Published in final edited form as:

J Med Virol. 2012 August ; 84(8): 1254–1266. doi:10.1002/jmv.23332.

Gene Expression Profile in Human Skeletal Muscle Cells Infected With Human Adenovirus Type 36

Zhong Q. Wang^{1,*}, Yongmei Yu¹, Xian H. Zhang¹, Jianhua Qin¹, and Elizabeth Floyd²

¹Nutrition and Diabetes Research Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana

²Ubiquitin Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana

Abstract

Human adenovirus type-36 (HAdV-36) is a specific pathogen that may lead to increased adiposity and obesity. In order to evaluate the effects of HAdV-36 on gene transcription, a microarray analysis of muscle cells infected with HAdV-36 was performed. Gene expression profile was determined by microarray analysis in cultured human skeletal muscle cells with or without HAdV-36 infection. Quantitative realtime PCR (qPCR) assay was performed in selected 35 genes to verify the results of the microarray analysis. A total of 13,060 unique genes were detected in the HAdV-36 infected muscle cells infected with HAdV-36. Among them, 1,004 genes were significantly altered by using a cut-off point at fold change 1.5 and *P* value <0.05. Most of the principal 100 altered genes were involved in development, immune response, signal transduction, transcriptional regulation as well as carbohydrate, lipid and protein metabolism. Thirty-two genes (91.4%) from the 35 selected genes were confirmed by qPCR assay. In addition, HAdV-36 altered 252 genes that are associated with cancer. The study showed HAdV-36 infection upregulated host cell antiviral defense. HAdV-36 also induces changes in gene expression related to cellular signaling pathways of signal transduction, transcriptional regulation as well as carbohydrate, lipid and protein metabolism. However, it remains to be investigated if HAdV-36 infection could lead to oncogenesis.

Keywords

microarray; gene expression; human skeletal muscle cells; obesity

INTRODUCTION

The etiology of obesity is multifactorial and infection is one cause [Dhurandhar et al., 2000]. Infection with specific pathogens may lead to increased adiposity and obesity [Dhurandhar et al., 2001]. The human adenovirus type 36 (HAdV-36) is a relatively new factor in promoting adipogenesis [Dhurandhar et al., 2000]. HAdV-36 is associated causally with increased adiposity and altered metabolic profile in animals and humans, respectively.

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*Correspondence to: Zhong Q. Wang, MD, Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808. wangzq@pbrc.edu.

The authors declare that they have no conflicts of interest.

Authorship: Z.Q.W. designed the study, Z.Q.W. wrote the manuscript. Y.Y. and X.H.Z. researched data. J.Q. performed statistic analysis for all data. E.F. reviewed manuscript. All authors read and approved the final version of the manuscript. Z.Q.W had primary responsibility for the final content.

Experimental infection of animals with HAdV-36 induces hypolipidemia and obesity without increasing food intake [Dhurandhar et al., 2001, 2002]. HAdV-36 was also observed to increase glucose uptake in cultured cells and lower plasma insulin concentration in animals [Dhurandhar et al., 2002; Pasarica et al., 2006]. HAdV-36 seropositive status appears to be a hallmark of a clinical-metabolic profile possibly preceding obesity and diabetes in non-obese patients [Trovato et al., 2009]. The presence of HAdV-36 antibodies in humans is associated with increased body mass index [Vangipuram et al., 2004; Greenway, 2006]. A recent report showed an association of obesity and higher body weight with the presence of neutralizing antibodies to HAdV-36 in children [Gabbert et al., 2010]. Other studies have also demonstrated that HAdV-36 may play a role in childhood obesity [Atkinson et al., 2010]. Infection of adipocytes with HAdV-36, but not Ad-2 leads to increased production of IL-6 which might contribute to chronic low-grade inflammation [Pasarica et al., 2008]. While down regulation of the cytotoxic immune response may allow HAdV-36-infected cells to survive [Rogers et al., 2008].

The mechanisms responsible for the increased adiposity may contribute to the alterations in gene expression of multiple enzymes and transcription factors by HAdV-36 [Vangipuram et al., 2007; Wang et al., 2008, 2010]. HAdV-36 infections in preadipocytes have been noted to induce cell differentiation in vitro, and remodel metabolically human adipose tissue by lowering glucose and lipid concentrations [Rogers et al., 2008]. The ability of HAdV-36 to infect primary cultured human skeletal muscle (hSKM) cells was confirmed by HAdV-36 E4 orf1 gene expression [Wang et al., 2008]. HAdV-36 infection increases glucose uptake in both diabetic and nondiabetic hSKM cells by activation of the Ras-PI 3K signaling pathway and increased glucose transporter 1 and 4 (GLUT1 and GLUT4) protein expression, but these alterations were independent of the insulin signaling pathway [Wang et al., 2008]. Recently, HAdV-36 was demonstrated to decrease fatty acid oxidation and increase de novo lipogenesis in primary cultured hSKM cells by promoting Cidec/fat specific protein 27 (FSP27) expression [Wang et al., 2010]. Therefore, the hypothesis was that HAdV-36 could infect multiple types of cells and alter host cellular signaling pathways by affecting gene expression. High density DNA microarray technology has been used extensively in gene and drug discovery, disease diagnosis, and toxicological research. Complete genomic sequences of microbial pathogens and their hosts offer sophisticated new strategies for studying host-pathogen interactions. Additionally, DNA microarrays can be used to exploit primary sequence data to measure transcript levels and detect sequence polymorphisms, for every gene, simultaneously [Cummings and Relman, 2000]. Recently, it was reported that HAdV-36, but not Ad-2, induced lipid accumulation and upregulated adipogenesis-related genes, such as PPAR-gamma signaling pathway, and inflammation, in comparison with Ad-2 infection in human mesenchymal stem cells (hMSCs) [Na et al., 2012]. However, there are currently no data available on the effects of HAdV-36 on gene expression in muscle cells. In order to evaluate the effects of HAdV-36 on skeletal muscle gene expression, which may provide evidence linking HAdV-36 induced diseases and the possible mechanisms of pathogenesis, the gene expression profile in HAdV-36 infected hSKM cells was measured using a DNA microarray assay.

