. Forty of the 43 proteins were downregulated in response to changing the medium, while only 3 of 43 were upregulated in 1 medium and downregulated in the other. Since these 43 proteins were identified on both lists, we decided to focus on these common proteins in order to understand the effect of medium change on SMCs. The complete list and categorization of these proteins is shown in Table 2.

### 3.4. Ingenuity Pathway Analysis

Based on their function, the 43 proteins were grouped as ones that play a role in energy metabolism (mainly glycolysis), that play a role in protein folding, that play a role in translation of proteins, that are regulatory, that are structural cytoskeleton proteins, or that belong to annexin family, as well as 3 others that do not fall into these categories. In order to understand how these proteins are associated with each other as well as with other signal transduction pathways, the data were further analyzed using Ingenuity Pathway Analysis (IPA version 8.5) software, which is a knowledge-based database containing biological interactions and functional annotations created from millions of modeled relationships between different types of biological samples and molecules. Interestingly, more than half of these proteins (23/43) were merged into a single network, with a score of 61 (Figure 3). 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 is, the lower the probability of randomness. The complete list of networks and scores is given in Table 3. Consistent with our manual protein characterization analysis based on function, IPA analysis showed that the top canonical pathway was the glycolysis/gluconeogenesis pathway ($P = 2.5 \times 10^{-9}$), which was followed by the 14-3-3 mediated signaling pathway ($P = 1.37 \times 10^{-5}$). The top 10 canonical pathways and biological functions are given in Figure 4.

## 4. Discussion

It is well known that change in environmental conditions may affect both the morphology and the differentiation state of cells (33–35). Not only the composition of the medium but also the replacement of culture media has been shown to affect gene expression in a periodic wave on differentiating cells (36). On the other hand, some other cell types appear to be less affected by such modifications, where medium change had been reported to have neither favorable nor harmful consequences on the development when cultured in different media, indicating that some factors have less impact on various cellular properties (37). Indeed, fewer changes are expected to occur when cells are already finally differentiated. The change will also probably be cell type-specific, and the response may be different when different cells are under study. Hence, there still remains a question as to what kind of intracellular molecular changes occur within the cells after changing the culture medium.

In this study, we wished to focus on vascular SMCs as they are frequently used as a model system in the study of cardiovascular diseases and there is a need for switching from one medium to another as a result of experimental protocols. In order to determine the impact of medium change on SMCs, we performed a label-free nano-LC-MS/MS analysis and examined the changes in the proteome. Before the analysis was performed, our hypothesis was that cells would behave similarly given that they are grown in the same medium. The expectation was that shifting from one medium to the other, i.e. from SMC-GM to DMEM/F12, would drive the cells to have similar proteome profiles as the ones that were always grown in DMEM/F12.

**Table 1.** Number of proteins identified.

| Medium change | Total proteins identified | Proteins with significantly altered expression | Proteins with PLGS score of >100 | Number of proteins common in both lists |
|---|---|---|---|---|
| SMC-GM → DMEM/F12 | 379 | 110 | 104 | 43 |
| DMEM/F12 → SMC-GM | 294 | 132 | 113 | |

**Table 2.** List and function of proteins that appear to be significantly altered on both lists. The direction of the arrow indicates whether the expression of the protein is up- or downregulated. Three arrows indicate unique proteins; 2 arrows indicate proteins whose expression is changed more than 5-fold. Note: The functions may include information from protein databases such as UniProt.

| Accession | Description | Regulation | Function |
|---|---|---|---|
| | | Energy metabolism | |
| P00338 | LDHA: L lactate dehydrogenase A chain | ↓ | Catalyzes the conversion of L-lactate and NAD+ to pyruvate and NADH in the final step of anaerobic glycolysis |
| P07195 | LDHB: L lactate dehydrogenase B chain | ↓ | Catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+ |
| P14618 | KPYM: Pyruvate kinase isozymes | ↓ | Glycolytic enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP<br>Plays a general role in caspase-independent cell death of tumor cells |
| P04406 | G3P: Glyceraldehyde 3 phosphate dehydrogenase | ↓↓ | Catalyzes step 6 of glycolysis |
| P18669 | PGAM1: Phosphoglycerate mutase 1 | ↓ | Catalyzes step 8 of glycolysis |
| P06733 | ENOA: Alpha enolase | ↓ | A glycolytic enzyme expressed in most tissues, one of the isozymes of enolase<br>Encodes the Myc-binding protein-1(MBP1), which downregulates the activity of c-myc<br>Plays a part in processes such as growth control, hypoxia tolerance, and allergic responses<br>May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell types such as leukocytes and neurons; stimulates immunoglobulin production |
| P60174 | TPIS: Triosephosphate isomerase | ↓ | Catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate |
| P37837 | TALDO: Transaldolase | ↓↓↓ | Generation of nicotinamide adenine dinucleotide phosphate (reduced NADPH), for reductive biosynthesis<br>Formation of ribose, which is an essential component of ATP, DNA, and RNA<br>Transaldolase links the pentose phosphate pathway to glycolysis |
| | | Protein folding | |
| P07237 | PDIA1: Protein disulfide isomerase | ↓ | Catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold |
| P30101 | PDIA3: Protein disulfide isomerase A3 | ↓ | Catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold |
| Q15084 | PDIA6: Protein disulfide isomerase A6 | ↓ | May function as a chaperone that inhibits aggregation of misfolded proteins<br>Plays a role in platelet aggregation and activation by agonists such as convulxin, collagen, and thrombin |

