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Spatial phenotyping of the endocardial endothelium as a function of intracardiac hemodynamic shear stress

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Abstract

Despite substantial evidence for the central role of hemodynamic shear stress in the functional integrity of vascular endothelial cells, hemodynamic and molecular regulation of the endocardial endothelium lining the heart chambers remains understudied. We propose that regional differences in intracardiac hemodynamics influence differential endocardial gene expression leading to phenotypic heterogeneity of this cell layer. Measurement of intracardiac hemodynamics was performed using 4-dimensional flow MRI in healthy humans ($n=8$) and pigs ($n=5$). Local wall shear stress (WSS) and oscillatory shear indices (OSI) were calculated in three distinct regions of the LV – base, mid-ventricle (midV), and apex. In both the humans and pigs, WSS values were significantly lower in the apex and midV relative to the base. Additionally, both the apex and midV had greater oscillatory shear indices (OSI) than the base. To investigate regional phenotype, endocardial endothelial cells (EEC) were isolated from an additional 8 pigs and RNA sequencing was performed. A false discovery rate of 0.10 identified 1051 differentially expressed genes

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Conflict of interest

The authors declare no conflicts of interest.

Uncited reference

(McCormick et al., 2016).

between the base and apex, and 321 between base and midV. Pathway analyses revealed apical upregulation of genes associated with translation initiation. Furthermore, tissue factor pathway inhibitor (TFPI; mean 50-fold) and prostacyclin synthase (PTGIS; 5-fold), genes prominently associated with antithrombotic protection, were consistently upregulated in LV apex. These spatio-temporal WSS values in defined regions of the left ventricle link local hemodynamics to regional heterogeneity in endocardial gene expression.

Keywords

Endocardial endothelium; 4D Flow MRI; Heart; Shear stress; RNA sequencing

1. Introduction

Endothelial cell (EC) structure and function is strongly influenced by the frictional force of wall shear stress (WSS) at the blood-EC interface. The effects of WSS on EC phenotype are well described to play an important role in blood vessel physiology and pathology. In regions where undisturbed WSS dominates, ECs are healthy. Conversely, ECs in regions of disturbed WSS, characterized by flow separation and transient flow reversals, have a pro-inflammatory, pro-oxidative stress phenotype and represent sites where atherosclerosis preferentially develops (Civelek et al., 2009; Davies et al., 2013).

Endocardial endothelial cells (EECs) line the heart chambers and represent an important barrier between the circulation and underlying myocardium. Studies in rat and pig demonstrate that EECs, which may originate from precursor vascular ECs, differ from mature vascular ECs with regard to morphology, gene and protein expression and labile molecule production (Hendrickx et al., 2004; Mebazaa et al., 1995). EECs are important in regulating cardiac contraction; endocardial denudation results in loss of contractile strength (Brutsaert et al., 1988; Mebazaa et al., 1993; Shen et al., 2013; Smith et al., 1991). This is attributed to the production and release of paracrine signaling agents such as nitric oxide (NO) (Smith et al., 1991). Unlike vascular ECs, the role of hemodynamics in the regulation of EEC biology has not been carefully studied.

Preservation of flow patterns throughout the LV is important to maintain its efficient function (Gharib et al., 2006). For example, diastolic vortex formation prevents the dissipation of blood kinetic energy and therefore reduces the myocardial force required to eject blood during systole (Kilner et al., 2000). Recent advances in cardiac imaging have enabled more detailed descriptions of intracardiac hemodynamics (Rodriguez Muñoz et al., 2012). In particular, the development of four-dimensional (4D) flow MRI enables 3D velocity encoding with ECG-gating, providing accurate spatial and temporal information. This technology has demonstrated that flow patterns within the LV change with age and gender and that blood residence times vary within the LV (Föll et al., 2013; Hendabadi et al., 2013). The influence of spatiotemporal differences of hemodynamics on EEC biology in the LV is poorly understood.

Here we calculated regional LV WSS using 4D flow MRI in humans and pigs to show that WSS varies regionally throughout the left ventricle of both species. We then isolated EECs

from sites matched to the regional hemodynamic characteristics in pig LV and profiled gene expression by RNA sequencing to demonstrate significant regional differences of EEC phenotypes.

