Quantitative profiling of the rat heart myoblast secretome reveals differential responses to hypoxia and re-oxygenation stress

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Corresponding Author: Dr. Siu Kwan Sze, Ph.D.

Corresponding Author's Institution: Nanyang Technological University

First Author: Xin Li, PhD

Order of Authors: Xin Li, PhD; Yan Ren, PhD; Vitaly Sorokin, MD; Kian Keong Poh, MD; Hee Hwa Ho, MD; Chuen Neng Lee, MD; Dominique de Kleijn, PhD; Sai Kiang Lim, PhD; James P Tam, PhD; Siu Kwan Sze, Ph.D.

Abstract: Secretion of bioactive mediators regulates cell interactions with the microenvironment in tissue homeostasis and wound healing processes. We assessed the cardiomyocyte secretory response to hypoxia with the aim of identifying key mediators of tissue pathology and repair after ischemic heart attack. We profiled the secretome of rat H9C2 cardiomyoblast cells subjected to 16h hypoxia followed by 24h re-oxygenation using iTRAQ and label-free quantitative proteomics. A total of 860 and 2007 proteins were identified in the iTRAQ and label-free experiments respectively. Among these proteins, 1363 were identified as being secreted proteins, including mediators of critical cellular functions that were modulated by hypoxia/re-oxygenation stress (SerpH1, Ppia, Attractin, EMC1, Postn, Thbs1, Timp1, Stip1, Robo2, Fat1). Further analysis indicated that hypoxia is associated with angiogenesis, inflammation, and remodeling of the extracellular matrix (ECM), whereas subsequent re-oxygenation was instead associated with modified secretion of proteins involved in suppression of inflammation, ECM modification, and decreased output of anti-apoptosis proteins. These data indicate that hypoxia and subsequent re-oxygenation modify the cardiomyocyte secretome in order to mitigate cellular injury and promote healing. The identified changes in cardiomyocyte secretome advance our current understanding of cardiac biology in ischemia/reperfusion injury and may lead to the identification of novel prognostic biomarker.

Significance

Cardiovascular diseases (CVDs) are the leading cause of death globally. Myocardial infarction (MI) resulting from ischemic heart disease represents a substantial component of CVD-associated mortality, and is associated with obstruction of blood flow to the myocardium. Restoration of blood flow through the occluded coronary artery is the current most effective therapy to limit infarct size and preserve cardiac function after acute myocardial infarction. However, this treatment does not prevent subsequent development of heart failure in some patients. Reperfusion following ischemia causes additional cell death and increase in infarct size, a phenomenon called myocardial ischemia/reperfusion (I/R) injury.

In order to advance our current understanding of cardiac biology in ischemia/reperfusion injury, we assessed the cardiomyocyte secretory response to hypoxia with the aim of identifying key mediators of tissue pathology and repair after ischemic heart attack. We profiled the secretome of rat H9C2 cardiomyoblast cells subjected to 16h hypoxia followed by 24h re-oxygenation using LC-MS/MS-based iTRAQ and label-free quantitative proteomics approaches. We identified many secreted proteins as mediators of critical cellular functions that were modulated by hypoxia and re-oxygenation stress. Further analysis of these modulated secretory proteins indicated that hypoxia is associated with angiogenesis, inflammation, and remodeling of the extracellular matrix (ECM), whereas subsequent re-oxygenation/reperfusion was instead associated with modified secretion of proteins involved in suppression of inflammation, ECM modification, and decreased output of anti-apoptosis proteins. These data indicate that hypoxia and subsequent re-oxygenation modify the cardiomyocyte secretome in order to mitigate cellular injury and promote healing. The identified changes in cardiomyocyte secretome may lead to the identification of novel prognostic biomarkers secreted from injured heart tissues into the circulation of patients with cardiovascular disease.

Graphical Abstract

Highlights

- Study secretome of cardiomyocytes responded to hypoxia and re-oxygenation.
- Hypoxia perturbed angiogenesis, inflammation and ECM remodeling pathways.
- Re-oxygenation modulated inflammation, ECM remodeling and apoptosis.
- Hypoxic cardiomyocytes secreted factors to mitigate injury and promote healing.

**Quantitative Profiling of the Rat Heart Myoblast Secretome Reveals Differential Responses
to Hypoxia and Re-oxygenation Stress**

Xin Li¹, Yan Ren¹, Vitaly Sorokin^{2,3}, Kian Keong Poh^{2,3}, Hee Hwa Ho⁴, Chuen Neng Lee^{2,5},
Dominique de Kleijn^{5,6}, Sai Kiang Lim⁷, James P. Tam¹ and Siu Kwan Sze^{1*}

¹School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore
637551.

² National University Heart Centre, Department of Cardiac, Thoracic & Vascular Surgery,
Singapore 119228.

³Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore.

⁴Department of Cardiology, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433.

⁵Department of Surgery, Yong Loo Lin School of Medicine, Singapore 119228.

⁶Experimental Cardiology Laboratory, Cardiology, University Medical Center Utrecht, the
Netherlands & Interuniversity Cardiovascular Institute of the Netherlands, Utrecht, the
Netherlands.

⁷Institute of Medical Biology, 8A Biomedical Grove, #05-505 Immunos, Singapore 138648.

Correspondence

Siu Kwan SZE

School of Biological Sciences,
Nanyang Technological University,
60 Nanyang Drive, Singapore 63755

Tel: (+65)6514-1006, Fax: (+65)6791-3856

Email: sksze@ntu.edu.sg

Abstract

Secretion of bioactive mediators regulates cell interactions with the microenvironment in tissue homeostasis and wound healing processes. We assessed the cardiomyocyte secretory response to hypoxia with the aim of identifying key mediators of tissue pathology and repair after ischemic heart attack. We profiled the secretome of rat H9C2 cardiomyoblast cells subjected to 16h hypoxia followed by 24h re-oxygenation using iTRAQ and label-free quantitative proteomics. A total of 860 and 2007 proteins were identified in the iTRAQ and label-free experiments respectively. Among these proteins, 1363 were identified as being secreted proteins, including mediators of critical cellular functions that were modulated by hypoxia/re-oxygenation stress (SerpinH1, Ppia, Attractin, EMC1, Postn, Thbs1, Timp1, Stip1, Robo2, Fat1). Further analysis indicated that hypoxia is associated with angiogenesis, inflammation, and remodeling of the extracellular matrix (ECM), whereas subsequent re-oxygenation was instead associated with modified secretion of proteins involved in suppression of inflammation, ECM modification, and decreased output of anti-apoptosis proteins. These data indicate that hypoxia and subsequent re-oxygenation modify the cardiomyocyte secretome in order to mitigate cellular injury and promote healing. The identified changes in cardiomyocyte secretome advance our current understanding of cardiac biology in ischemia/reperfusion injury and may lead to the identification of novel prognostic biomarker.