**Table 2.** (continued).

| | | | |
|---|---|---|---|
| Q8NBS9 | TXND5: Thioredoxin domain containing protein 5 | ↓ | A protein disulfide-isomerase; its expression is induced by hypoxia<br>Its role may be to protect hypoxic cells from apoptosis |
| P14625 | ENPL: Endoplasmin | ↓ | Molecular chaperone that functions in the processing and transport of secreted proteins; has ATPase activity |
| P50454 | SERPH: Serpin H1 | ↓ | Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1,<br>human chaperone protein for collagen |
| P08238 | HS90B: Heat shock protein HSP 90 beta | ↓ | |
| P27797 | CALR: Calreticulin | ↓ | Multifunctional protein that binds $Ca^{2+}$ ions, rendering them inactive<br>Binds to misfolded proteins and prevents them from being exported<br>from the endoplasmic reticulum to the Golgi apparatus |
| P62988 | UBIQ: Ubiquitin | ↓↓ | Binds to proteins and labels them for destruction |
| | | Cytoskeleton | |
| | | Actin cytoskeleton | |
| P60709 | ACTB: Actin cytoplasmic 1 | ↓↑ | Highly conserved proteins that are involved in various types of cell motility |
| P26038 | MOES: Moesin | ↓ | Probably involved in connections of major cytoskeletal structures to the plasma membrane |
| P07737 | PROF1: Profilin | ↓↓ | Regulates actin polymerization in response to extracellular signals |
| Q562R1 | ACTBL: Beta-actin-like protein 2 | ↑ | Beta-actins coexist in most cell types as components of the cytoskeleton and as mediators of internal cell motility |
| | | Tubulin cytoskeleton | |
| P07437 | TBB5: Tubulin beta chain | ↓ | Major constituent of microtubules |
| Q13748 | TBA3C: Tubulin alpha 3C D chain | ↓↓↓ | One of the alpha tubulin genes |
| P68363 | TBA1B: Tubulin alpha 1B chain OS | ↓ | Tubulin binds 2 moles of GTP, 1 at an exchangeable site on the beta chain |
| | | Intermediate filaments | |
| P04264 | K2C1 Keratin type II cytoskeletal 1 | ↓↓↓ | May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein kinase C (RACK1/ GNB2L1). |
| P17661 | DESM Desmin | ↓ | type III intermediate filament found near the Z line in sarcomeres |
| P41219 | PERI Peripherin | ↓ | a type III Intermediate filament (IF) protein expressed mainly in neurons |
| | | Translation | |
| P62424 | RL7A: 60S ribosomal protein L7a | ↓↑ | One of the proteins in the 60S large subunit of the eukaryotic ribosome |
| P05387 | RLA2: 60S acidic ribosomal protein P2 | ↓↓↓ | A ribosomal phosphoprotein that is a component of the 60S large subunit |