2. Methods

2.1. 4D Flow MRI acquisition

Four-dimensional (4D) flow MRI (4D flow MRI) was performed to assess intracardiac hemodynamics in humans and pigs. For human MRI, IRB approval and informed consent were obtained from all 4D flow MRI study participants. The specifics of image acquisition and analysis were as previously described (Markl et al., 2012, 2016). Studies were performed on 8 healthy volunteers (mean age: 24 ± 1.8 years; four females and four males, heart rate $63.5 \pm$ beats per minute, LV ejection fraction (EF) % 65.3 ± 2.1). Animal experiments were performed in compliance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” (NIH publication 85–23, revised 1996) and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. For MRI analysis, healthy adult pigs ($n=5$, Yorkshire swine, weight= 61.6 ± 2.9 kg; range: 59–67 kg) were used. 4D flow MRI was performed with a dual cardiac and respiratory prospectively-gated cine phase-contrast MRI sequence with the following parameters: temporal resolution=20.8 ms, spatial resolution= $2 \times 2 \times 2$ mm³, ip angle= 8° , field of view= $320 \text{ mm} \times 320 \text{ mm}$, pixel bandwidth 460 Hz/pixel. The velocity encoding (V_{enc}) sensitivity was adjusted for each animal to minimize velocity aliasing during diastole ($V_{\text{enc}}=75\text{--}185$ cm/s) as previously described (Witschey et al., 2015).

2.2. Endocardial endothelial cell sample isolation and RNA sequencing

Endocardial endothelial cells (EEC) were isolated from 8 pigs. Incisions were made 5 mm from the apex of the heart to expose the apical endothelium. A longitudinal incision was made 0.5–1 cm left of the left anterior descending (LAD) artery to expose the main LV chamber where base and midV EEC samples were collected. Approximately 1–2 cm² of endothelium were gently scraped from each region (Fig. 5A) and placed in RNA isolation buffer (mirVana Isolation Kit, ThermoFisher, Waltham, MA). Nucleic acid isolation, purification, QC and concentration were performed as previously described (Jiang et al., 2015). Pure populations of EECs were confirmed by Western blot and immunofluorescence.

RNA samples from base, midV and apex in 8 male pigs were submitted to the Children's Hospital of Philadelphia High-Throughput Sequencing Center for quality control, library construction and sequencing using the Illumina HiSeq 2500 with 100 base pair paired-end reads and yielding an average of about 32 million read pairs per sample. Reads were aligned to the Sscrofa10.2.73 genome with STAR (Dobin et al., 2013) and the PORT pipeline (<https://github.com/itmat/Normalization>) was used for normalization and quantification. Differential gene expression (with comparisons run in paired-by-pig mode) was performed using edgeR (Robinson et al., 2010) and PADE (<https://github.com/itmat/pade>), an extension of PaGE (Grant et al., 2005). For analysis, we used genes identified by both approaches at FDR 0.10. Additionally, genes with average normalized counts less than 50 in each of the two regions compared were excluded from analysis.

2.3. Pathway analysis

Data were analyzed by QIAGEN Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Gene Ontology (GO) enrichment analysis (GO Biological Process and Molecular Function) was performed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) web server (<http://david.abcc.ncifcrf.gov/>) (Huang et al., 2009a, b). We selected an FDR of 0.10 (10%) for gene list analysis. Identified canonical pathways were ranked according to *p*-value.

Quantitative real-time polymerase chain reaction (qPCR), Western blotting and immunofluorescence procedures were as previously described (Jiang et al., 2015). Primers used for qPCR are shown in Supplemental Table 1.

2.4. Statistical analysis

Data are shown as mean $\pm$ standard deviation unless otherwise specified. Comparisons were performed using either the Wilcoxon rank sum test for comparison of two groups (qPCR data) or analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. An adjusted *p*-value of <0.05 was considered statistically significant. Statistical analysis was performed using Graphpad Prism6 (Graphpad Software, La Jolla, CA, USA).

2.5. Accessibility of data

The RNASeq data and metadata have been deposited into the ArrayExpress public repository (<https://www.ebi.ac.uk/arrayexpress/>) with accession E-MTAB-3669.