Keywords

Cardiac, Hypoxia, Re-oxygenation, I/R injury, Secretome, Quantitative Proteomics, H9C2 Cell

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally and account for a greater proportion of human mortality than cancer, diabetes and asthma combined¹. Myocardial infarction (MI) resulting from ischemic heart disease represents a substantial component of CVD-associated mortality, and is associated with obstruction of blood flow to the myocardium. Restoration of blood flow through the occluded coronary artery is the current most effective therapy to limit infarct size and preserve cardiac function after acute myocardial infarction². However, this treatment does not prevent subsequent development of heart failure in some patients, and reperfusion following ischemia causes additional cell death and increased infarct size: a phenomenon known as ischemia/reperfusion (I/R) injury³.

I/R injury induces inflammation and extracellular matrix (ECM) remodeling of the damaged myocardium that eventually result in the replacement of dead cells by fibrous collagen-based scar tissue⁴. Scar tissue formation occurs in three distinct stages; initially, plasma proteins infiltrate the injured tissue to form a fibronectin/fibrin-based matrix that serves as a scaffold for the recruitment and proliferation of pro-inflammatory leukocytes⁵. Next, this provisional matrix is lysed by proteolytic enzymes secreted by granular cells prior to intermediary matrix deposition by fibroblasts⁶. Finally, scar tissue is formed when fibrillar collagen is deposited onto the matrix and completes the replacement of dead myocardium with fibrous acellular material. However, the lack of tensile strength in the scar tissue promotes left ventricular remodeling and subsequent dysfunction that can lead to progressive loss of cardiac output⁷.

The self-repair of heart tissue after I/R injury depends largely on replacing lost myocardium with acellular collagen-based scar tissue. Various strategies have been explored to enhance cardiac healing after MI including transplantation of bone marrow-derived mononuclear cells (MNCs)⁸, mesenchymal stem cells (MSCs)⁹ and skeletal myoblasts (SkMb)¹⁰. Surprisingly, many of these transplanted cells have been reported to improve cardiac healing via release of paracrine factors that influence neovascularization, myocardial protection, cardiac remodeling, and contractility¹¹. A previous study of the secreted proteome (secretome) of human MSCs identified 201 unique

78 proteins involved in key signaling pathways in cardiovascular biology, bone development, and
79 hematopoiesis, indicating considerable potential for MSCs secretome to mediate tissue repair
80 and replacement processes¹². However, a major barrier to the further development of therapies
81 for MI is the current lack of data on the cardiomyocyte secretory response to I/R injury.

82 Previous studies have demonstrated that cellular secretion of bioactive mediators can influence
83 cardiac repair through dynamic modulation of the local microenvironment. To better understand
84 the influence of cellular secretion on tissue repair, we investigated whether injured
85 cardiomyocytes secrete factors that can influence the healing process. We exploited recent
86 advances in proteomics and bioinformatics technology to conduct temporal qualitative and
87 quantitative secretome profiling of embryonic rat H9C2 cardiomyocytes subjected to
88 normoxia/hypoxia/re-oxygenation protocols^{13, 14}. H9C2 cells have been widely used as an
89 alternative to primary cardiomyocytes since they are morphologically similar to immature
90 embryonic cardiocytes but also possess several features of adult cardiac cells¹⁵. We used the
91 stable isotope label-based method of 'isobaric tag for relative and absolute quantitation' (iTRAQ)
92 in conjunction with label-free LC-MS/MS methods to quantify secreted proteins in the conditioned
93 medium of cultured H9C2 cells. In total, 2165 proteins were identified (false discovery rate <1%)
94 and data mining using public databases revealed that 1363 of these proteins were known
95 secretory products. Many of the novel secreted proteins comprising the secretome of hypoxia-
96 stressed rat heart myoblast cells were identified by mass spectrometry-based quantitative
97 proteomics and shed new light on cardiac cell responses to hypoxic injury.

98

99 **Material and methods**

100 *Cell culture and conditioned medium*

101 Rat heart myoblast cell line H9C2 was purchased from American Type Culture Collection
102 (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing
103 10% fetal bovine serum. Upon reaching 60% confluence, the cells were washed at least three
104 times with PBS and incubated in serum-free DMEM for 6h. After 6h incubation, the medium was
105 aspirated and replaced with fresh serum-free DMEM. Conditioned medium (CM) was collected
106 from H9C2 cells after 16h culture under normoxic condition (95% Air, 5% CO₂). For hypoxia
107 experiments, the fresh serum-free DMEM had been pre-treated by bubbling in hypoxic gas (95%
108 N₂, 5% CO₂, <0.1% O₂) for 30min to expel dissolved oxygen. The cells were then transferred into
109 a humidified, airtight MIC-101 chamber (Billups-Rothenberg, Inc., Del Mar, CA) and then flushed
110 with hypoxic gas before being incubated for 16h. In some experiments, the hypoxia-conditioned
111 medium was then replaced with fresh DMEM and incubated in normoxic condition for a further 2h
112 or 24h in order to obtain 're-oxygenation stress' medium. For each condition assessed, n=30 x
113 10cm Petri dishes of cells were subjected to the normoxia, hypoxia or re-oxygenation protocols
114 respectively in MIC-101 chambers (the total complement of 30 dishes per condition was
115 considered as a single 'batch'). Two sample batches were generated in total; one batch was used
116 for the iTRAQ and label-free experiments, while the second batch was used for Western
117 validation. LC-MS/MS analysis of each sample was run in triplicates by three injections. The CMs
118 from separate Petri dishes cultured under the same conditions were pooled together, centrifuged
119 at 500Xg for 5min, and passed through 0.2µm filters. The filtered medium was then concentrated
120 using 10kDa Amicon Ultra-15 centrifugal filter units (Millipore, Carrigtwohill, Ireland) and
121 lyophilized to obtain secreted proteins. Each condition tested generated a total of ~250ml pooled
122 CM from which we extracted protein masses of 1.08mg (16h normoxia), 0.778mg (16h hypoxia),
123 0.182mg (2h re-oxygenation), and 1.226mg (24h re-oxygenation). The profile of secreted proteins
124 detected in the short re-oxygenation condition was highly variable (likely due to the secretome not
125 being stabilized within 2h of re-oxygenation) therefore we excluded these data from our analyses.