MATERIALS AND METHODS

Isolation and Culture of hSKM Cells

Human skeletal muscle cells were isolated from the vastus lateralis muscle biopsy of volunteers and grown as described previously [Wang et al., 2008]. The protocol to obtain muscle biopsies was approved by the human investigations committee of the Pennington Biomedical Research Center, and informed consent was signed by the volunteer individual. Briefly, muscle biopsies were obtained with a needle from the vastus lateralis muscle from three healthy lean men (age 31.8 ± 4.3 years; BMI 23.2 ± 1.04 kg/m²; mean $\pm$ SE).

Approximately, 50 mg muscle tissue was minced with surgical scissors and digested by a solution containing 0.05% trypsin and 0.045 mmol/L of EDTA with constant shaking at 37°C. After centrifugation to remove fat and debris, myoblasts were grown in monolayer culture in SkGM with 10% (v/v) FBS, 1% (v/v) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin), 2 mmol/L glutamine, and 25 mmol/L HEPES (pH 7.4).

Infection of hSKM Cells With HAdV-36

HAdV-36 was kindly provided by Dr. Nikhil Dhurandhar (Pennington Biomedical Research Center). Cells were maintained in SkGM medium (Cambrex, MD) with 10% FBS. Antibiotics were removed before HAdV-36 infection. Unless otherwise indicated in the figure legends, 80% confluent hSKM cells were inoculated with HAdV-36 at a dose of 3.8 PFU/cell. After 1 hr, the HAdV-36 virus was removed and following rinsing three times with PBS buffer, complete medium was added. Successful infection was ascertained by determining expressions of respective viral genes (E4 orf-1) by quantitative real-time PCR assay (qPCR).

RNA Extraction

At Day 5 post-infection, total RNA was extracted from the cultured skeletal muscle cells for microarray and qPCR assays as described previously [Wang et al., 2010]. Total RNA was isolated from the tissue powder using TRIZOL reagent (Carlsbad, CA). After DNase I digestion, RNA was further purified with RNeasy Mini Kit (Germantown, MD). RNA concentration and quality were measured by RNA 6000 Nano LabChip kit (Santa Clara, CA).

Gene Expression Profiling

DNA microarray profiling was assessed in HAdV-36 infected and control hSKM cells from three independent experiments. The Illumina Human-6 v2 Expression BeadChips were purchased from Illumina Inc (San Diego, CA) and contained a total of 47,296 oligonucleotide probes (gene-specific 50mer) or extended sequence tag sequences as well as housekeeping genes used for gene expression profiling. Biotin-labeled cRNA was generated and amplified linearly from 1 µg of total RNA from each sample TotalPrep RNA Amplification Kit, Ambion (Austin, TX). After the cRNA was fragmented by heating at 60°C for 30 min, the biotin-labeled targets were hybridized overnight to the Illumina Expression BeadChip. Following hybridization, the BeadChip was washed and streptavidin-Cy3 conjugate staining protocol. Once the BeadChips had been processed, they were scanned by the Genomics Resource Laboratory with an Illumina BeadArray Reader. Then, chemiluminescence detection, image acquisition and analysis were performed according to the manufacturer's protocol. Signals were quantified, corrected for background, and the final images as well as feature data and gene analysis were processed using Illumina BeadStudio software (San Diego, CA).

Quantitative Real-Time PCR

The primer sequences of candidate genes were designed using Primer Express Software version 3.0 (Foster, CA). Primer sequences of selected genes are shown in Table I. All primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). A 1-µg aliquot of total RNA for each sample was reverse transcribed in a 100-µl reaction volume with a commercial High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The quantitative PCR was conducted in 384-well microtiter plates on the ABI Prism Sequence Detector 7900 (Applied Biosystems) with Bio-Rad iTaq SYBR Green Supermix with ROX Kits. For each gene, PCR amplification was performed in duplicate and the results were normalized by β-actin.

Statistical Analysis

The effects of HAdV-36 on the muscle gene expression, metabolic parameters, cellular signaling and functional parameters were determined using analysis of covariance. For gene expression, within-array normalization was done based on housekeeping genes on each array. Global normalization among arrays was accomplished using quantile normalization method [Bolstad et al., 2003]. ANOVA analysis with Bonferroni adjustment was used for detecting significantly differentiated genes. Differentially expressed genes between HAdV-36 and control cells were determined based on the following criteria: (1) Bonferroni-adjusted $P < 0.05$ and (2) fold change of HAdV-36 over control group ≥ 1.5 . All data analyses were carried out on SAS (Cary, NC). Several public databases including David/Ease, GenMAPP, Panther and GOTM, TreeView version 1.6, and Ingenuity system software (Redwood, CA) were used to assess functional gene cluster analysis [Eisen et al., 1998], and details were described in previous studies [Wang et al., 2009].

RESULTS

Global Gene Expression in HAdV-36 Infected hSKM Cells

The signals of 13,060 genes were detected in both HAdV-36 infected and control muscle cells in the BeadChips, which contain 47,296 genes (e.g., 27.61% of all the genes were detected). The correlations between control muscle cells as well as between HAdV-36 infected and control cells are shown in the Figure 1A,B. The distribution of fold changes of 13,060 genes in HAdV-36 infected muscle cells is shown in Figure 1C. There were 1,004 genes at fold change ≥ 1.5 . Among them, 441 genes were down-regulated and 563 genes were up-regulated. Using the cut-off point at fold change ≥ 2.0 , 595 genes were altered. Among them, 223 genes were down-regulated and 372 genes were up-regulated in HAdV-36 infected hSKM cells when compared with control cells. The fold changes at >2 to <3 , >3 to <4 , >4 to <5 , and >5 were 371, 111, 56, and 57, respectively. The maximal upregulated gene was human myxovirus (influenza virus) resistance 1 (MX1) with fold change 44.35, and the most down-regulated genes were human gremlin 1, cysteine knot superfamily (GREM1) with fold change -12 . Among the 1,004 genes with a fold change ≥ 1.5 , 801 genes were mapped using Ingenuity analysis. The data showed that the 121 biological processes and partial biological process (42 biological processes) were observed (Fig. 2). Fifty-four genes were found to be associated with molecular mechanisms of cancer, hereditary breast cancer signaling, and breast cancer regulation by stathmin 1, pancreatic adenocarcinoma signaling and nonsmall cell lung cancer signaling, as seen in Figure 2. There were 252 oncological genes, which account for one fourth of the 1,004 altered genes (Table II).