**Table 2.** (continued).

| | | | |
|---|---|---|---|
| P13639 | EF2: Elongation factor 2 | ↓ | Catalyzes the GTP-dependent ribosomal translocation step during translation elongation<br>Catalyzes the coordinated movement of the 2 tRNA molecules, the mRNA, and conformational changes in the ribosome |
| Q05639 | EF1A2: Elongation factor 1 alpha 2 | ↓ | Is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome |
| P06748 | NPM: Nucleophosmin | ↓ | Is involved in the biogenesis of ribosomes<br>May assist small basic proteins in their transport to the nucleolus |
| P62937 | PPIA: Peptidyl prolyl cis trans isomerase A | ↓ | Catalyzes the interconversion of peptidylprolyl imide bonds in peptide and protein substrates |
| | Regulatory protein | | |
| P62258 | 1433E: 14-3-3 protein epsilon | ↓ | Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways<br>Interacts with CDC25, RAF1, and IRS1, suggesting its role in diverse biochemical activities related to signal transduction, such as cell division and regulation of insulin sensitivity |
| P63104 | 1433Z: 14-3-3 protein zeta delta | ↓ | Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways<br>Binding generally results in the modulation of the activity of the binding partner |
| P09382 | LEG1: Galectin | ↓ | May regulate apoptosis, cell proliferation, and cell differentiation<br>Inhibits CD45 protein phosphatase activity and therefore the dephosphorylation of Lyn kinase<br>Beta-galactoside-binding protein, modulates cell–cell and cell–matrix interactions |
| P06703 | S10A6: Protein S100 A6 | ↓ | Involved in the regulation of a number of cellular processes, such as cell cycle progression and differentiation |
| | Annexin family | | |
| P07355 | ANXA2: Annexin A2 | ↓ | Is involved in diverse cellular processes such as cell motility (especially that of the epithelial cells), linkage of membrane-associated protein complexes to the actin cytoskeleton, endocytosis, fibrinolysis, ion channel formation, and cell matrix interactions<br>Is a calcium-dependent phospholipid-binding protein whose function is to help organize exocytosis of intracellular proteins to the extracellular domain<br>Annexin II is a pleiotropic protein, meaning that its function is dependent on place and time in the body |
| P04083 | ANXA1: Annexin A1 | ↓ | May have potential antiinflammatory activity<br>May act as a tumor suppressor gene and modulate the proliferative functions of estrogens |

**Table 2.** (continued).

| | | | |
|---|---|---|---|
| P08758 | ANXA5: Annexin A5 | ↓ | The function of the protein is unknown<br>Annexin A5 has been proposed to play a role in the inhibition of blood coagulation by competing for phosphatidylserine binding sites with prothrombin and also to inhibit the activity of phospholipase A1 |
| | | Others | |
| P13489 | RINI: Ribonuclease inhibitor | ↓ | Leucine-rich repeat protein that forms extremely tight complexes with certain ribonucleases |
| P20962 | PTMS: Parathymosin | ↓↓↓ | Parathymosin may mediate immune function by blocking the effect of prothymosin alpha, which confers resistance to certain opportunistic infections |
| Q14192 | FHL2: Four and a half LIM domains protein 2 | ↓↑ | Inhibits transcriptional activation of vSMC-specific genes mediated by the bone morphogenetic protein signaling pathway<br>May function as a molecular transmitter linking various signaling pathways to transcriptional regulation |