3. Results

3.1. Regional differences in LV wall shear stress and oscillatory shear index

To investigate regional WSS in healthy humans and pigs, we selected three heart regions: base, inferior to the aortic valve on the posterior heart wall, mid-ventricle (midV), inferior to the mitral valve on the free wall, and the apex (Fig. 1A). Following selection of two-dimensional planes, the images were segmented to define the LV lumen and 12 reference points were positioned. Hemodynamic characteristics were calculated as previously described (Stalder et al., 2008; Markl et al., 2012; Dyverfeldt et al., 2015). Using velocity values obtained from the 4D MRI images, we calculated WSS including both WSS magnitude and its two components: axial and circumferential. Additionally, the oscillatory shear indices (OSI) were calculated for each of the 12 reference points. In Fig. 1B, green lines represent WSS magnitude and direction and purple lines show OSI magnitude. These images illustrate several points regarding the complexity of flow within the LV including the regional differences in WSS magnitude. They also show the complex differences in WSS direction in the different LV regions. Furthermore, they illustrate that in a given 2D plane, the 12 points selected experience highly variable WSS and OSI values. We therefore focused our analysis on specific points within each region to correlate with the downstream endocardial phenotyping.

Shown in Figs. 2 and 3 are the time-averaged WSS_{mag} and axial WSS for individual human and pig subjects, respectively. Red lines indicate the averages for each subject group. For

both humans and pigs, the WSS_{mag} values were higher in the LV base, specifically during systole, compared to either midV or apex. Similar trends were observed for axial WSS. The maximum WSS_{mag} , axial and circumferential WSS values are listed in Table 1. We observed significant differences between base and apex for time-averaged magnitude, axial and circumferential WSS. It is important to note that the axial WSS values were different only in systole.

Regional OSI values, an indicator of oscillatory WSS that allow for quantification of the change in direction and magnitude of WSS, were calculated (Table 2). Regional OSI trends were similar to those observed for WSS; the apex recorded significantly higher 3D and axial OSI than the base and midV (Table 2). Of note, high OSI in arteries is frequently associated with vascular pathologies (Davies, 1995; Isoda et al., 2010).

3.2. Endocardial regions have distinctive patterns of gene expression

To investigate phenotype differences in the EECs from base, midV and apex we performed RNA sequencing (RNAseq) in 8 pigs, 3 regions per animal (Fig. 4A). At an FDR = 0.10 the following comparisons were made: base:apex, base:midV and apex:midV. This analysis revealed 1051 differentially expressed genes (DEGs) between base:apex and 325 DEGs between base:midV (Fig. 4B). No DEGs were observed in the apex:midV comparison, consistent with comparable WSS values for these two regions. Hierarchical clustering (method = average, similarity measure = Pearson correlation) of each region further illustrates broad similarities in DEGs between midV and apex in contrast to the base region (Fig. 4C).

To assess changes in biological and/or signaling pathways associated with differential gene expression Ingenuity Pathway Analysis (IPA) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) were used (Huang et al., 2009a, b). For both base:apex and base:midV comparisons, IPA and DAVID ranked translational pathways at the top of the list including eukaryotic initiation factor 2 (eIF2) and mammalian target of rapamycin (mTOR) signaling, translation elongation and regulation of cell growth (Tables 3 and 4, respectively). Despite classification as distinct pathways, these are all part of the broader translation machinery, specifically translation initiation.

3.3. Increased expression of anticoagulant genes in apical EECs

Our calculations of lower WSS and elevated OSI in the apex coupled with previous measurements of increased residence time of blood components in the apex (Hendabadi et al., 2013) suggest that the apical region may be predisposed to thrombus formation. However, thrombi are generally not observed in healthy individuals, an outcome that may in part be attributable to regional expression of anti-coagulation genes. For these and subsequent experiments, we focused on the base-apex comparison.

RNAseq data revealed the upregulation of several anticoagulant genes in the apex relative to the base (Fig. 5) including tissue factor pathway inhibitor (TFPI), the principal inhibitor of tissue factor (Wood et al., 2014) and the key initiator of the coagulation cascade. By RNAseq, TFPI was upregulated 20-fold in the LV apex compared to base. Validation by qPCR showed a mean increase of 50-fold (range 15–100 fold) in apex EECs relative to base

EECs (Fig. 5A). Furthermore, RNAseq identified differential expression of prostacyclin synthase (PTGIS) in the apex. PTGIS, the primary enzyme responsible for prostacyclin (PGI₂) production, an integral inhibitor of platelet activation (Moncada et al., 1976), was 5-fold higher in the LV apex than base EECs (Fig. 5B).