The Principal Affected Genes in HAdV-36 Infected Cells

The principal 100 genes (about 10% of total 1,004 affected genes) altered by HAdV-36 are listed in Table III. These genes were involved in 10 biological processes that include carbohydrate and lipid metabolism (4 genes), development (20 genes), unknown processes (9 genes), immune response (9 genes), protein metabolism (7 genes), other processes or metabolism (2 genes), signal transduction (13 genes), transcription regulation (23 genes), transportation (8 genes), and unknown genes (2 genes). Interestingly, all of nine genes involved in immune response and most of the genes associated with the transcription regulation process (19/23 genes; 82%) were up-regulated by HAdV-36. In particular, gene expression of MX1, ribonucleotide reductase M2 (RRM2), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), ISG15 ubiquitin-like modifier (ISG15), chromatin licensing and DNA replication factor 1 (CDT1), MYB and E2F transcription factor 7 (E2F7) were up-regulated significantly in HAdV-36 versus control muscle cells ($P < 0.001$). However, mRNA levels of KIAA119, integrin alpha 11 (ITGA11), connective tissue growth

factor (CTGF), stimulator of chondrogenesis1 (SCRG1), thrombospondin 2 (THBS2), serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1), cartilage oligomeric matrix protein [Maisonpierre et al., 1997], inhibin, beta E (INHBE) and TSC22 domain family, member 3 (TSC22D3) were significantly down-regulated in HAdV-36 infected cells in comparison with control cells ($P < 0.001$).

Heat Map Analysis

The heat map generated from the DNA microarray data reflects gene expression values in HAdV-36 infected muscle cells in comparison with control muscle cells. The heat map analysis of 1,004 genes is illustrated in Figure 3. Green color indicates down-regulation and red indicates up-regulation of gene expression in the HAdV-36 infected muscle cells in comparison with control cells. Notably, as indicated by the arrows, KIAA119 was downregulated and HERC6 was upregulated. KIAA119 and hect domain and RLD 6 (HERC6) were down-regulated 11.90-fold and upregulated 7.89-fold in HAdV-36 infection when compared to control cells, respectively.

qPCR Verifications

In order to verify DNA microarray results, qPCR analyses were performed on 35 selected genes as shown in Table IV. Thirty-two of them (91.4%) were confirmed, but not IGFBP4, PEPN13, and LPR1. Linear regression analysis showed there was a significant correlation between the results of the microarray assay and the PCR assay in the selected 35 genes ($r = 0.8944$, $P < 0.001$, Fig. 4).

Cellular Signaling Pathways in HAdV-36 Infected Muscle Cells

Cellular signaling pathway analysis showed HAdV-36 infection resulted in altered E2F, NF κ B and minichromosome maintenance complex component 10 (MCM10) signaling pathways in the muscle cells (Fig. 5).

HAdV-36 Infection-Related Diseases

HAdV-36 infection altered associated network functions, molecular and cellular functions as well as physiological system development and function. Gene expression in HAdV-36 infected hSKM cells links to a number of disorders and diseases such as cancer, gastrointestinal disease, and genetic disorders (Table II). Among the total of 252 oncogenic genes, 115 genes were down-regulated and 137 genes were upregulated by HAdV-36.

DISCUSSION

Previous studies demonstrated that HAdV-36 infects human skeletal muscle cells and results in viral gene expression in muscle cells [Wang et al., 2008, 2010]. HAdV-36 infection causes alterations in the gene expression of multiple enzymes and transcription factors [Cummings and Relman, 2000; Vangipuram et al., 2007; Wang et al., 2008, 2010]. Non-adipogenic human adenovirus 2 (HAdV-2) was not used as a negative control in this study, because an earlier study showed that only 76 genes (with significantly changed expression ratios at fold change ≥ 1.5) were identified in Ad-2 infected HeLa cells, suggesting HAdV-2 modulates a limited set of cellular gene expression [Zhao et al., 2003], and it also confirmed that Ad-2 did not alter cellular signaling pathways and gene expression in human mesenchymal stem cells [Wang et al., 2008, 2010; Na et al., 2012]. Another factor in our decision to not include HAdV-2 was the cost/efficiency consideration. This study showed that HAdV-36 profoundly affected gene expression in muscle cells; 1,004 genes met the criteria of fold change ≥ 1.5 with P value < 0.05 . It is not surprising that HAdV-36 induced significant changes in the expression of genes that mediate reprogramming of host cellular

signaling pathways. This reprogramming allows for virus multiplication, and also results in host cell dysfunction.

HAdV-36 infection also caused up-regulation of the immune response in muscle cells. For example, it significantly up-regulated human myxovirus (influenza virus) resistance 1 (MX1) gene. MX1 is an interferon - induced GTP-binding protein [Haller et al., 2007]. MxA, the product of the human MX1 gene, inhibits a step of the virus replication cycle that follows primary transcription [Pavlovic et al., 1992]. In mice the interferon-inducible MX protein is responsible for a specific antiviral state against influenza virus infection. MxA protein is localized in the cytoplasm, whereas Mx1 protein accumulates in the nucleus. The protein encoded by the MX1 gene is similar to the mouse protein as determined by its antigenic relatedness, induction conditions, physicochemical properties, and amino acid analysis [Pavlovic et al., 1992]. It was observed that HAdV-36 upregulated MX1 gene expression 44-fold, a finding that was confirmed by qPCR analysis. It was reported that mice carrying a wildtype MX1 gene (MX1^{+/+}) exhibit a greater resistance to infection with common laboratory strains of influenza A virus, when compared with standard laboratory mice (MX1^{-/-}). MX1 also protects mice against the pandemic human 1918 influenza virus and a highly lethal human H5N1 strain from Vietnam. Resistance to H5N1 of MX1^{+/+}, but not MX1^{-/-} mice was enhanced if the animals were treated with a single dose of exogenous alpha interferon before infection [Tumpey et al., 2007]. Thus, HAdV-36 induced robust upregulation of MX1 in human muscle cells, supporting the notion that interferon-induced resistance factor MX1 represents a key component of the murine innate immune system that mediates protection against epidemic and pandemic influenza viruses [Lai et al., 2008]. HAdV-36 significantly upregulated interferon-induced protein with tetratricopeptide repeats 2, 3 (IFIT2, IFIT3), and G1P2 gene expression (fold changes were 10.17, 10.16, and 10.09, respectively). IFIT2 and IFIT3 are members of the IFIT family proteins and contain 6 TPR repeats. These are induced by interferons and significantly upregulated in reprogramming the peripheral blood of children during the acute phase of influenza [Schmeisser et al., 2010]. In mice, IFIT2 protein has been shown to interact with microtubules in vitro, co-localize with beta-tubulin in vivo, and become enriched in the mitotic spindle of nonneuronal cells undergoing mitosis. IFIT2 was also found to have an inverse correlation between IFIT2 expression and cell migration [Schmeisser et al., 2010].

