# SUPPLEMENTARY MATERIAL

# Tianma Modulates Blood Vessel Tonicity

Lin Feng[1,#], Arulmani Manavalan[1,2], Manisha Mishra[1,2], Siu Kwan Sze[1], Jiang-Miao Hu[3] and Klaus Heese[1,2,*]

[1]*School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551, Singapore*

[2]*Institute of Advanced Studies, Nanyang Technological University, 60 Nanyang View, 639673, Singapore*

[3]*Kunming Institute of Botany, Chinese Academy of Science, Kunming 650204, Yunnan, People's Republic of China*

## MATERIALS AND METHODS

### iTRAQ Protocol

#### *Sample Preparation - Acetone Precipitation*

Each sample condition had 600 µg of total protein lysate transferred to a new tube. Six volumes of 100 % -20 °C-chilled acetone were added to each tube and vortexed thoroughly at regular intervals. The tubes were incubated overnight at -20 °C and the following day, vortexed and centrifuged at 16,000 x g for 30 min to pellet down all proteins. The supernatant was discarded and the pellets were disturbed and washed in 500 µl of 90 % -20 °C-chilled acetone. Subsequently, the tubes were centrifuged at 16,000 x g for 20 min and the supernatant discarded. The washed pellets were allowed to air-dry at RT for 15 min, then dissolved in 100 µl of 200 mM TEAB (0.5 M Triethyl ammonium bicarbonate buffer), and 2 % SDS and then incubated at 50 °C for 5-10 min with simple agitation using a thermomixer (Eppendorf, Hamburg, Germany). Following which the tubes were centrifuged at 16,000 x g for 30 min. The supernatant was collected and protein concentration re-quantified using the 2-D Quant kit.

#### *SDS-PAGE and in-gel Digestion*

Each sample had 200 µg of acetone-precipitated proteins prepared (mixed with loading dye), denatured for 10 min in a thermo bath (Fine PCR, Seoul, Korea) and resolved up to 60 %. The gels were washed twice with autoclaved Milli-Q Water (MQW) for 5 min each. Fixing solution (50 % methanol and 10 % Acetic Acid (AcOH)) was added till the gels were submerged and kept overnight on a SH30L reciprocating shaker (Fine PCR). The gels were then washed with MQW thrice for 15 min each. In-gel digestion was performed in a laminar flow hood (Gelman, Singapore). The gels were diced into 1 – 2 mm pieces and transferred into tubes. 5 ml of 25 mM TEAB in 50 % Acetonitrile (ACN) buffer was added to the tubes, vortexed and left at RT for 10 min after which the buffer was discarded and the step repeated four times. Finally, 80 % ACN in 20 mM TEAB was added, vortexed and the tubes were left at RT for 10 min. The supernatant was discarded and the sample tubes were left to air-dry for 30 min.

#### *Reduction, Alkylation, Trypsin Digestion and Extraction*

Stock solutions of 200 mM tris (2-carboxyethyl) phosphine (TCEP) in HPLC water (J.T. Baker, Mallinckrodt, Inc., Phillipsburg, NJ, USA) and 200 mM S-methyl methanethiosulfonate (MMTS) in isopropanol were prepared. 5 mM of TCEP in 25 mM TEAB buffer was added to the dried gel pieces, vortexed and briefly spun before being incubated at 65 °C for 1 hr to allow a reduction reaction to take place. Following this, 10 mM MMTS in 25 mM TEAB buffer (tube was covered with aluminum foil) was added to gel pieces vortexed and briefly spun. The alkylation reaction was then allowed to proceed for 45 min in the dark at RT. The supernatant was removed and discarded. The gel pieces were again washed with 25 mM TEAB in 50 % ACN buffer as described above. The gel was dehydrated by 100 % ACN. Finally, the tubes were air-dried for 30 min. First, trypsin (4 µg of trypsin in 25 mM TEAB) was added to each set of the gel pieces and incubated at 4 °C for 15 min for proper rehydration. Then 10 ml of 2.5 µg trypsin solution was again added to tubes and incubated overnight in a 37 °C incubator.

Subsequently, the tubes were spun briefly and the aqueous extract of the digested solution was collected. To the remaining gel pieces, 50 % ACN and 1 % AcOH was added, vortexed and incubated in a water bath sonicator for 30 min. The supernatant was transferred and combined to the main sample tube. The extraction step was repeated 5 times. The trypsin digested peptides were pooled and dried completely in the SpeedVac (Concentrator 5301, Eppendorf) at 30 °C and stored at -20 °C.