In addition to soluble and membrane-bound factors produced by endothelial cells, the endothelial glycocalyx provides electrical repulsion of activated platelets via its strong negative charge (Reitsma et al., 2007). The glycocalyx components heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) and neuropilin-1 (NRP1) were both upregulated 5-fold in the apex versus base (Fig. 5C,D).

3.4. Upregulation of NRF2 and downstream antioxidant genes in LV apex

The unfolded protein response (UPR) is a cellular adaption to endoplasmic reticulum (ER) stress and is activated in atherosusceptible regions such as the aortic arch where WSS is low and OSI values are elevated (Civelek et al., 2009). We measured regional expression of UPR-related genes to determine if similar trends existed in EEC of the LV apex. By qPCR there was no difference in X-box binding protein (XBP1) expression (Fig. 6A). Unlike low flow arterial regions, we observed significantly decreased apical expression of kinase-like extracellular signal related kinase (PERK), activating transcription factor 4 (ATF4), and Binding Protein/GRP78 (glucose-regulated protein 78 kDa, HSPA5) (Fig. 6B–D). Collectively, these data suggest that in contrast to arterial ECs, low WSS/high OSI in the apex does not lead to upregulation of the UPR.

Activation of PERK signaling leads to expression of nuclear factor erythroid-2 related factor 2 (NRF2/NFE2L2), a key gene involved in the oxidative stress/antioxidant response (Cullinan and Diehl, 2004). Despite reduced expression of PERK in apex, NRF2 was identified in the DEG lists as upregulated in the apex along with the downstream target NAD(P)H:quinone oxidoreductase 1 (NQO1). This expression was confirmed by qPCR (Fig. 6E). The elevated expression observed in the apex suggests that endocardial cells in the apex are protected from antioxidant stress.

4. Discussion

The endocardial lining of the heart represents a greatly understudied endothelial compartment, particularly with regard to its phenotypic relationship with WSS. Here we report regional WSS and oscillatory (OSI) flow dynamics throughout the human and pig cardiac cycles, and establish corresponding spatial relationships with endocardial endothelial cell gene expression profiles.

Preservation of blood flow patterns within the LV is important in maintaining its function (Gharib et al., 2006). The etiology of most major cardiomyopathies and valve insufficiencies, as well as corrective surgical procedures and deployment of assist devices, progressively or acutely alters LV hemodynamics (Al-Wakeel et al., 2015; Eriksson et al., 2013; Witschey et al., 2015; Wong et al., 2014). Research has naturally focused on cardiomyocyte remodeling in these pathologic contexts. However, despite substantial evidence for the central role of hemodynamic shear stress in the functional integrity of

vascular endothelial cells (Davies, 2009; Davies et al., 2013) hemodynamic regulation of the LV endocardial endothelium remains undetermined. The convergence of advances in high resolution MRI flow imaging (Markl et al., 2012) and greatly improved precision in cell and molecular phenotyping by gene sequencing (Jiang et al., 2015) has recently allowed greater access to the quantitative spatio-temporal relationships between LV hemodynamics and the endocardial endothelium.

Although we show an association between regional WSS and endocardial gene expression, we have not established the mechanisms governing this relationship. It is possible, and entirely likely, that other factors associated with flow, including transport effects at the blood-EC interface as well as contractility of the underlying myocardium influence the endocardial phenotype. Other possibilities include circumferential stretch (strain) (CS) attributed to the volumetric changes of blood. Recently, Qiu and Tarbell have shown that the spatio-temporal interaction between WSS and CS, termed stress phase angle (SPA), has unique effects on endothelial function (Qiu and Tarbell, 2000). Therefore it is possible that regional SPA contributes to spatial differences in EEC phenotype.