The effects of HAdV-36 on DNA replication and transcript regulation in muscle cells were observed. Ribonucleotide reductase M2 (RRM2) subunit is one of two subunits that constitute ribonucleotide reductase, the enzyme that catalyzes the conversion of ribonucleotide 5'-diphosphates into 2'-deoxyribonucleotides, which are required for DNA synthesis. It has been reported that RRM2 is associated with gastric cancer [Morikawa et al., 2010]. siRNA-mediated RRM2 knockdown inhibits significantly proliferation of melanoma cell lines with different oncogenic mutations, with synergistic enhancement when in combination with temozolomide [Zuckerman et al., 2011]. To date, there is no evidence indicating HAdV-36 infection can result in cancer. However, this study showed HAdV-36 significantly increased RRM2 gene expression. Thus, the relationship between HAdV-36 infection and cancer needs to be further investigated. Interestingly, most of the genes involved in transcriptional regulation were upregulated in HAdV-36 infected muscle cells (80.2% of 91 genes at fold change ≥ 2.5). A qPCR assay verified that HAdV-36 significantly upregulated selected genes such as E2F2, hairy and enhancer of split 4 (HES4), histone cluster 2, H2aa3 (HIST2H2AA3), chromatin licensing and DNA replication factor 1 (CDT1), and v-myb myeloblastosis viral oncogene homolog (MYB) genes. E2F family transcription activator binds DNA cooperatively with DP proteins through the E2 recognition site, 5'-TTTC[CG]CGC-3', found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. The Homo sapiens transcription factor Dp-1 (DRTF1)/E2F complex functions in the control of cell-

cycle progression from the G1 to S phase [Girling et al., 1993]. E2F-2 binds specifically to RB1 protein, in a cell-cycle dependent manner. For genomic integrity to be maintained, the cell cycle and DNA damage responses must be linked. CDT1 (upregulated over ninefold by HAdV-36), a G1-specific cell-cycle factor, is targeted for proteolysis by the Cul4-Ddb1(Cdt2) ubiquitin ligase following DNA damage [Roukos et al., 2011]. HAdV-36 significantly down regulated CTGF, IGFBP4, ITGA11, SERPINE1, THBS2 and steroid sensitive gene 1 (URB) in muscle cells, but increased gene expression of enolase2 (ENO2) and heat shock 70 kDa protein 2 (HSPA2).

Using microarray analysis, Na and coworkers [Na et al., 2012] recently reported that there was no difference in viral growth between HAdV-36 infection and Ad-2 infection in human mesenchymal stem cells (hMSCs) and demonstrated that HAdV-36 infection is associated more frequently with activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α , 8.33-fold), Uncoupling protein 1 (UCP1, 116.88-fold), SORBS1 and SH3 domain containing 1 (SORBS1, 19.58-fold), CCAAT/enhancer binding protein alpha (C/EBP α) and inflammatory pathways compared with Ad-2 infection [Bolstad et al., 2003; Na et al., 2012]. However, this study found PPAR γ (-1.78-fold, $P < 0.01$) and SORBS1 (-1.81, $P < 0.01$) were significantly downregulated, PGC-1 α (1.64-fold, $P < 0.05$) and C/EBP α (4.18-fold, $P < 0.001$) were significantly upregulated, but UCP 3 was only slightly upregulated (1.37-fold, $P \frac{1}{4}$ NS) in HAdV-36 infection in comparison with control hSKM cells. These differences between studies may be due to different cell types (hMSCs cells vs. hSKM cells) and various time points of HAdV-36 infection (1–2 days vs. 6 days) [Bolstad et al., 2003; Na et al., 2012]. On the other hand, it showed that 252 oncologic genes were regulated by HAdV-36. Thus, it may be worth-while to further investigate if there is a potential risk of developing cancer in HAdV-36 infection conditions.

Cross-talk between signal transduction pathways in HAdV-36 infected muscle cells is also a possibility. Based on the Ingenuity pathway analysis, it was observed that HAdV-36 altered NF κ B signaling which indirectly interacts with metallothionein 3 (MT3), mitogen-activated protein kinase kinase kinase 2 (MAP4K2), solute carrier family 2 (facilitated glucose transporter), member 6 (SLC2A6), pentraxin 3 (PTX3), and WD40 domain repeat protein 34 (WDR34) [Gao et al., 2009]. NF κ B also indirectly and directly upregulated expression of the target genes of the E2F family of transcription factors (E2F7 and E2F2). This in turn affected minichromosome maintenance signaling in the muscle cells [Wu et al., 2009; Kothandaraman et al., 2010] (Fig. 5). Recently, it was reported that MCM6 may become a target for identification of novel anticancer drugs [Zhang et al., 2010].

CONCLUSION

This study showed that HAdV-36 targets the genes involved in immune response, regulation of cell development, transcription, signaling transduction, as well as transport and protein metabolism. This viral infection upregulated the skeletal muscle cell antiviral defense response, and induced changes in the expression of genes that mediate the reprogramming of multiple cellular signaling pathways, allowing virus survival and multiplication as well as impairing host cell function. Since HAdV-36 infection altered many ontological genes, further investigation to evaluate the relationship between HAdV-36 infection and oncogenesis is warranted.

Acknowledgments

We thank Dr. Nikhil Dhurandhar for kindly providing adenovirus 36. We also thank Mr. Russell Tipton (Pennington Biomedical Research Center) for the help of preparing this manuscript.