[#]*Current address: Department of Obstetrics and Gynecology, University of California Irvine, Irvine, CA 92697, USA*

### *Labeling of Peptides with iTRAQ Tags (4 Plex)*

Each iTRAQ reagent tubes (tags- 114, 115, 116, 117) had 70 µl of 100 % ethanol added and vortexed thoroughly. The dried peptides were dissolved in 30 µl of 500 mM TEAB (dissolution buffer). Each iTRAQ tag was transferred to the respective peptide tubes and the tubes were incubated at RT for 2 hr with gentle shaking (thermomixer). All samples were then combined and kept in the SpeedVac at 30 °C to dry completely.

### *Desalting*

The dried peptide samples were reconstituted in 500 µl of 0.1 % formic acid (FA) and kept in the water bath sonicator for 5 min. 50 mg C18 cartridge (Sep-Pak® Vac C18 cartridges, Waters, Milford, MA, USA) was conditioned thrice with 100 % methanol pushed through at a rate of 2 to 3 drops per second via a syringe. The stationary phase was acidified three times with 0.1 % FA (following the same method as conditioning). The samples were loaded into the column and allowed to flow via gravitational force and the flow-through was reloaded three times. Next, the sample loaded columns were desalted twice with 0.1 % FA. Elution buffer (75 % ACN + 0.1 % FA) was added and, using a syringe, the buffer was pushed through the columns and the samples were collected. This C18 desalting protocol was performed thrice with the desalting wash's solution and the flow-through combined together. The samples were pooled and placed in the SpeedVac to dry and stored at -20 °C.

### *Electrostatic Repulsion-hydrophilic Interaction Chromatography (ERLIC)*

Eight hundred µg of iTRAQ-labeled peptides were fractionated using PolyWAX LP weak anion-exchange column (4.6 × 200 mm, 5 µm, 300 Å; PolyLC, Columbia, MD, USA), within the Shimadzu HPLC system (Kyoto, Japan). The HPLC gradient used composed of 100 % solvent A (85 % ACN, 0.1 % AcOH, 10 mM ammonium acetate, 1 % FA, pH 3.5) for 5 min, 0 %–36 % solvent B (30 % ACN, 0.1 % FA, pH 3.0) for 15 min, and 36 %–100 % solvent B for 25 min, and finally 100 % solvent B for 10 min, running for a total of 1 hr at a flow rate of 1.0 ml $min^{-1}$. A total of 29 fractions were collected and was later reduced to 16 fractions by pooling of samples. The 16 sample tubes were kept in SpeedVac to dry completely. The dried peptides in each sample tube were reconstituted in 100 µl 0.1 % FA for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

### *LC-MS/MS Analysis*

The samples were analyzed thrice using a Q-Star Elite mass spectrometer (Applied Biosystems/MDS SCIEX; Applied Biosystems, Foster City, CA, USA) coupled with an online microflow HPLC system (Shimadzu). 30 µl of peptide mixture was injected and separated on a home-packed nanobored C18 column with a picofrit nanospray tip (75 µm i.d. × 15 cm, 5 µm particles) (New Objectives, Wubrun, MA, USA) for each analysis (Multiple injections give a better coverage of the target proteome with superior statistical consistency. This is especially true for single peptide proteins as more MS/MS spectral evidence was obtained from multiple injections leading to higher confidence of peptide identification and quantification.). The samples were separated at a constant flow rate of 30 µl/min with a splitter achieving an effective flow rate of 0.3 µl/min. Data acquisition was performed in the positive ion mode, with a selected mass range of 300-1600 m/z, and peptide ions with +2 to +4 charge states were subject to MS/MS. The three most abundant peptide ions above 5 count threshold were selected for MS/MS and each selected target ion was dynamically excluded for 30 s with 30 mDa mass tolerance. Automatic collision energy and automatic MS/MS accumulation were used to activate smart information-dependent acquisition (IDA). With maximum accumulation time being 2 s, the fragment intensity multiplier was set to 20. The relative abundance of the proteins in the samples was reflected by the peak areas of the iTRAQ reporter ions.