MRI analysis and RNA sequencing identified the greatest differences between base and apex. For both WSS and RNAseq, the midV represents an intermediate region and the pathways differentially expressed in the midV are similar to those in the apex. We therefore prioritized RNA sequencing analysis to the base-apex comparison. Pathway analysis identifies two major biological processes from the base-apex DEG lists: translation initiation and oxidative phosphorylation. Both of these pathways have significant effects on metabolic homeostasis; their identification raises the possibility that the metabolic environment of the LV apex is distinct than that of the base, the consequences of which warrant further investigation.

Regions of the vasculature exposed to low, disturbed WSS such as the aortic arch are primed for disease due to activation of inflammatory and oxidative pathways as well as ER stress (Civelek et al., 2011). It is thought that upregulation of the UPR serves as an adaptive mechanism in these regions (Civelek et al., 2009). We therefore investigated whether ER stress/UPR genes were upregulated in apical EECs exposed to low WSS. Surprisingly, the expression of XBP1, ATF4 or HSPA5 was decreased in the apex relative to base suggesting that the UPR is downregulated in this region and that low WSS in the apex may not lead to ER stress.

Despite a decrease in the expression of other UPR genes, expression of NRF2 was elevated in the apex. The transcription factor NRF2 regulates the expression of target genes involved in the antioxidant response, including NQO1, heme oxygenase (HMOX1), and glutathione S-transferases (GSTs) among others (Gorrini et al., 2013). Both RNAseq and qPCR showed increased expression of NQO1 (Fig. 6B) and HMOX1 (data not shown) suggesting activation of the NRF2 pathway. NRF2 is known to be important in maintaining cardiac health through upregulation of its target genes (Li et al., 2009).

The contribution of regional differences in endocardial gene expression to cardiac function remains to be tested. Using isolated myocardium, studies have demonstrated a crucial role

for endocardium in cardiac contractility (Mebazaa et al., 1993; Shen et al., 2013). This is attributed to endothelial production of signalling molecules such as nitric oxide and endothelin (Brutsaert, 2003). Based on the integral role shear stress plays on endothelial gene expression and function, it is possible that communication between endocardial ECs and CMs varies by region within the LV. Furthermore, perturbation of LV hemodynamics (following mitral annuloplasty, myocardial infarction, etc) may result not only in altered endocardial EC function, but also altered CM and myocardial function. With these considerations, the endocardial endothelium represents a new target for the treatment of cardiomyopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jbiomech.2016.11.018>.

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Fig. 1. Analysis of 4D Flow MRI. A, Representative human 4D flow MRI image showing base, midV, and apical two-dimensional plane selection. Planes are color coded for velocity. B, Representative results of time-averaged WSS_{mag} and OSI calculations for base, midV and apex. Following manual segmentation of the LV luminal area, WSS and OSI values were extracted from intraventricular velocities for 12 discrete points. Luminal colors indicate intraventricular blood flow velocities (m/s). The green lines emanating from the 12 points indicate WSS magnitude (line length) and direction. Purple lines show OSI magnitude only (line length) at the same 12 points and each is linked to the same position (1–12) as the WSS data (i.e. there is no directional information in the OSI data). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Regional differences in WSS_{mag} and axial WSS in HUMANS. A, Time-averaged WSS_{mag} and B, Time-averaged axial WSS values are shown for individual volunteers ($n = 8$) (gray lines) and average (red line) for the regions of interest. Diastole is represented by the first 60% of the cardiac cycle and systole the second 40% of the cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Regional differences in WSS_{mag} and axial WSS in PIGS. A, Time-averaged WSS_{mag} and B, Time-averaged axial WSS values are shown for individual volunteers ($n=5$) (gray lines) and average (red line) for the regions of interest. Diastole is represented by the first 60% of the cardiac cycle and systole the second 40% of the cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Analysis of whole genome sequencing reveals spatial heterogeneity in endocardial gene expression. A, Regions of LV selected for RNAseq sample collection. B, Venn diagram of DEGs between comparisons at FDR = 0.10. C, Heat map showing hierarchical clustering of the DEGs for each region (method=average, similarity measure=Pearson correlation). Ao, aorta; LA, left atrium; LV, left ventricle; midV, mid-ventricle; RA, right atrium; RV, right ventricle.

Fig. 5.