Grant sponsor: COBRE center; Grant number: (NIH P20-RR021945); Grant sponsor: NORC center; Grant number: (NIH 1P30-DK072476).

Abbreviations

hSKM	human skeletal muscle
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
UCP1	Uncoupling protein 1

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Figure 1.

Correlation of microarray data between controls and between HAdV-36 infected cells and control cells as well as gene distribution in HAdV-36 infected muscle cells. Panel A shows correlation of detected genes between control cells, panel B is the correlation between HAdV-36 and control cells. Panel C shows gene distribution in HAdV-36 infected muscle cells. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jmv>]

Figure 2.
Biological processes of genes in HAdV-36 infected muscle cells.

Figure 3.

Heat map illustrated all altered genes in HAdV-36 infected muscle cells. Dark color stands for up-regulated genes and light one means down-regulated genes in HAdV-36 infected muscle cells.

Figure 4.

The correlation between q-PCR and microarray results in HAdV-36 infected cells. Thirty five genes were chosen to run qPCR for verifying the results of microarray. Correlation of qPCR and microarray shows there is closely correlation between qPCR and microarray assay in HAdV-36 infected muscle cells. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jmv>]

Figure 5.

The relationships between the cellular signal pathways in HAdV-36 infected human muscle cells. NFκB pathway, cross-talking between signaling, E2F and MCM pathways. -----> stands for indirect interaction, and ———> is direct interaction between signaling pathways. Beneath each gene, up number is fold change and bottom number is *P* value. Dark user input molecule that is upregulated (a positive [+]) expression value) and whose expression value meets the user defined cutoff. Light user input molecule that is downregulated (a negative [-] expression value) and whose expression value meets the user defined cut-off.

TABLE I

Sequence of Primers From Selected Gene List

#	Accession	Gene symbol	Forward	Reverse
1	NM_002462	MX1	GGCCAGCAAGCGCATCT	TGGAGCATGAAGAACTGGATGA
2	NM_001549	IFIT3	GGCCCCAACCTGGGATT	AAATGTACCTTAGAGCAACATGCAA
3	NM_001547	IFIT2	AATTGAGGTGGCAACATAGTTGA	CCCGTCGCTTCTAGCTATGTATC
4	NM_005101	G1P2	TCCTGCTGGTGGTGACAA	TTGTTATTCCTCACCAGGATGCT
5	NM_001034	RRM2	TCTGGCTTTCTTTGCAGCAA	GCTAAATCGTCCACCAAGTTT
6	NM_004091	E2F2	GCCTATGTGACTTACCAGGATATCC	CCTTGACGGCAATCACTGTCT
7	NM_021170	HES4	CTCAGCTCAAAACCCTCATCCT	CGCCTTCTCCAGCTTCGA
8	NM_003516	HIST2H2AA	CGCCAGGCAGGAGTTTCTC	TTGCCACGACCAGACATGAC
9	NM_030928	CDT1	GGTCAAGAGATCGAGACCATCCT	CCCGGCTAATTTTTTGTATTTTAGT
10	NM_005375	MYB	TCGCCAGCAAGGTGCAT	AGAGGTTCTTAACATTATCCAGAATGG
11	NM_021244	RRAGD	GGCACAGAGGAGGGAGTTCTG	GGATTCTCGGCTTCACTTCAGT
12	NM_004474.2	FOXD2	TCATCACCATGGCCATCCT	GAAGTTCGAGATCTCGCTCAA
13	NM_003713.3	PPAP2B	CCATGCCTCCTTCTCCATGT	AAGCGGGCCTGCAGGTATA
14	NM_012140.3	SLC25A10	GCTGGCATCCGCCTCAT	CGTAGCTGTTCCAGAAACACAAA
15	NM_001901	CTGF	TGCACCGCCAAAGATGGT	GAAGTTCGCTGCGGTACAC
16	NM_001975	ENO2	TTGCTCTTGTCACGTGTCT	CCCAGCACTATGCACAGTTCA
17	NM_021979	HSPA2	GGGACCCACCATCGAAGAA	GGCAAAGAGGTTTACGCTGACT
18	NM_001552	IGFBP4	TGGACTGAATGTGCCTAATGGA	CCCAGGAAGCCCCTCATC
19	NM_012211.3	ITGA11	AATATCTCGCAGTCAGCAACCT	ATGCTACCGTCTGAGTCTCCTT
20	NM_000602	SERPINE	CCGCCGCCTCTTCCA	GCCATCATGGGCACAGAGA
21	NM_003247	THBS2	CAATATCACACGCATCCGTCTCT	GCCACTCCCTTTGCAATTCTT
22	NM_199512	URB	CAGTGGTCAGGCGTGCAAT	ACGCCTAAAAGCTTCAGAATGG
23	NM_005281	GPR3	CCATTGCCTTCTTCATGGTGTT	CGATGCGGCAGATTTGG
24	NM_013355	PKN3	AAACCCCTCGGCTTCAG	GTATTTCCCTGTCCCCTTGAAGT
25	NM_014353	RAB26	TCGCACTAGCCCAAGCAATA	CGAAGCCGCTGCACAAG
26	NM_005012	ROR1	CAGCCTGGACCACGGAGAT	GTATTCCATGCCAGCTGCAA
27	NM_003881	WISP2	GAAGAGAAGGCACACAGAGATTCTG	GGCTTGATTTCAGGAACAATTTTACA
28	NM_003475	RASSF7	TCGCACTAGCCCAAGCAATA	CGAAGCCGCTGCACAAG
29	NM_080685	PTPN13	TGAGGATGTTATTTACGCTCGAGTT	ATGTGGGCACTGCAAGCA
30	NM_032512	PDZD4	TGGTGGACAGTGGCACTCA	CCCAGCGCCATGATATGC
31	NM_003954	MAP3K14	CGACACCCCAAGCTATTTCAA	GGATGTGCAGGTGTTCACCAT
32	NM_152331.1	ACOT4	GCCGTATGCTACATGCTTCAAC	GAAATGCCCAAAAGCCCAAT
33	NM_002332	LRP1	GGATTGACGCCAGGTCAGA	CCCGAAGCACCTCCATGT
34	NM_001146.3	ANGPT1	CAAGGTCTTGACCGTGAATCTG	GAGCACTGTCACCCCAAGTAGAG
35	NM_138440	SLITL2	CACTCGCCAACCTCAGCAA	CGGAAGGTCTCATTGGTGATTT