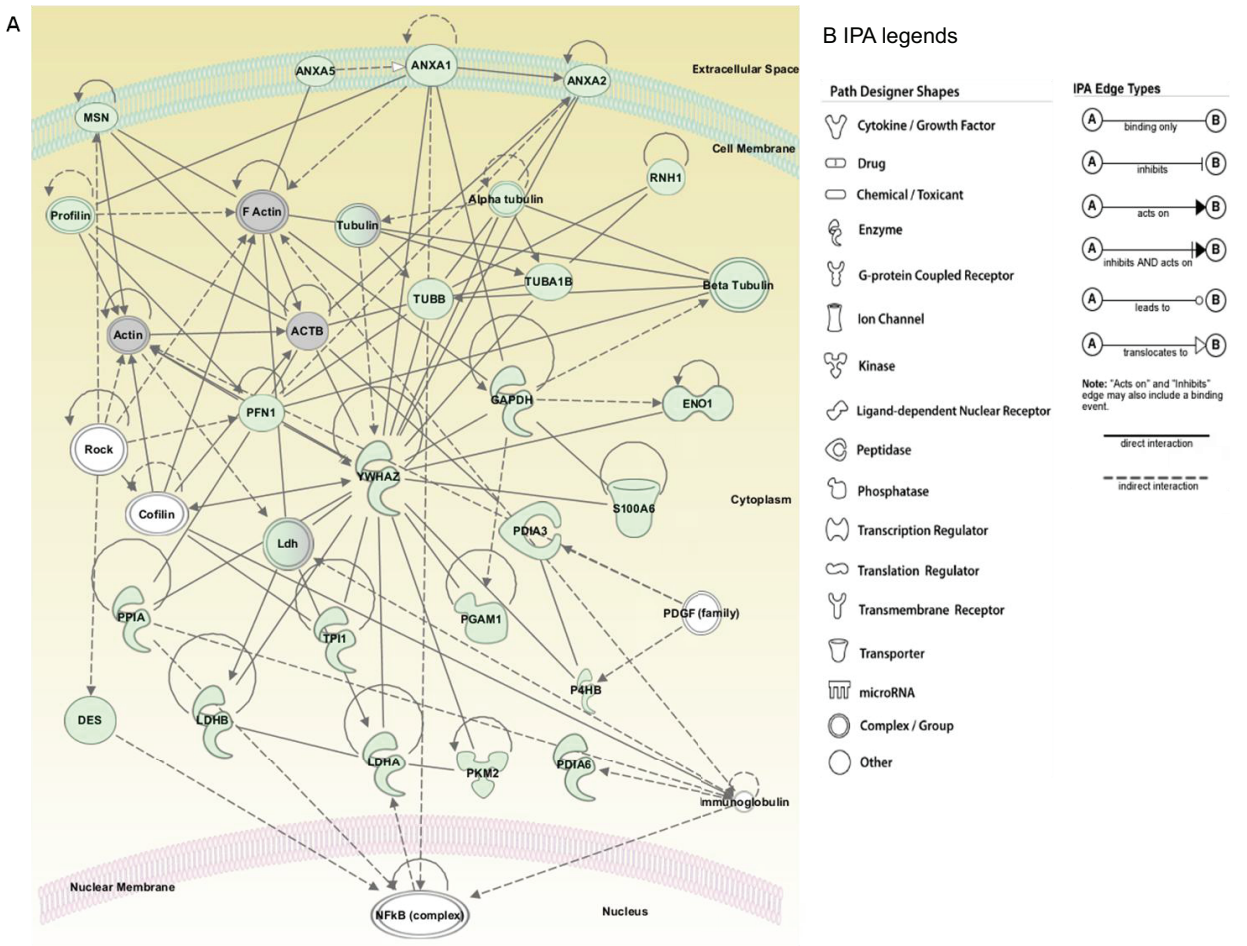


**Figure 3.** A) Most high-scored network (Network 1): pathway generated by IPA with proteins identified as specific target antigens for the common proteins on both lists in response to changing medium (see text for details). Proteins with no color were not detected by LC-MS, yet were added to the network by the IPA algorithm. Green indicates downregulation and gray indicates down- or upregulation (based on the medium change direction). The intensity of the color correlates with the degree of fold change in expression. Note that most proteins are downregulated and were fitted in a single network. B) IPA legends for path designer shapes and edge types.

**Table 3.** The high-scored biological networks in human aortic SMCs in response to medium change. Molecules in bold were identified in the analysis, while others were added by the software program to create a network.

| Network ID | Molecules in network | Top functions | Score | Focus molecules |
|---|---|---|---|---|
| 1 | ACTB, Actin, Alpha tubulin, ANXA1, ANXA2, ANXA5, Beta tubulin, Cofilin, DES, ENO1, F-actin, GAPDH, Immunoglobulin, Ldh, LDHA, LDHB, MSN, NFkB (complex), P4HB, PDGF (family), PDIA3, PDIA6, PFN1, PGAM1, PKM2, PPIA, Profilin, RNH1, Rock, S100A6, TPI1, TUBA1B, TUBB, Tubulin, YWHAZ | Hematological disease, immunological disease, inflammatory disease | 61 | 23 |
| 2 | 26S Proteasome, Akt, Calmodulin, CALR, CD3, Ck2, Collagen(s), Creb, EEF2, EEF1A2, ERK1/2, FHL2, Histone h3, Hsp70, Hsp90, HSP, HSP90AB1, HSP90B1, Insulin, Integrin, Jnk, KRT1, LGALS1, MHC class I (complex), NPM1, P38 MAPK, PI3K (complex), Pkc(s), Ras, RNA polymerase II, RPL7A, SERPINH1, TCR, Vegf, YWHAE | Cellular compromise, cellular function and maintenance, protein synthesis | 26 | 12 |
| 3 | ACTBL2, AP2A1, CCDC15, CHD3, EIF6, G6PD, GET4, HAUS1, HAUS2, HAUS3, HAUS4, HAUS5, HAUS6, HAUS7, HAUS8, IRF3, MYB, NAA38, PA2G4, PDS5A, PLK4, PRPH, PTMS, PXK, RELB, RPLP0, RPLP1, RPLP2, RXRB, TALDO1, TUBG1, TUBG2, TXNDC5, UBC, ZNF24 | Cellular assembly and organization, cellular function and maintenance, cell cycle | 11 | 6 |