iTRAQ quantitative proteomics

A total of 160µg protein from each condition was used for iTRAQ labeling. These proteins from the 4-plex labeled samples, 640µg in total, were combined and used for fractionation. The protein samples were reduced using tris(2-carboxyethyl)phosphine (TCEP), alkylated with methyl methanethiosulfonate (MMTS) and then digested with trypsin as previously reported^{16, 17}. The tryptic peptides were labeled with 4-plex iTRAQ tags 114 (normoxia), 115 (hypoxia for 16h), 116 (re-oxygenation for 2h), and 117 (re-oxygenation for 24h), according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The peptides were labeled with the respective isobaric tags, incubated for 2h, then combined into a single sample and vacuum centrifuged to dryness. The vacuum-dried samples were reconstituted in Buffer A (10mM KH₂PO₄, 25% acetonitrile, pH 2.85) and the iTRAQ-labeled peptides fractionated using PolySULFOETHYL ATM SCX columns (200 x 4.6mm, 5µm particle size, 200Å pore size) together with a HPLC system (Shimadzu, Japan) at flow rate of 1ml/min. The 50min HPLC gradient consisted of 5min in 100% Buffer A; 15min in 0-20% Buffer B (10mM KH₂PO₄, 25% ACN, 500mM KCl, pH 3.0); 10min in 20-40% Buffer B; 5min in 40-100% Buffer B; 5min in 100% Buffer B; and finally 10min in 100% buffer A. Chromatograms were recorded at 214nm during sample fractionation. In total, 50 fractions were collected, concentrated to dryness using vacuum centrifugation, and then pooled into 20 separate fractions based on 214nm reading in the LC spectrum. The fractions were subsequently reconstituted in 0.1% trifluoroacetic acid and desalted with Sep-Pak Vac C18 cartridges (Waters, Milford, Massachusetts). After desalting, the samples were again concentrated to dryness using vacuum centrifugation and reconstituted in 100µl formic acid (0.1%) for LC-MS/MS analysis.

Analysis of the iTRAQ-labeled samples by mass spectrometry was performed in triplicate by three injections using a QStar Elite mass spectrometer (Applied Biosystems; MDS-Sciex, USA), coupled with online micro-flow HPLC system (Shimadzu, JAPAN). The peptides were separated using nano-bored C18 columns with a picofrit nanospray tip (75µm ID x 15cm, 5µm particles) (New Objectives, Wubrun, MA). The HPLC flow-rate was maintained at 30µl/min and the separation was performed with a splitter to achieve an effective flow rate of 0.3µl/min. Data

acquisition was performed using Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). The mass spectrometer data were acquired in the positive ion mode with a selected mass range of 300-2000m/z. Peptides with +2 to +4 charge states were selected, and the three most abundantly charged precursor peptides above a 10 count threshold were used for MS/MS and dynamically excluded for 30sec with ± 30 mDa mass tolerance. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2s. The peak areas of the iTRAQ reporter ions were used to quantify proteins in the samples.

iTRAQ database search: Protein identification and quantification were performed using ProteinPilot software version 3 (revision number 67476; Applied Biosystems, Foster City, CA). The Paragon algorithm in the ProteinPilot software was used for peptide identification, which was further processed by Pro Group algorithm for isoform-specific quantification. User defined parameters were as follows: (i) Sample Type, iTRAQ 4-plex (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 rat database (target: Rat refseq protein database 59026 sequences; 30608194 residues) and the corresponding reverse sequence [decoy: for FDR estimation, $FDR = \text{decoy_hits} / (\text{target_hits} + \text{decoy_hits})$]; (x) Search effort, thorough. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm for calculation of the reporter peak area, error factor (EF) and p-value. The resulting data were automatically bias-corrected to correct for potential reporter variations due to the possible unequal quantities of proteins in the different labeled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantitation results. Bias correction is only performed when there are at least 20 proteins having an average ratio of iTRAQ reporters. The proteins and peptides identified and quantified by ProteinPilot database searching were exported to Excel files (Supplementary_Data_1 - iTRAQ_ProteinsSummary.xlsx and Supplementary_Data_2 - iTRAQ_PeptideSummary.xlsx) for further analysis and data mining.

Label-free quantitative proteomics

A total of 500µg freeze-dried secreted protein was reconstituted in SDS running buffer and separated on a 1.5mm thick SDS-PAGE gel followed by silver staining (gel image shown in Supplementary Figure 1). The gel lanes corresponding to each condition (normoxia, hypoxia and re-oxygenation) were cut into 6 separate pieces, then de-stained, and the proteins reduced using dithiothreitol (DTT) and alkylated by iodoacetamide (IAA). The proteins were cleaved by overnight digestion in porcine trypsin (Sequencing Grade Modified, Promega, Wisconsin). The tryptic peptides were extracted using 5% formic acid in 50% acetonitrile and vacuum-dried by speedvac.

Each dried protein fraction was reconstituted in 100µl formic acid (0.1%) and analyzed using an LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany) coupled with a ProminenceTM HPLC unit (Shimadzu). Samples were injected from an autosampler (Shimadzu) and concentrated in a Zorbax peptide trap (Agilent, Palo Alto, CA). The peptide separation was performed in a capillary column (75µm inner diameter x 15cm) packed with C₁₈ AQ (5µm particles, 300Å pore size; Michrom Bioresources, Auburn, CA). Mobile phase A (0.1% formic acid in H₂O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish the 90min gradient which comprised 3min of 0-5% B; 52min of 5-25% B; 19min of 25-80% B; 8min at 80% B; and 8min at 5% B. The HPLC system was operated at a constant flow rate of 30µl/min and a splitter was used to create an effective flow rate of ~500nl/min at the electrospray emitter. The sample was injected into an LTQ-FT through an ADVANCETM CaptiveSprayTM source (Michrom Bioresources) with an electrospray potential of 1.5kV. The gas-flow was set at 2, ion transfer tube temperature was 180°C, and collision gas pressure was 0.85 millitorr. The LTQ-FT was set to perform data acquisition in the positive ion mode as described previously¹⁸. Briefly, a full MS scan (350-1600 *m/z* range) was acquired in the FT-ICR cell at a resolution of 100,000 and a maximum ion accumulation time of 1000ms. The automatic gain control target for FT was set at 1e+06, and precursor ion charge state screening was activated. The linear ion trap was used to collect peptides and measure peptide fragments generated by collision-activated dissociation (CAD). The default automatic gain control setting was used in the linear ion trap (full MS target at 3.0e+04, MSⁿ at 1e+04). The 10 most intense ions above a 500 count threshold were selected for

fragmentation by CAD (MS^2) which was performed concurrently with a maximum ion accumulation time of 200ms. For CAD, the activation Q was set at 0.25, isolation width (m/z) was 2.0, activation time was 30ms, and normalized collision energy was 35%.