TABLE II

Summary of Microarray Analysis in HAdV-36 Infected Muscle Cells

Top networks		
Associated network functions		Score
Cardiovascular system development and function, organismal development, cancer		46
Gene expression, cellular growth and proliferation, RNA post-transcriptional modification		42
DNA replication, recombination, and repair, gene expression, carbohydrate metabolism		38
Cell cycle, cellular assembly and organization, cancer		36
Cellular development, infection mechanism, cancer		35
Diseases and disorders		
Name	<i>P</i> value	Number of molecules
Cancer	5.00E−14 to 2.88E−02	252
Gastrointestinal disease	5.00E−14 to 2.62E−02	124
Genetic disorder	5.40E−11 to 2.88E−02	161
Dermatological diseases and conditions	4.48E−06 to 2.51E−02	96
Reproductive system disease	9.30E−05 to 2.88E−02	138
Molecular and cellular functions		
Name	<i>P</i> value	Number of molecules
Cell cycle	4.14E−10 to 2.88E−02	127
DNA replication, recombination, and repair	7.05E−07 to 1.98E−02	94
Cell death	1.35E−07 to 2.88E−02	185
Cellular growth and proliferation	1.57E−06 to 2.88E−02	223
Cellular movement	6.96E−05 to 2.35E−02	103
Physiological system development and function		
Name	<i>P</i> value	Number of molecules
Connective tissue development and function	6.03E−05 to 2.88E−02	79
Skeletal and muscular system development and function	1.09E−03 to 2.88E−02	46
Cardiovascular system development and function	1.61E−03 to 2.77E−02	58
Embryonic development	2.18E−03 to 1.98E−02	21
Endocrine system development and function	2.18E−03 to 2.18E−03	2
Top canonical pathways		
Name	<i>P</i> value	Ratio
Cell cycle control of chromosomal replication	2.19E−06	9/31 (0.29)
Role of CHK protein in cell cycle checkpoint control	1.06E−04	8/35 (0.229)
Mismatch repair in Eukaryotes	6.21E−04	5/24 (0.208)

Cyclins and cell cycle regulation	2.71E-03	10/89 (0.112)
Cell cycle: G1/S checkpoint regulation	2.99E-03	8/61 (0.131)

TABLE III

HAdV-36 Regulated Top 100 Gene List (Down or Upregulated)

Accession	Symbol	Fold change	Gene name
Carbohydrate and lipid metabolisms			
NM_003713	PPAP2B	-3.89	Homo sapiens phosphatidic acid phosphatase type 2B (PPAP2B), transcript variant 1, mRNA
NM_198207	LASS1	5.27	Homo sapiens LAG1 longevity assurance homolog 1 (<i>S. cerevisiae</i> ; LASS1), transcript variant 2, mRNA
NM_001975	ENO2	7.70	Homo sapiens enolase 2 (gamma, neuronal; ENO2), mRNA
NM_182908	DHRS2	7.06	Homo sapiens dehydrogenase/reductase (SDR family) member 2 (DHRS2), transcript variant 1, mRNA
Development			
NM_004585	RARRES3	8.21	Homo sapiens retinoic acid receptor responder (tazarotene induced) 3 (RARRES3), mRNA
NM_004335	BST2	6.93	Homo sapiens bone marrow stromal cell antigen 2 (BST2), mRNA
NM_021147	UNG2	5.59	Homo sapiens uracil-DNA glycosylase 2 (UNG2), transcript variant 1, mRNA
NM_080668	CDCA5	5.54	Homo sapiens cell division cycle associated 5 (CDCA5), mRNA
NM_021979	HSPA2	5.46	Homo sapiens heat shock 70kDa protein 2 (HSPA2), mRNA
NM_000138	FBN1	-3.70	Homo sapiens fibrillin 1 (Marfan syndrome; FBN1), mRNA
NM_002581	PAPPA	-3.84	Homo sapiens pregnancy-associated plasma protein A, pappalysin 1 (PAPPA), mRNA
NM_003277	CLDN5	-3.93	Homo sapiens claudin 5 (transmembrane protein deleted in velocardiofacial syndrome; CLDN5), mRNA
NM_003062	SLIT3	-4.30	Homo sapiens slit homolog 3 (<i>Drosophila</i> ; SLIT3), mRNA
NM_019035	PCDH18	-4.58	Homo sapiens protocadherin 18 (PCDH18), mRNA
NM_003247	THBS2	-5.36	Homo sapiens thrombospondin 2 (THBS2), mRNA
NM_000602	SERPINE1	-5.42	Homo sapiens serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
NM_080645	COL12A1	-5.76	Homo sapiens collagen, type XII, alpha 1 (COL12A1), transcript variant short, mRNA
NM_003246	THBS1	-5.89	Homo sapiens thrombospondin 1 (THBS1), mRNA
NM_007281	SCRG1	-6.55	Homo sapiens scrapie responsive protein 1 (SCRG1), mRNA
NM_012211	ITGA11	-9.13	Homo sapiens integrin, alpha 11 (ITGA11), transcript variant 2, mRNA
NM_001901	CTGF	-9.37	Homo sapiens connective tissue growth factor (CTGF), mRNA
NM_080629	COL11A1	-9.81	Homo sapiens collagen, type XI, alpha 1 (COL11A1), transcript variant B, mRNA
NM_003013	SFRP2	-10.61	Homo sapiens secreted frizzled-related protein 2 (SFRP2), mRNA
NM_013372	GREM1	-12.21	Homo sapiens gremlin 1, cysteine knot superfamily, homolog (<i>Xenopus laevis</i>) (GREM1), mRNA
Genes with unknown process			
NM_138440	SLITL2	-5.83	Homo sapiens slit-like 2 (<i>Drosophila</i>) (SLITL2), mRNA
NM_006207	PDGFRL	-5.29	Homo sapiens platelet-derived growth factor receptor-like (PDGFRL), mRNA
NM_031479	INHBE	-4.93	Homo sapiens inhibin, beta E (INHBE), mRNA
NM_031455	CCDC3	-4.82	Homo sapiens coiled-coil domain containing 3 (CCDC3), mRNA
NM_024866	ADM2	-4.32	Homo sapiens adrenomedullin 2 (ADM2), mRNA
NM_181724	TMEM119	-4.00	Homo sapiens transmembrane protein 119 (TMEM119), mRNA
NM_194293	CMYA1	-3.64	Homo sapiens cardiomyopathy associated 1 (CMYA1), mRNA
NM_014109	ATAD2	5.32	Homo sapiens ATP ase family, AAA domain containing 2 (ATAD2), mRNA
NM_016448	DTL	5.56	Homo sapiens denticleless homolog (<i>Drosophila</i> ; DTL), mRNA