### *Mass Spectrometric Data Analysis*

The data was acquired with the Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). Using ProteinPilot Software 3.0, Revision Number: 114732 (Applied Biosystems), protein identification and quantification were performed. The peptides were identified by the Paragon algorithm in the ProteinPilot software and the differences between expressions of various isoforms were traced by Pro Group algorithm using isoform-specific quantification. The parameters used for database searching were defined as follows: (i) Sample Type: iTRAQ 4plex (Peptide Labeled); (ii) Cysteine alkylation: MMTS; (iii) Digestion: Trypsin; (iv) Instrument: QSTAR Elite ESI; (v) Special factors: None; (vi) Species: None; (vii) Specify Processing: Quantitate; (viii) ID Focus: biological modifications, amino acid substitutions; (ix) Database: concatenated 'target' (IPI rat; version 3.40; 40389 sequences and 20, 549, 266 residues) and 'decoy' (the corresponding reverse sequences for false discovery rate (FDR) estimation); (x) Search effort: thorough. Pro Group algorithm was used to automatically select the peptide for iTRAQ quantification, where the reporter peak area, error factor (EF) and *p*-value were calculated. Auto bias-correction was carried out on the acquired data to remove variations imparted as a result of unequal mixing during the combination of the differently labeled samples. To minimize the false positive identification of proteins, a strict cutoff of unused ProtScore $\geq 2$ was used as the qualification criteria, which corresponds to a peptide confidence level of 99 %. A FDR of 0.33% (<1.0%) was applied. The cutoff for up- or down-regulation (pre-defined at 1.2 and 0.83 respectively) was determined by using the *p*-value cut-off (0.05) to obtain the list of proteins with significant ratios. The *p*-value assigned by the ProteinPilot software measures the confidence of the real change in the protein expression level. Data analysis and functional classification were conducted using online databases such as NCBI, UniProt, and Panther.

### *Post-proteomic Data Verification by SDS-PAGE - Western Blot Analysis*

The same pooled extracts were used for post-proteomics data validation using western blot analysis. Twenty micrograms of cell lysates were resolved by 8-12 % SDS-PAGE at 0.02 Ampere (A) of constant current and transferred to a polyvinylidine fluoride (PVDF) membrane (0.22 μm; Amersham) using the 'semi-dry' transfer method (BioRad, Singapore) for 60 min at 0.12 A in buffer containing 25 mM Tris, 192 mM glycine, 20 % methanol, and 0.01 % (wt/vol) SDS. The membrane was blocked with 5 % BSA (BioRad) in Phosphate-buffered saline (PBS) plus 0.1 % Tween-20 (PBS-T) for 2 hrs at RT, washed three times in PBS-T for 10 min each, and incubated with primary antibody (diluted in 2 % BSA in PBS-T) for overnight at 4 °C. The membranes were washed as described above, incubated with HRP-conjugated secondary antibody for 1 hr at RT, and developed using the ECL plus western blot detection reagent (Amersham). X-ray films (Konica Minolta Inc., Tokyo, Japan) were exposed to the membranes before film development in a Kodak X-OMAT 2000 processor (Kodak, Ontario, Canada). For equal sample loading, protein concentration was quantified with '2D Quant' kit (Amersham) with at least two independent replicates. BSA was used as the standard. To re-probe the same membrane with another primary antibody, Pierce's (Pierce Biotechnology, Inc., Rockford, IL, USA) 'stripping solution' was used to strip the membranes. In addition, equal sample loading was confirmed using Gapdh (Glyceraldehyde-3-phosphate dehydrogenase) as a reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses (n = 4), and representative blots are shown. Values (= relative protein expression) represent the ratio of densitometric scores (GS-800 Calibrated Densitometer and Quantity One quantification analysis software version 4.5.2; BioRad) for the respective western-blot products (mean ± SD (standard deviation)) using the Gapdh bands as a reference.

**Supplementary Table 1. Functional Classification of Differentially Expressed Proteins between Control and Tianma-treated Rat Aorta Quantified by iTRAQ Proteomics**