Mascot database search: The extract_msn (version 4.0) program in Bioworks Browser 3.3 (Thermo Electron, Bremen, Germany) was used to extract tandem MS spectra in the dta format from the raw data of LTQ-FT ultra. These dta files were then converted into the MASCOT generic file format using an in-house program. Intensity values and fragment ion m/z ratios were not manipulated. The data were then used to obtain protein identities by searching against the Refseq rat protein database (59026 sequences) by means of an in-house MASCOT server (version 2.2.03) (Matrix Science, Boston, MA), with the inclusion of 59026 decoy sequences (reverse sequences) in order to estimate the FDR. The search was limited to a maximum of 2 missed trypsin cleavages; $\delta^{13}C$ of 2; mass tolerances of 10ppm for peptide precursors; and 0.8Da mass tolerance for fragment ions. Fixed modification was carbamidomethyl at Cys residue, whereas variable modifications were oxidation at Met residue and phosphorylation on Ser, Thr and Tyr residues. The peptides identified by MASCOT search were exported to an Excel file (Supplementary_Data_3 - Mascot Identified_Peptides_with_FDR.xlsx) for FDR calculation and further analysis. The MASCOT DAT files were retrieved from the server for calculating the normalized spectral index (S_{IN}) for label-free quantitation. FDR of the identified peptides was controlled at below 1%.

Label free quantitation: We applied three different algorithms to estimate protein quantities based on the label-free LC-MS/MS results; normalized spectral index (S_{IN}), normalized spectral abundance factor (NSAF), and exponentially modified protein abundance index (emPAI). Only proteins with similar secretion trends in all three label-free methods as well as in iTRAQ were shortlisted for further analysis. The S_{IN} was calculated using MASCOT search results as previously described¹⁹. Briefly, for each protein we calculated the spectral index (SI) which incorporated fragment ion intensity values with spectrum count and peptide number. SI was then normalized relative to the sum of SI of each identified protein in the experiment, and then further

normalized by protein length. The fragment ion intensity of the spectrum assigned to a specific peptide was obtained from the MS2 section in DAT MASCOT search results using the in-house Ruby script through the spectrum title. The spectra for SI_N were selected from the spectrum list with FDR <1%. NSAF was calculated using an in-house program based on a published algorithm (as previously described²⁰), and empAI value was calculated by MASCOT database search program²¹. The MASCOT identified proteins with three label-free quantification results are shown in Supplementary_Data_4 - Label_Free_Quantified_Proteins.xlsx.

Secreted protein determination

Bioinformatic tools were employed to identify secreted proteins as described in previous studies²². The fasta format sequence information of the identified proteins was used to submit to web-based bioinformatics tools SignalP²³, TMHMM²², and SecretomeP²⁴ to determine the probability of being secreted proteins; only those proteins with secretion probability above the threshold values determined by the tools were used. Protein matching was conducted using the Exocarta database that records proteins identified as being secreted products based on exosome data from previous studies²⁵. Text mining was employed to provide supporting information on protein secretion based on data from primary publications (as described in Supplementary Table 1).

Western blot

A total of 50µg protein corresponding to each condition was dissolved in gel loading buffer and then heated for 10min at 95°C prior to loading onto a SDS-PAGE gel. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and probed with primary antibodies anti-Ppia: (rabbit polyclonal anti-serum IgG, dilution 1:500, Millpore #07-313); anti-Park7 (mouse monoclonal, dilution 1:500, Santa Cruz sc-55572); anti-Stip1 (goat polyclonal, dilution 1:500, Santa Cruz sc-27962); anti-SerpinH1 (mouse monoclonal, dilution 1:500, Santa Cruz sc-5293); anti-VEGF (rabbit polyclonal IgG, dilution 1:1000, Santa Cruz sc-152); and anti-Actin (Santa Cruz sc-8432 mouse monoclonal, dilution 1:2000). Secondary antibodies were polyclonal HRP-conjugated goat anti-mouse (DakoCytomation P0447, dilution 1:2000), rabbit anti-goat

(DakoCytomation P0449, dilution 1:2000) or goat anti-rabbit (DakoCytomation P0448, dilution 1:2000). The protein blots are shown in Supplementary Figures 2-6.

Results and discussion

Hypoxia induction in H9C2 cardiomyocytes

To confirm that our experimental protocol successfully induced hypoxic stress in H9C2 cardiomyocytes, we used Western blots to monitor vascular endothelial growth factor (VEGF) secretion into the culture supernatants of cells subjected to 16h normoxia, 16h hypoxia, or 16h hypoxia followed by 24h re-oxygenation. VEGF gene transcription is activated by the binding of hypoxia-inducible factor-1 α (HIF-1 α) to a hypoxic response element (HRE) in the VEGF promoter^{26,27}, and our Western blot analyses confirmed that VEGF secretion was significantly increased in our low oxygen cell cultures (Figure 1a), indicating that hypoxic responses were successfully induced in our assays.

Proteomic analysis of rat myoblast secretome

In order to determine the H9C2 cell secretory response to low oxygen stress, we next collected the conditioned culture medium of cells subjected to 16h normoxia, 16h hypoxia, or 16h hypoxia followed by 24h re-oxygenation for proteomic analysis. Using an iTRAQ quantitative approach, we identified a total of 860 proteins in the culture supernatants (FDR <1%). All proteins were identified as comprising at least 2 peptides ($\geq 95\%$ confidence) and the protein unused score was >2.08 (with a score of 2.0 being equivalent to 99% confidence of successful protein identification). Only proteins meeting these very high stringency criteria were short-listed for further analysis. The 860 proteins identified by iTRAQ are listed in the Supplementary Data 1 as Excel file, and the individual peptides identified and quantified are listed in Supplementary Data 2 as Excel file.

Protein redundancy due to assignment of the same set of peptides to multiple different proteins by database search software can often complicate proteomic analysis. In order to overcome this

problem, we analyzed our data using ProteinPilot software, which employs an Pro Group algorithm to group proteins based on shared peptide spectral characteristics. Only one protein representative of each group is then reported in the results dataset (thereby minimizing redundancy). If two or more proteins can be assigned to the same set of peptides with equal confidence, the first protein is arbitrarily designated 'representative' and the other proteins are classed as 'equivalent'. The representative proteins from each group are included in the summary report shown in Supplementary Data 1. The protein summary report also displays the respective iTRAQ ratios, p-values, and error factors corresponding to the iTRAQ ratios (where $p < 0.05$ indicates >95% confidence of a change in protein concentration irrespective of the magnitude of the change²⁸).