Accession	Symbol	Fold change	Gene name
Immune response			
NM_001710	CFB	5.50	Homo sapiens complement factor B (CFB), mRNA
NM_006417	IFI44	6.85	Homo sapiens interferon-induced protein 44 (IFI44), mRNA
NM_002038	G1P3	8.07	Homo sapiens interferon, alpha-inducible protein (clone IFI-6-16; G1P3), transcript variant 1, mRNA
NM_001549	IFIT3	10.04	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA
NM_001547	IFIT2	10.09	Homo sapiens interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA
NM_005101	G1P2	10.43	Homo sapiens interferon, alpha-inducible protein (clone IFI-15K; G1P2), mRNA
NM_003733	OASL	10.70	Homo sapiens 2'-5'-oligoadenylate synthetase-like (OASL), transcript variant 1, mRNA
NM_005532	IFI27	10.90	Homo sapiens interferon, alpha-inducible protein 27 (IFI27), mRNA
NM_002462	MX1	43.46	Homo sapiens myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse; MX)
Protein metabolism			
NM_014905	GLS	-4.70	Homo sapiens glutaminase (GLS), mRNA
NM_052947	HAK	-3.66	Homo sapiens heart alpha-kinase (HAK), mRNA
NM_080685	PTPN13	-3.55	Homo sapiens protein tyrosine phosphatase, nonreceptor type 13 (APO-1/CD95(PTPN13))
NM_001013005	HERC6	7.75	Homo sapiens hect domain and RLD 6 (HERC6), transcript variant 4, mRNA
NM_024625	ZC3HAV1	7.77	Homo sapiens zinc finger CCCH-type, antiviral 1 (ZC3HAV1), transcript variant 2, mRNA
NM_003954	MAP3K14	16.85	Homo sapiens mitogen-activated protein kinase kinase kinase 14 (MAP3K14), mRNA
NM_016323	HERC5	21.66	Homo sapiens hect domain and RLD 5 (HERC5), mRNA
Other process or metabolism			
NM_018689	KIAA 1199	-11.71	Homo sapiens KIAA 1199 (KIAA 1199), mRNA
NM_004105	EFEMP1	-5.25	Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), transcript variant 1
Signal transduction			
NM_012242	DKK1	-5.35	Homo sapiens dickkopf homolo 1 (Xenopus laevis; DKK1), mRNA
NM_016651	DACT1	-5.06	Homo sapiens dapper, antagonist of beta -catenin, homolog 1 (Xenopus laevis; DACT1), mRNA
NM_020311	CMKOR1	-4.89	Homo sapiens chemokine orphan receptor 1 (CMKOR1), mRNA
NM_002133	HMOX1	-4.72	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA
NM_006209	ENPP2	-4.41	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin; ENPP2), mRNA
NM_024767	DLC1	-4.25	Homo sapiens deleted in liver cancer 1 (DLC1), transcript variant 3, mRNA
NM_003714	STC2	-3.87	Homo sapiens stanniocalcin 2 (STC2), mRNA
NM_003881	WISP2	-3.66	Homo sapiens WNT1 in ducible signaling pathway protein 2 (WISP2), mRNA
NM_001033047	NPNT	-3.62	Homo sapiens nephronectin (NPNT), mRNA
NM_005458	GABBR2	5.62	Homo sapiens gamma-aminobutyric acid (GABA) B receptor, 2 (GABBR2), mRNA
NM_014736	KIAA0101	5.99	Homo sapiens KIAA0101 (KIAA0101), transcript variant 1, mRNA
XM_938838	CNIH2	6.28	PREDICTED: Homo sapiens cornichon homolog 2 (Drosophila; CNIH2), mRNA
NM_002220	ITPKA	7.34	Homo sapiens inositol 1,4,5-trisphosphate 3-kinase A (ITPKA), mRNA
Transcriptional regulation			
NM_181353	ID1	-5.42	Homo sapiens inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1), transcript variant 2
NM_021158	TRIB3	-5.09	Homo sapiens tribbles homolog 3 (Drosophila; TRIB3), mRNA