| Accession No. | Proteins Name/Genes Symbol | Molecular Function | No. of Peptides (>95%)* | T : C iTRAQ-Ratio | *P*-value |
|---|---|---|---|---|---|
| **Extracellular Matrix Proteins** | | | | | |
| IPI00200594.2 | Elastin; Eln | Extracellular matrix structural constituent | 22 | 2.54 | 0.2453 |
| IPI00326179.3 | Fibulin-5; Fbln5 | Receptor activity | 98 | 1.89 | 0.0284 |
| IPI00190287.1 | Prolargin; Prelp | Receptor activity | 41 | 2.40 | 0.1253 |
| IPI00191090.1 | Biglycan; Bgn | Receptor activity | 89 | 1.38 | 0.0243 |
| IPI00200225.1 | Fibromodulin; Fmod | Receptor activity | 9 | 1.37 | 0.1538 |
| IPI00231982.1 | Fibronectin; Fn1 | Receptor binding | 107 | 0.45 | 1.61E-07 |
| IPI00190088.2 | Periostin, osteoblast specific factor; Postn | Heparin binding | 45 | 0.53 | 0.0001 |
| Cytoskeletal proteins | | | | | |
| IPI00189819.1 | Actin, cytoplasmic 1; Actb | Structural constituent of cytoskeleton | 474 | 0.69 | 0.0549 |
| IPI00197129.1 | Actin, aortic smooth muscle; Acta2 | Structural constituent of cytoskeleton | 938 | 0.54 | 0.0017 |
| IPI00195673.1 | Tubulin beta 6; Tubb6 | Structural constituent of cytoskeleton | 25 | 0.54 | 0.4937 |
| IPI00211206.7 | PDZ and LIM domain protein 1; Pdlim1 | Structural constituent of cytoskeleton | 12 | 0.58 | 0.1224 |
| IPI00421517.7 | Desmin; Des | Structural constituent of cytoskeleton | 32 | 0.58 | 0.0255 |
| IPI00393787.2 | Alpha-parvin; Parva | Structural constituent of cytoskeleton | 18 | 0.54 | 0.3748 |
| IPI00393867.4 | Myo1c; Myo1c | Structural constituent of cytoskeleton | 33 | 1.53 | 0.0170 |
| IPI00231418.5 | Lamin-B1; Lmnb1 | Structural constituent of cytoskeleton | 10 | 1.53 | 0.0450 |
| IPI00779779.1 | Microtubule-associated protein 4; Map4 | Protein binding | 4 | 0.36 | 0.01108 |
| IPI00365286.3 | Vinculin; Vcl | Rho GTPase binding | 245 | 0.53 | 1.24E-06 |

**Supplementary Table 1. Contd…..**

| Accession No. | Proteins Name/Genes Symbol | Molecular Function | No. of Peptides (>95%)* | T : C iTRAQ-Ratio | *P*-value |
|---|---|---|---|---|---|
| **Extracellular Matrix Proteins** | | | | | |
| IPI00388015.3 | Coro1c protein; Coro1c | Actin binding | 30 | 0.70 | 0.1123 |
| IPI00421523.2 | Echinoderm microtubule-associated protein-like 2; Eml2 | Catalytic activity | 2 | 0.40 | 0.2610 |
| Angiogenesis, VEGF signalling pathway | | | | | |
| IPI00421523.2 | Paxillin; Pxn | Structural constituent of cytoskeleton | 2 | 3.70 | 0.1846 |
| IPI00215465.1 | Alpha-crystallin B chain; Cryab | Structural molecule activity | 5 | 0.59 | 0.4790 |
| IPI00559274.2 | Milk fat globule-EGF factor 8 protein; Mfge8 | Integrin binding | 4 | 0.58 | 0.2335 |
| Cell proliferation regulation | | | | | |
| IPI00554039.1 | RGD1565368_predicted similar to glyceraldehyde-3-phosphate dehydrogenase | Protein binding | 26 | 0.64 | 0.4613 |
| IPI00568616.2 | Rho-associated coiled-coil containing protein kinase 1; Rock1 | Protein serine/threonine kinase activity | 2 | 1.36 | 0.5680 |
| IPI00366079.1 | Uncharacterized protein; LOC100362805 | Isomerase activity | 4 | 1.68 | 0.2714 |
| IPI00568511.2 | RGD1560049_predicted similar to Dual specificity protein phosphatase 3 | MAP kinase phosphatase activity | 5 | 0.76 | 0.5426 |
| Carbohydrate metabolism | | | | | |
| IPI00197555.6 | Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial; Suclg1 | Catalytic activity | 2 | 2.07 | 0.4318 |
| IPI00200659.1 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial; Sdha | Oxidoreductase activity | 5 | 0.37 | 0.0133 |
| IPI00421428.9 | Phosphoglycerate mutase 1; Pgam1 | Intramolecular transferase activity | 5 | 0.67 | 0.3276 |
| IPI00361524.3 | LOC679990_similar to phosphoglucomutase 5 | Magnesium ion binding | 51 | 0.62 | 0.0727 |
| Protein metabolism | | | | | |
| IPI00204703.5 | Serpin H1; Serpinh1 | Protein binding | 19 | 1.84 | 0.0113 |
| IPI00471645.1 | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit; Ddost | Transferase activity | 3 | 1.27 | 0.0846 |
| IPI00471577.1 | Cytochrome b-c1 complex subunit 1, mitochondrial; Uqcrc1 | Oxidoreductase activity | 7 | 1.79 | 0.2200 |