Although relative quantitation by iTRAQ analysis is reasonably effective at identifying increases versus decreases in protein level, this technique is known to underestimate the true magnitude of changes in protein concentration²⁹. In order to validate the iTRAQ quantified proteins and to select for hypoxia-modulated proteins for subsequent Western blot analysis and data mining, we used the complementary label-free LC-MS/MS quantitative proteomics to further analyze the secreted proteins generated under each condition (16h normoxia, 16h hypoxia, and 24h re-oxygenation). Three LC-MS/MS replicates were performed for each condition. The conditioned media were fractionated by 1D-gel and then subjected to LC-MS/MS profiling for protein quantification using 3 different algorithms for data analysis (normalized spectral index, SIN; normalized spectral abundance factor, NSAF; exponentially modified protein abundance index, emPAI). The 1D gel-based MS data were then used to conduct a MASCOT search and peptide lists were generated from the CSV results files (FDR <1%; Supplementary Data 3). A total of 8933 peptides were identified in normoxic medium, 8462 in hypoxic medium, and 10829 in the re-oxygenated culture medium. From these peptides we identified a total of 2007 proteins that were quantified using the SIN, NSAF and emPAI algorithms (Supplementary Data 4). Taken together, we were able to identify a total 2165 proteins by combining data from the iTRAQ experiment (n=860) with data from the label-free experiment (n=2007), as shown in Figure 1b.

317 For subsequent analyses, we used bioinformatic tools to select for proteins that had been
318 identified as secretory products with a high level of confidence. Four methods were used; the
319 signal peptide was identified by SignalP algorithm²³, proteins with trans-membrane helices were
320 identified by TMHMM²², and we used SecretomeP²⁴ to identify non-classical secreted proteins.
321 The proteins were also screened using the Exocarta database²⁵ of experimentally-detected
322 exosome proteins. Of the 2165 proteins detected by our analyses, 1363 were recognized as
323 being secreted proteins by one of the 4 bioinformatic methods. Exocarta screening identified 631
324 exosome proteins, while SignalP identified 540 proteins that exhibited a signal peptide at the N-
325 terminus, SecretomeP analysis identified 911 proteins as non-classical secreted products, and
326 TMHMM identified 288 proteins as having trans-membrane helices (Figure 1c).

327 In order to provide further confidence that the putative secreted proteins had been correctly
328 identified, we next used a text mining technique to scan for previous publications that already
329 identified these proteins as being present in the extracellular space. For this purpose we
330 downloaded the *Gene2pubmed* database (created 05-Jun-2010) from NCBI and scanned this for
331 abstracts that included the keyword 'secret' (truncation of 'secreted' and 'secretion') in association
332 with the specific protein identified by our MS experiment as being a non-classical secreted
333 product.

334 After manual checking, 10 more proteins were found to have been identified or treated as
335 secreted proteins in previous studies (Supplementary Information 2). Secretion of chaperonin 60
336 (Hspd1) is reportedly induced by IL-1 β and TNF- α stimulation of osteoclasts³⁰.
337 Phosphatidylethanolamine-binding protein 1 (Pebp1) was identified as a secreted protein
338 released by adult rat hippocampal progenitor cells in a previous 2-DE-coupled mass spectrometry
339 experiment³¹. In a study conducted by Konrad *et al.*, Nidogen-1 (Nid1) was found to be
340 exclusively produced and secreted by mesenchymal peritubular cells and influenced their
341 adhesive properties in an autocrine manner³². The antioxidant enzyme CuZn superoxide
342 dismutase (Sod1) is typically considered to be a cytoplasmic protein, but previous reports have
343 observed Sod1 secretion in correlation with depolarization-dependent calcium influx³³. Intriguingly,

a previous study has also identified that nicotinamide phosphoribosyltransferase (Nampt) is secreted by L6 rat skeletal muscle cells, supporting the concept that this protein may be a myokine³⁴. For the remaining proteins with no published evidence of secretory status, it is possible that our report represents the first evidence that these molecules are components of the cardiomyocyte secretome.

Minimal contamination by the cellular proteome

To determine the extent of secretome contamination by cellular proteins from dead cell lysate, we quantitatively compared the proteins detected in the secretome with the cellular proteome of the H9C2 cell line using the SI_N label-free quantification method (wherein protein concentration is normalized by protein length and the sum of abundance index). We therefore plotted the SI_N of each protein in order to estimate changes in relative abundance across the different experiments.

As shown in Figure 2a, the proteins that comprised the rat myoblast H9C2 secretome under normoxic conditions could be grouped into 6 abundance categories using SI_N profiling; the graph shows the sum of the abundance of the proteins in each category (left side), and the total number of proteins in each category (right side). Just 12.5% of these proteins accounted for ~84.2% of the total abundance. Excluding the 3 proteins that were not identified as secreted products by the bioinformatics tools/text mining (Enolase 3, Eno1 protein-like, Actin gamma 1 propeptide-like), the 16 most highly abundant proteins were all recognized as being secreted products. We next estimated the abundance of H9C2 cellular proteins by calculating the SI_N value of each intracellular protein and compared these with the secretome data as shown in Figure 2b. The comparisons of all three datasets of secreted proteins versus the cellular proteome are displayed in Supplementary_Data_5 - Compare Abundance Distribution Secreted vs Cellular Proteomes.xlsx. As shown in Figure 2b and Supplementary Data 5, the relative abundance of proteins comprising the secretomes generated under normoxic, hypoxic, and re-oxygenation conditions were distinct from those that comprised the intracellular proteome, thus indicating minimal contribution from cellular proteins to the composition of the secretomes studied here.

Quantification of the secretome under hypoxia/re-oxygenation stress

Among the 860 proteins quantified in the iTRAQ experiment, 130 proteins displayed significant changes in abundance during hypoxia (ratio 115/114, $P < 0.05$), including 87 proteins that were decreased and 43 proteins that were increased under low oxygen conditions. Subsequent re-oxygenation for 24h was associated with modulated secretion of 106 proteins (ratio 117/114, $P < 0.05$), including 27 proteins that were decreased and 79 proteins that were increased during this phase.