Accession	Symbol	Fold change	Gene name
NM_004089	TSC22D3	-4.70	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA
NM_022658	HOXC8	-4.42	Homo sapiens homeobox C8 (HOXC8), mRNA
NM_004083	DDIT3	-4.20	Homo sapiens DNA-damage-inducible transcript 3 (DDIT3), mRNA
NM_006397	RNA SEH2A	5.41	Homo sapiens ribonuclease H2, large subunit (RNA SEH2A), mRNA
NM_021030	ZNF14	5.54	Homo sapiens zinc finger protein 14 (KOX 6; ZNF14), mRNA
NM_003504	CDC45L	5.72	Homo sapiens CDC45 cell division cycle 45-like (S. cerevisiae; CDC45L), mRNA
NM_006479	RAD51AP1	5.82	Homo sapiens RAD51 associated protein 1 (RAD51AP1), mRNA.
NM_005189	CBX2	6.04	Homo sapiens chromobox homolog 2 (Pc class homolog, Drosophila) (CBX2), transcript variant 1, mRNA
NM_021058	HIST1H2BJ	6.09	Homo sapiens histone 1, H2bj (HIST1H2BJ), mRNA
NM_004091	E2F2	6.19	Homo sapiens E2F transcription factor 2 (E2F2), mRNA.
NM_018154	ASF1B	7.39	Homo sapiens ASF1 anti-silencing function 1 homolog B (S. cerevisiae; ASF1B), mRNA
NM_003517	HIST2H2AC	7.92	Homo sapiens histone 2, H2ac (HIST2H2AC), mRNA
NM_005441	CHAF1B	8.01	Homo sapiens chromatin assembly factor 1, subunit B (p 60; CHAF1B), mRNA
NM_021170	HES4	8.28	Homo sapiens hairy and enhancer of split 4 (Drosophila; HES4), mRNA
NM_005319	HIST1H1C	8.63	Homo sapiens histone 1, H1c (HIST1H1C), mRNA
NM_003516	HIST2H2AA	8.69	Homo sapiens histone 2, H2aa (HIST2H2AA), mRNA
NM_005375	MYB	9.00	Homo sapiens v-myb myeloblastosis viral oncogene homolog (avian; MYB), mRNA
NM_030928	CDT1	9.49	Homo sapiens DNA replication factor (CDT1), mRNA
NM_016817	OAS2	10.30	Homo sapiens 2'-5'-oligo adenylate synthetase 2, 69/71kDa (OAS2), transcript variant 1, mRNA
NM_001034	RRM2	12.44	Homo sapiens ribonucleotide reductase M2 polypeptid (RRM2), mRNA
NM_004789	LHX2	27.55	Homo sapiens LIM homeobox 2 (LHX2), mRNA
Transportation			
NM_001856	COL16A1	-4.37	Homo sapiens collagen, type XVI, alpha 1 (COL16A1), mRNA
NM_018477	ACTR10	-4.22	Homo sapiens actin-related protein 10 homolog (S. cerevisiae) (ACTR10), mRNA
NM_000727	CACNG1	-4.13	Homo sapiens calcium channel, voltage-dependent, gamma subunit 1 (CACNG1), mRNA
NM_006934	SLC6A9	-3.96	Homo sapiens solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A 9)
NM_020351	COL8A1	-3.96	Homo sapiens collagen, type VIII, alpha 1 (COL8A1), transcript variant 2, mRNA
NM_021244	RRAGD	6.65	Homo sapiens Ras-related GTP binding D (RRA GD), mRNA
NM_000558	HBA1	7.07	Homo sapiens hemoglobin, alpha 1 (HBA 1), mRNA
NM_006688	C1QL1	9.36	Homo sapiens complement component 1, q subcomponent-like 1 (C1QL1), mRNA
Unknown genes			
NM_199511	URB	-3.77	Homo sapiens steroid sensitive gene 1 (URB), transcript variant 1, mRNA

TABLE IV
qPCR Assay Verified Gene Alterations of Microarray in Human Skeletal Muscle Cells

Pathway and gene		Accession	Microarray		qPCR Mean $\pm$ SEM	HADV-36 vs. control P value
#	Immune response		Fold change			
1	MX1	NM_002462	44.35		44.4 $\pm$ 3.94	0.001
2	IFT3	NM_001547	10.17		6.81 $\pm$ 0.21	0.001
3	IFT2	NM_001549	10.16		17.92 $\pm$ 0.77	0.001
4	GIP2	NM_005101	10.09		13.39 $\pm$ 0.08	0.001
	DNA replication					
5	RRM2	NM_001034	12.98		9.27 $\pm$ 0.15	0.001
	Transcriptional regulation					
6	E2F2	NM_004091	6.19		5.36 $\pm$ 0.20	0.001
7	HES4	NM_021170	8.28		7.17 $\pm$ 0.16	0.001
8	HIST2H2AA3	NM_003516	8.69		4.73 $\pm$ 0.11	0.01
9	CDT1	NM_030928	9.68		9.48 $\pm$ 0.29	0.001
10	MYB	NM_005375	9.55		4.6 $\pm$ 0.05	0.001
	Transportation					
11	RRAGD	NM_021244	6.65		14.1 $\pm$ 0.20	0.001
12	FOXO2	NM_004474.2	3.67		4.5 $\pm$ 0.04	0.001
13	PPAP2B	NM_003713.3	-3.87		-2.51 $\pm$ 0.04	0.01
14	SLC25A10	NM_012140.3	4.93		5.82 $\pm$ 0.13	0.001
	Development					
15	CTGF	NM_001901	-9.29		-3.81 $\pm$ 0.11	
16	ENO2	NM_001975	7.7		9.31 $\pm$ 0.26	0.001
17	HSPA2	NM_021979	5.46		7.58 $\pm$ 0.38	0.001
18	IGFBP4	NM_001552	-2.54		1.2 $\pm$ 0.03	NS
19	ITGA11	NM_012211	-9.74		-2.61 $\pm$ 0.01	0.01
20	SERPINE1	NM_000602	-5.35		-2.24 $\pm$ 0.02	0.05
21	THBS2	NM_003247	-5.36		-2.07 $\pm$ 0.04	0.05
22	URB	NM_199511	-3.73		-2.43 $\pm$ 0.03	0.01
	Signal transduction					

#	Pathway and gene	Accession	Microarray		qPCR		HAdV-36 vs. control
			Fold change	Mean $\pm$ SEM	Mean $\pm$ SEM	P value	
23	GPR3	NM_005281	3.58	5.31 $\pm$ 0.34		0.001	
24	PKN3	NM_013355	3.93	7.56 $\pm$.063		0.001	
25	RAB26	NM_014353	3.15	3.25 $\pm$ 0.13		0.01	
26	ROR1	NM_005012	-3.21	-3.29 $\pm$ 0.02		0.01	
27	WISP2	NM_003881	-3.66	-3.4 $\pm$ 0.04		0.001	
28	RASSF7	NM_003475	3.48	3.25 $\pm$ 0.07		0.01	
	Protein metabolism						
29	PTPN13	NM_080685	-3.55	-1.16 $\pm$ 0.09		NS	
30	PDZD4	NM_032512	2.63	1.53 $\pm$ 0.11		0.05	
31	MAP3K14	NM_003954	16.85	5.62 $\pm$ 0.23		0.001	
	Lipid metabolism						
32	ACOT4	NM_152331	3.33	9.56 $\pm$ 0.16		0.001	
33	LRP1	NM_002332	-2.51	-1.6 $\pm$ 0.20		NS	
	Angiogenesis						
34	ANGPT1	NM_001146	-2.83	-4.51 $\pm$ 0.06		0.001	
	Cell migration						
35	SLITL2	NM_138440	-5.93	-3.1 $\pm$ 0.03		0.001	