**Supplementary Table 1. Contd…..**

| Accession No. | Proteins Name/Genes Symbol | Molecular Function | No. of Peptides (>95%)* | T : C iTRAQ-Ratio | *P*-value |
|---|---|---|---|---|---|
| **Extracellular Matrix Proteins** | | | | | |
| IPI00215384.1 | Nucleolin-related protein NRP; Norp | RNA splicing factor activity | 2 | 0.30 | 0.2006 |
| Protein folding | | | | | |
| IPI00568118.2 | Calnexin; Canx | Calcium ion binding | 5 | 1.50 | 0.1243 |
| Cell signalling | | | | | |
| IPI00363395.2 | Ras-related protein Rap-1b; Rap1b | GTPase activity | 5 | 0.62 | 0.44244 |
| IPI00364932.2 | Uncharacterized protein; Rsu1 | Protein binding | 24 | 0.63 | 0.0040 |
| IPI00205208.1 | androgen regulated protein, Cystatin-related protein 1 precursor; Andpro | Cysteine-type endopeptidase inhibitor activity | 4 | 6.08 | 0.0013 |
| IPI00358175.3 | Uncharacterized protein, Predicted similar to Filamin-C (Gamma-filamin) (Filamin-2) (Protein FLNc) (Actin-binding-like protein) (ABP-L) (ABP-280-like protein) isoform 2; Flnc | Actin binding | 33 | 1.38 | 0.0770 |
| Transcription regulation | | | | | |
| IPI00201300.2 | Polymerase I and transcript release factor; Ptrf | DNA binding | 62 | 1.59 | 0.0994 |
| IPI00765168.1 | Pura_predicted similar to Transcriptional activator protein Pur-alpha | Activator | 5 | 1.50 | 0.3129 |
| Glutathione metabolism | | | | | |
| IPI00231229.9 | Glutathione S-transferase P; Gstp1 | Transferase activity | 5 | 0.60 | 0.4761 |
| IPI00411230.3 | Glutathione S-transferase mu 2; Gstm2 | Transferase activity | 17 | 0.61 | 0.0264 |
| Transport | | | | | |
| IPI00200466.3 | ADP/ATP translocase 2; Slc25a5 | Amino acid transmembrane transporter activity | 8 | 1.46 | 0.0726 |
| IPI00207890.1 | Fatty acid-binding protein, adipocyte; Fabp4 | Lipid binding | 2 | 0.51 | 0.4527 |
| IPI00231927.11 | ADP/ATP translocase 1; Slc25a4 | Amino acid transmembrane transporter activity | 11 | 1.64 | 0.2829 |
| IPI00325146.6 | Annexin A2; Anxa2 | Calcium ion binding | 41 | 2.99 | 0.1695 |
| IPI00565637.1 | Sideroflexin 3; Sfxn3 | Cation transmembrane transporter activity | 4 | 0.45 | 0.2976 |
| IPI00231966.5 | ADP-ribosylation factor 4; Arf4 | GTPase activity | 2 | 0.63 | 0.4578 |
| Others | | | | | |
| IPI00199980.1 | Proteasome subunit beta type-7; Psmb7 | Peptidase activity | 2 | 3.53 | 0.3583 |

**Supplementary Table 1. Contd…..**

| Accession No. | Proteins Name/Genes Symbol | Molecular Function | No. of Peptides (>95%)* | T : C iTRAQ-Ratio | *P*-value |
|---|---|---|---|---|---|
| **Extracellular Matrix Proteins** | | | | | |
| IPI00766820.1 | LOC686753 similar to nephronectin isoform a | Calcium ion binding | 3 | 0.70 | 0.5337 |
| IPI00361346.2 | IgG-2a gamma-2a immunoglobulin heavy chain; IgG-2a | Antigen binding | 5 | 0.64 | 0.3144 |
| IPI00392384.1 | Tensin 1, Tns1 | Focal adhesion phospho-protein that binds to F-actin | 8 | 8.40 | 0.0376 |
| IPI00776962.1 | LOC500040 54 kDa protein | - | 3 | 0.71 | 0.1917 |

The list contains quantitative information of the proteins from tianma-stimulated rat aorta compared with control. To minimize the false positive identification of proteins, a strict cutoff of unused ProtScore ≥ 2 was used as the qualification criteria, which corresponds to a peptide confidence level of 99 %. A FDR (false discovery rate) of 0.33% (<1.0%) was applied and a change in expression levels of at least 1.2-fold (up-regulation) or at least <0.833-fold (down-regulation) was set as defined in the experimental procedures. Then data analysis and functional classification were conducted using online databases such as NCBI, UniProt, and Panther.

*the total number of peptides identified with >95% confidence; C = control cells, T = tianma-treated cells.