For the label-free experiment, we compared differences in secretome protein abundance between normoxia, hypoxia and re-oxygenation conditions using the respective SI_N values expressed as a ratio of the normoxia SI_N values (or listed as not applicable/NA if the protein was not identified in either condition). We also used NSAF³⁵ and emPAI³⁶ approaches to quantitate the 1D gel-based LC-MS/MS data for cross-reference (Supplemental Data 4). These label-free datasets were compared with the iTRAQ dataset to facilitate identification of proteins that were similarly modulated in both experiments (listed in Supplementary_Data_6 - Significant changed proteins by iTRAQ, emPAI, NASF and SIN.xlsx). None of the algorithms we used for the label-free analyses is able to provide 100% accurate quantitation, so we selected proteins for further analysis only where two or more of the label-free measurements were consistent and were also associated with a significant iTRAQ ratio ($P < 0.05$; Supplemental data 6). In total, 87 secretome proteins associated with hypoxia and 71 secretome proteins associated with re-oxygenation exhibited similar changes in abundance when assessed by both the label-free and iTRAQ experiments.

To gain a systematic view of secreted protein modulation during hypoxia and re-oxygenation, we next analyzed the involvement of specific biological processes in the secretome responses using DAVID tools³⁷. The proteins most highly secreted in hypoxia were associated with biological processes including extracellular matrix organization, cell adhesion, and collagen fibril organization (Figure 3). The secretome proteins most substantially down-regulated in hypoxia were instead associated with organization of cellular components, intracellular transport, and

protein localization (as listed in the Supplementary_Data_7 - Hypoxia_Reox perturbed biological processes.xlsx). During 24h re-oxygenation, the up-regulated secretome proteins were mediators of cell metabolism, polarity, and protein complex assembly. In contrast, the proteins decreased during re-oxygenation were involved in regulation of cell adhesion and apoptosis.

Proteins modulated during hypoxia

Our data indicated that hypoxia alters cardiomyocyte release of extracellular matrix (ECM)-related proteins including Serpin H1 (Serpinh1) which binds specifically to collagen and may function as a chaperone molecule during collagen biosynthesis. A previous study has shown that even modest reductions in Serpinh1 expression lead to a significant decrease in synthesis of procollagen³⁸, suggesting a key role for Serpinh1 in ECM regulation. Other investigators have observed cell surface expression of Serpinh1 and identified a putative role for this protein in platelet aggregation mediated by collagen binding³⁹. In our study, levels of secreted Serpinh1 were found to be increased in conditioned medium from cells cultured under hypoxic conditions (iTRAQ: 1.34 P=1.04e-3; emPAI: 1.4; NASF: 1.1; SI_N: 1.2), perhaps indicating up-regulation of collagen deposition pathways. Indeed, hypoxia has previously been reported to promote HIF-1-mediated expression of a cluster of hydroxylase genes that are indispensable for collagen fiber formation⁴⁰, and we detected substantial hypoxia-induced increases in secretome content of procollagen-lysine (iTRAQ: 1.12 P=2.40e-3; emPAI: 1.0; NASF: 1.4; SI_N: 2.0) and 2-oxoglutarate 5-dioxygenase 1/2 (Plod1, Plod2) (iTRAQ: 1.12 P=3.24e-2; emPAI: 2.0; NASF: 1.1; SI_N: 2.4), which is required for stable collagen cross-linking. Consistent with these data, we also observed hypoxia-induced increases in cardiomyocyte secretion of collagen alpha-1(VIII) chain (Col8a1) (iTRAQ: 1.25 P=2.23e-2; emPAI: increased; NASF: 4.3; SI_N: 7.76), collagen alpha-1(XII) chain (Col12a1) (iTRAQ: 1.26 P=2.85e-9; emPAI: increased; NASF: 4.3; SI_N: 2.6), collagen type IV alpha 2 (Col4a2) (iTRAQ: 1.28 P=4.95e-2 SI_N: 2.3), collagen type V alpha 2 (Col5a2) (iTRAQ: 1.20 P=4.73e-4 SI_N: 1.9) and collagen alpha-3(V) chain (Col5a3) (iTRAQ: 1.28 P=4.97e-2 SI_N: 2.8). Furthermore, formation of collagen-based scar tissue is known to provide mechanical support to the injured myocardium⁴¹, and we detected increased abundance of Lysyl oxidase homolog1 (Loxl1) under hypoxic conditions (iTRAQ: 1.47 P=6.28e-4; emPAI: 2.1; NASF: 1.4; SI_N:

1.4). Expression of cross-linking enzymes such as Loxl1 supports matrix cross-linking in the infarcted myocardium as the scar tissue matures⁴². Dense cross-linked collagen in the mature scar enhances tensile strength of the infarct, but also increases passive stiffness and can lead to diastolic dysfunction.

Restriction of oxygen supply during hypoxia promotes angiogenesis in order to restore normal gas exchange in the affected tissues. Extracellular matrix protein 1 (Ecm1) has been reported to interact with the potent anti-angiogenic factor perlecan domain V⁴³, and we observed that levels of Ecm1 were increased in the hypoxic secretome (iTRAQ: 1.30 P=3.24e-2; emPAI: 2.0; NASF: 1.1; SI_N: 2.9). In addition, the ectoenzyme attractin (Atrn) supports recall responses by peripheral blood mononuclear cells (PBMC) challenged with antigens such as tetanus toxoid *in vitro*⁴⁴, and we detected increased abundance of Atrn in the hypoxic secretome (iTRAQ: 1.37 P=6.86e-3; emPAI: 1.3; NASF: 1.8; SI_N: 5.69). It has been reported by a previous study that recombinant Atrn mediates monocyte/macrophage spreading and T cell clustering⁴⁵, hence increased Atrn secretion during hypoxia may represent an important component of the inflammatory response to low oxygen stress. Biglycan (Bgn) is a member of the small leucine-rich proteoglycan family that regulates collagen fibrillogenesis and fibril diameter⁴⁶. Previous data have demonstrated that expression of biglycan is progressively increased over 2 weeks after MI in wild-type mice, and that collagen matrix formation of infarct scars is impaired in Bgn-knockout mice⁴⁷. Exogenous administration of Bgn has also been reported to protect myocardial cells from hypoxia/re-oxygenation injury via an NO-dependent mechanism⁴⁸, and we observed decreased abundance of Bgn in the hypoxic secretome (iTRAQ: 0.85 P=1.23e-2; emPAI: 1.0; NASF: 0.8; SI_N: 0.7), indicating a potential deficit in cardio-protective responses under low oxygen conditions. Vitamin k-dependent protein s (Pros1) is an anticoagulant plasma protein implicated in protection against MI and arterial thrombosis⁴⁹. Accordingly, we detected decreased Pros1 secretion by cardiomyocytes after hypoxia (iTRAQ: 0.60 P=1.96e-2; emPAI: decrease; NASF: decrease; SI_N: decrease). Stress-induced-phosphoprotein 1 (Stip1) is an adaptor protein that coordinates the functions of HSP70 and HSP90, and we observed that Stip1 abundance was decreased in the secretome after hypoxia (iTRAQ: 0.75, P=1.28e-5; emPAI: 0.55; NASF: 0.46; SI_N: 0.42).