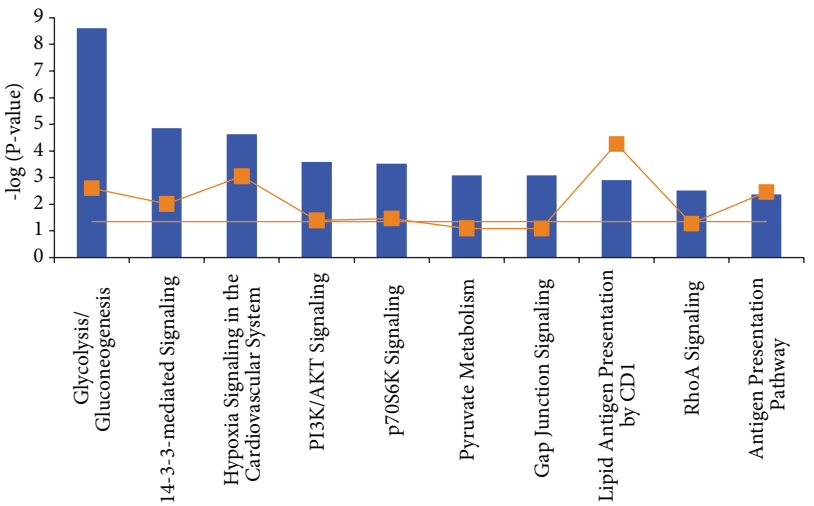


**Figure 4.** Top canonical pathways. The 43 significant genes identified by LC-MS/MS were used for the analysis of canonical pathways. The bars represent the various pathways or functions described on the left of the graph. The height of the bars indicates the P-values. The yellow boxes indicate # of genes in the list / total # of genes in the pathway. The yellow line shows the threshold of significance.

Interestingly, the list of differentially expressed proteins was quite similar: 43 out of approximately 100 proteins were the same on both lists. Such an overlap is quite striking, especially when completely independent samples and different experiments are analyzed. Furthermore, 40/43 of the overlapping proteins had the same direction of expression change (up- or downregulation). Hence, contrary to our expectations, our data indicated that the proteomic differences that we detected were a response to medium shift per se rather than a proteome profile for the cells grown in a specific medium.

The presence of growth factors has been shown to result in protein expression changes in SMCs in several previous studies (38–40). However, in our study, although we observed changes, the changes were not in response to the presence of growth factors. The fact that we failed

to find alterations specific to a growth medium could be explained as follows: 1) the changes are not immediate, i.e. longer incubation times are needed; 2) the altered proteins are of low abundance; or 3) limitations arose, derived from our equipment.

Based on our analysis, the glycolytic pathway appeared to be important with several proteins being differentially regulated after a medium change. The 2 formulations have different concentrations of glucose levels (1 g/L in SMC-GM and 3.15 g/L in DMEM/F12), and hence it is not surprising that a change is detected. Interestingly, however, switching to higher glucose levels did not stimulate the glycolytic pathway. Since most of these proteins are downregulated in both switches, we interpreted this result as a response of stalling cellular processes until the cells adapt to new conditions leading to less need for energetic processes. In a similar study, the number of medium changes was shown to affect the proliferation and metabolism of cultured cells (41). Although in this study we only changed the medium once (yet to a different formulation), both studies indicate that factors regarding the metabolism may be a common denominator as a result of medium change. Indeed, these suggestions should be verified via functional assays and additional immunoblotting experiments to strengthen and draw more rigid conclusions. Interestingly, proteins involved in translation and protein folding were also significantly downregulated, suggesting that synthesis and concurrently folding of new proteins were diminished in the time frame of the experiment. Reduction in the synthesis of new proteins can also explain the decreased requirement for energy production pathways. These 2 data sets show correlation with each other.

Surprisingly, although there were no apparent alterations in the morphology of cells after a medium change, our proteomic analysis revealed that several cytoskeletal proteins with examples from all types (including actin filament components, microtubules, and intermediate filaments) were in fact differentially expressed. This finding is further proof that molecular changes are not always reflected at the morphological level. Other identified proteins include regulatory proteins that function in several signaling pathways, suggesting the presence of additional expression changes that we failed to detect. Hence, additional studies are needed to identify more proteins to have a better understanding of the effect of medium.

In summary, our results suggest a general downregulation of proteins in glycolysis and in processes requiring energy such as protein synthesis and folding. With these findings, most importantly, we wish to raise awareness for the type of expression changes that might occur in response to shifting medium, albeit with no significant change in morphology, and draw scientists' attention to the need for careful interpretation of their results.

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