Upregulation of anticoagulant-associated genes in the LV apex. The expression of anticoagulant genes was measured by qPCR. Fold change of gene expression relative to base = 1. Expression was normalized to GAPDH. A, TFPI; B, PTGIS; C, HS6ST2; D, NRP1. For each gene assayed $n = 10$. Data represent mean $\pm$ SEM. *** $P < 0.005$.

Fig. 6.

Increased NRF2 expression in the LV apex. The expression of candidate UPR genes was measured by qPCR. Fold change of gene expression relative to base = 1. Expression was normalized to GAPDH. A, XBP1; B, PERK/EIF2AK3; C, ATF4; D, HSPA5/BiP/Grp78; E, NRF2/NFE2L2; F, NQO1. For each gene assayed $n=12$. Data represent mean $\pm$ SEM.

* $P < 0.05$, ** $P < 0.005$.

Table 1

Regionally averaged wall shear stress (WSS) (Pa).

Human			Pig		
Magnitude	Axial	Circumferential	Magnitude	Axial	Circumferential
0.59±0.11	Dias: 0.32±0.11 Syst: -0.56±0.11	Max: 0.16±0.08 Min: -0.09 ± 0.02	Base	Dias: 0.12±0.04 Syst: -0.36±0.05	Max: 0.07±0.05 Min: -0.13±0.06
0.26±0.08***	Dias: 0.24±0.07	Max: 0.08±0.03* Min: -0.05±0.03	MidV	Dias: 0.21±0.09 Syst: -0.14±0.005**	Max: 0.07±0.03 Min: -0.04±0.01*
0.26±0.06***	Dias: 0.23±0.08 Syst: -0.12±0.02***, #	Max: 0.04±0.01***, ## Min: -0.02±0.008***, #	Apex	Dias: 0.14±0.04 Syst: -0.06±0.01***, #	Max: 0.04±0.02# Min: -0.03±0.005*

Dias, diastole; Syst, systole; Max, maximum; Min, minimum; MidV, mid-ventricle; Pa, pascals.

To Base

To MidV

^{*} $P<0.05$,^{**} $P<0.01$,^{***} $P<0.005$,[#] $P<0.05$,^{##} $P<0.01$.

Table 2

Regionally averaged oscillatory shear index (OSI) (%).

Human			Pig		
3D	Axial	Circumferential	3D	Axial	Circumferential
23.4±6.4	23.9±7.3	23.4±6.4	Base	11.8±4.1	9.35±5.0
27.4±6.2	26.8±5.9	25.8±8.7	MidV	30.2±7.8	31.6±10.9
31.0±4.2 ^{*,#}	32.1±5.0 ^{*,##}	27.2±4.0	Apex	31.3±2.1 ^{***}	31.7±3.0 ^{***}
					29.7±2.4 [*]

3D, 3-dimensional; MidV, mid-ventricle.

To Base

To MidV

^{*} $P<0.05$,

^{***} $P<0.005$,

[#] $P<0.05$,

^{##} $P<0.01$.

Table 3

Pathway analysis of DEGs from Base:Apex Comparison.

IPA analysis		DAVID overrepresented biological functions		
Canonical pathway	Molecules represented	<i>P</i> *	Functional category	No. of genes
EIF2 signaling	59	8.3E-28	Translational elongation	46
mTOR signaling	39	4.8E-14	Translation	66
Regulation of eIF4 and p70S6K signaling	30	1.1E-10	Oxidation reduction	71
Fatty acid β -oxidation	10	5.3E-06	Regulation of cell growth	28
LPS/IL-1 mediated inhibition of RXR function	27	9.4E-06	Regulation of developmental growth	12

* Fisher's exact probability.

Table 4

Pathway analysis of DEGs from Base:MidV comparison.

IPA analysis		DAVID overrepresented biological functions		
Canonical pathway	Molecules represented	<i>P</i> *	Functional category	No. of genes
EIF2 signaling	15	3.6E-08	Muscle contraction	18
Regulation of eIF4 and p70S6K signaling	9	1.7E-04	Muscle system process	18
mTOR signaling	10	2.5E-04	Translational elongation	13
Agranulocyte adhesion and diapedesis	10	2.6E-04	Striated muscle contraction	9
Hepatic fibrosis/hepatic stellate cell activation	10	3.7E-04	Actin filament-based movement	7

* Fisher's exact probability.