453 According to the UniProt gene ontology annotation, stip1 locates to the cytoplasm and nucleus,
454 and the TMHMM algorithm used in our report did not predict any trans-membrane helices in this
455 protein. However, our detection of stip1 in the secretome is consistent with other reports that this
456 protein locates to the cell membrane⁵⁰ and can be detected in the exosome fraction⁵¹.

457 *Proteins modulated during re-oxygenation*

458 Cardiomyocyte re-oxygenation after hypoxia was associated with increased secretion of proteins
459 related to cell adhesion including Roundabout 2 (Robo2) (iTRAQ: 1.19 P=1.91e-2), Periostin
460 (Postn) (iTRAQ: 1.22 P=1.06e-3 ; emPAI: 1.3; NASF: 0.8; SI_N: 1.7), Thrombospondin 1 (Thbs1)
461 (iTRAQ: 1.15 P=2.71e-2; emPAI: 1.1; NASF: 0.9; SI_N: 1.6), and Proto-cadherin Fat1 (Fat1)
462 (iTRAQ: 1.25 P=3.08e-2; emPAI: 2.0; NASF: 1.5; SI_N: 3.2). Robo is a novel member of the Ig
463 superfamily of cell adhesion molecules (CAM)⁵² and exhibits high levels of homology to other
464 CAMs including NCAM, L1 and DCC⁵³. Postn is a protein involved in fibrosis and ECM
465 metabolism in a range of different diseases and exhibits genomic associations with ventricular
466 hypertrophy⁵⁴ and heart failure⁵⁵. Expression of Postn in cardiac tissue correlates with myocardial
467 pressure levels and is associated with ventricular remodeling⁵⁶. Accordingly, abundant expression
468 of Postn can be detected in the border zone in both mouse and human myocardial infarction, and
469 Postn secretion in infarcted myocardial tissue modulates the phenotype and function of local
470 fibroblasts and protects against cardiac rupture⁵⁷. Similarly, adhesive glycoprotein Thbs1
471 mediates cell-cell and cell-matrix interactions, and this secretome component has been found
472 to promote migration of human coronary artery smooth muscle cells and promote thickening of
473 the arterial wall⁵⁸. Expression of Thbs1 is thought to be induced by hypoxia and mediated by HIF-
474 1 α , and consistent with these concepts, a previous study has reported that Thbs1 is induced in
475 healing myocardial infarcts and contributes to suppression of local inflammatory responses and
476 angiogenic processes⁵⁹. Furthermore, in an epithelial cell wound model, knockdown of cell
477 membrane protein Fat1 impaired lamellipodial dynamics as well as cell polarization and
478 migration⁶⁰. Increased abundance of these proteins is likely to contribute to cell-ECM interaction,
479 cell adhesion, anti-inflammatory responses and ECM remodeling during cardiomyocyte re-
480 oxygenation after hypoxia. Indeed, where we had previously observed depletion of bgn in the

481 hypoxic secretome, the cardiomyocyte re-oxygenation secretome was enriched in this
482 cardioprotective protein that regulates collagen fibril formation⁴⁶ (iTRAQ: 1.45 P=7.53e-5; emPAI:
483 1.6; NASF: 1.1; SI_N: 1.2).

484 Peptidyl-prolyl cis-trans isomerase (Ppia) is an 18kDa protein that possesses multiple distinct
485 functions. A previous study has indicated a role for Ppia as a secreted growth and survival factor
486 for vascular smooth muscle cells⁶¹. During hypoxia/re-oxygenation of rat cardiac myocytes, the
487 autocrine effects of secreted Ppia protect against oxidative stress-induced apoptosis⁶².
488 Accordingly, over-expression of Ppia in cancer cells renders them resistant to hypoxia- and
489 cisplatin-induced cell death⁶³. In the current report, we observed that Ppia secretion by
490 cardiomyocytes was increased during re-oxygenation after hypoxia (iTRAQ: 1.26 P=3.01e-2;
491 emPAI: 2.1; NASF: 1.4; SI_N: 2.6), as has also been reported in a previous study⁶². The proteins
492 that were significantly decreased during the 24h re-oxygenation protocol included glyoxalase I
493 (Glo1), trans-membrane protein 132A (Tmem132a), and metalloproteinase inhibitor 1 (Timp1),
494 which are known to exert potent anti-apoptotic effects. Over-expression of Glo1 attenuates renal
495 ischemia-reperfusion injury in rats⁶⁴, and knockdown of Tmem132a by RNA interference
496 facilitates serum starvation-induced cell death in Neuro2a cells⁶⁵. Similarly, Timp1 prevents
497 radiation-induced apoptosis of capillary endothelial cells⁶⁶. Down-regulated secretion of these
498 anti-apoptosis proteins suggests increased induction of programmed cell death among re-
499 oxygenated cardiomyocytes after hypoxia.

500 In addition to exerting anti-apoptotic effects, Timp1 also inhibits the activity of metalloproteinases.
501 Reduced secretion of Timp1 by carcinoma cells subjected to hypoxia and re-oxygenation stress
502 has been reported by a previous study⁶⁷. Down-regulation of Timp1 secretion would therefore
503 suggest increased activity of matrix metalloproteinases in the re-oxygenated cardiomyocyte
504 cultures, consistent with increased rates of collagen degradation. Cardiac collagen deposition and
505 degradation is maintained in dynamic balance in healthy tissues but is dysregulated in fibrotic
506 disease⁶⁸. Accordingly, we observed that re-oxygenation was associated with decreased
507 secretome abundance of collagen type V alpha 2 (iTRAQ: 0.87 P=3.76e-4; emPAI: 0.2; NASF:

0.4; SI_N : 0.1) and collagen cross-linking enzyme Lox11 (iTRAQ: 0.80 $P=9.29e-3$; emPAI: decrease; NASF: decrease; SI_N : decrease).

Validation of the regulated secreted proteins

Western blots were used to validate the identified changes in cardiomyocyte protein secretion during hypoxia and re-oxygenation protocols. Representative proteins Stip1, SerpinH1, Ppia and DJ-1 (Park7) were blotted to compare secreted levels in normoxia, hypoxia and re-oxygenation (Figure 4 and Supplementary Figures 2-6). The Western blot analyses confirmed that Stip 1 was decreased and that SerpinH1 and Ppia were increased after hypoxia and re-oxygenation, whereas there was no significant change in secretion of Park7 under these conditions. These findings were consistent with our proteomics data, so we next sought to determine whether the observed changes were a consequence of altered protein secretion or could be attributed to changes in protein expression level. We therefore performed Western blot analysis of cell lysate samples from cultures subjected to normoxia, hypoxia or re-oxygenation and were able to confirm that cellular expression levels of Stip1, SerpinH1 and Ppia were not significantly altered under these conditions (Figure 4). Although Park7 and SerpinH1 (both secreted and cellular) were blotted and exposed for the same duration on a single membrane, SerpinH1 was detected as being abundant in cell lysate and minimally present in the secretome, whereas the reverse pattern was observed for Park7 (Figure 4). These data provided further confirmation that the composition of the cellular proteome is distinct from that of the secretome, and that our preparations of secretome samples contained minimal contribution from the cellular lysate.

Conclusion

In present study, we provide evidence that the cardiomyocyte secretory response to hypoxia is associated with angiogenesis and inflammation, whereas subsequent re-oxygenation/reperfusion is instead associated with suppression of inflammation and decreased output of cell survival factors. In addition to these oxygenation-specific changes, we also observed that secretion of proteins involved in extracellular matrix (ECM) remodeling is dynamically regulated over the course of hypoxia/re-oxygenation stress, indicating that this process is likely a key component of the cardiac tissue response to ischemia/reperfusion injury.

In order to obtain high-quality quantitative data, we prepared two independent batches of biological samples for analysis and used three different methods of protein quantitation to delineate secretome composition. Two MS-based quantitative methods (iTRAQ and label-free) were used to profile the first batch of secretome samples derived from rat cardiomyoblast H9C2 cells subjected to normoxia, hypoxia and/or re-oxygenation stress. Only secreted proteins that were identified by both iTRAQ and label-free methods as being modulated by hypoxia/re-oxygenation were short-listed for further investigation. A representative subset of the differentially secreted proteins was then confirmed by Western blot using the second, independent batch of biological samples. Using this approach, we successfully identified numerous secreted proteins that were differentially regulated by hypoxia and re-oxygenation stress, including SerpinH1, Ppia, Attractin, EMC1, Postn, Thbs1, Timp1, Stip1, Robo2 and Fat1. Further analysis of these data revealed that putative pathways involved in mitigating cellular injury and promoting healing (angiogenesis, cellular inflammatory responses, ECM re-modeling and apoptosis) were substantially modified by hypoxia and/or re-oxygenation. Our data elucidate cardiomyocyte secretome composition and modulation in response to hypoxia and re-oxygenation, and provide an overview of molecular events and potential mechanisms that could influence the extent of ischemia/reperfusion injury in human patients with heart disease. These findings may also lead to the identification of novel prognostic biomarkers secreted from injured heart tissues into the circulation of patients with cardiovascular disease.

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560

Supplementary Materials:

Supplementary information: S. Table 1 and S. Figures 1 to 6

Supplementary_Data_1 - iTRAQ_ProteinsSummary.xlsx

List of iTRAQ identified and quantified proteins as exported from ProteinPilot v.3 (with FDR calculation).

Supplementary_Data_2 - iTRAQ_PeptideSummary.xlsx

List of iTRAQ identified and quantified peptides as exported from ProteinPilot v.3

Supplementary_Data_3 - Mascot Identified_Peptides_with_FDR.xlsx

List of peptides identified by MASCOT based on LC-MS/MS data (with FDR calculation).

Supplementary_Data_4 - Label_Free_Quantified_Proteins.xlsx

List of proteins as quantified by 3 label-free algorithms: Normalized spectral index (SIN); Normalized spectral abundance factor (NSAF); and The Exponentially Modified Protein Abundance Index (emPAI).

Supplementary_Data_5 - Compare Abundance Distribution of Secreted vs Cellular Proteomes.xlsx

Secretome and cellular protein abundance distribution in the normoxia, hypoxia and re-oxygenation cultures.

Supplementary_Data_6 - Significantly changed proteins by iTRAQ, emPAI, NASF and SIN.xlsx

List of secreted proteins significantly changed by hypoxia/re-oxygenation stress as determined by 4 separate quantitative proteomics methods.

Supplementary_Data_7 - Hypoxia_Reox perturbed biological processes.xlsx

Biological processes modified by hypoxia/re-oxygenation stress as identified by DAVID bioinformatic tools.

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Figure Legends

Figure 1 a) VEGF secretion in cultures of rat H9C2 cardiomyocytes subjected to 16h normoxia (No), 16h hypoxia only (Hy), or 16h hypoxia followed by 24h re-oxygenation (Re) as assessed by Western blot. b) Venn diagram showing the proteins identified by iTRAQ and label-free LC-MS/MS experiments. c) Secreted proteins as identified by SignalP, SecretomeP, TMHMM and Exocarta algorithms.

Figure 2. a) Distribution of secreted protein abundance and protein number. The left side of the graph shows cumulative protein abundance and the right side shows cumulative protein number (expressed as a percentage of the total). b) Abundance distribution of proteins identified in the secretome versus cellular proteome of H9C2 cells. The abundance of each secreted protein and cellular protein was plotted against the protein length (with individual protein abundance expressed as a percentage of total abundance).

Figure 3. Biological processes enriched in the secreted proteins modulated by hypoxia/re-oxygenation of H9C2 cells. a) Up-regulated during 16h hypoxia; b) Down-regulated during 16h hypoxia; c) Up-regulated during 24h re-oxygenation; d) Down-regulated during 24h re-oxygenation. Keywords with $P < 0.05$ were plotted and P-values were transformed to -Log (high values indicate high confidence of keyword enrichment).

Figure 4. Western blot of Stip1, SerpinH1, Park7, and Ppia proteins in the secretome and cellular proteome of H9C2 cells. The whole blots of the proteins are shown in the Supplementary Figures 2 to 6.

Figures:

Figure 1

Figure 2

Figure 3

Figure 4

Supplementary_Data_2 - iTRAQ_PeptideSummary

[Click here to download Supplementary material: Supplementary_Data_2 - iTRAQ_PeptideSummary.xlsx](#)

Supplementary_Data_4 - Label_Free_Quantified_Proteins

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Supplementary_Data_7 - Hypoxia_Reox perturbed biological process

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