RNA-seq Reveals Novel Transcriptome of Genes and Their Isoforms in Human Pulmonary Microvascular Endothelial Cells Treated with Thrombin

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Abstract

The dysregulation of vascular endothelial cells by thrombin has been implicated in the development of a number of pathologic disorders such as inflammatory conditions, cancer, diabetes, coronary heart disease. However, transcriptional regulation of vascular endothelial cells by thrombin is not completely understood. In the present study, Illumina RNA-seq was used to profile the transcriptome in human pulmonary microvascular endothelial cells (HMVEC-L) treated with thrombin for 6 h to gain insight into thrombin’s direct effects on the endothelial function. Out of 100 million total reads from a paired end sequencing assay, 91–94% of the reads were aligned to over 16,000 genes in the reference human genome. Thrombin upregulated 150 known genes and 480 known isoforms, and downregulated 2,190 known genes and 3,574 known isoforms by at least 2 fold. Of note, thrombin upregulated 1,775 previously unknown isoforms and downregulated 12,202 previously unknown isoforms by at least 2 fold. Many genes displayed isoform specific differential expression levels and different usage of transcriptional start sites after the thrombin treatment. The cross comparisons between our RNA-seq data and those of DNA microarray analysis of either 6 h thrombin treated HUVEC or 5 h TNFα treated HMVEC have provided a significant overlapping list of differentially expressed genes, supporting the robust utility of our dataset. Further in-depth follow-up analysis of the transcriptional regulation reported in this study may shed light on molecular pathogenic mechanisms underlying thrombin mediated endothelial dysfunction in various diseases and provide new leads of potential therapeutic targets.

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Introduction

Endothelial cells lining the inner surface of microvessels form a semipermeable barrier that actively participates in blood–tissue exchange of plasma fluid, proteins and cells. The precise regulation of endothelial barrier and function is essential for maintaining circulatory homeostasis and the physiological function of different organs. The dysregulation of microvascular barrier and function represent crucial events in the development of a variety of disease processes, such as adult respiratory distress syndrome, ischemia–reperfusion injury, diabetic vascular complications, and tumor metastasis [1].

It is now understood that while increased circulating levels of soluble mediators such as thrombin, pro-inflammatory cytokines such as TNFα, and immune cells such as neutrophils are a hallmark of host defense against injury, most of these mediators target the vascular endothelium to produce vasoactive and cytotoxic effects [1]. Thrombin is a serine protease. Besides its central role in the coagulation cascade acting as both a coagulant factor and an anticoagulant factor, thrombin can trigger important cellular effects via a protease-activated receptor pathway or receptor independent pathways [2]. Thrombin can stimulate endothelial cells and regulate the expression, release and activation of a number of biological mediators. Thrombin is known for its endothelial hyperpermeability effect. Thrombin can protect endothelial cells from apoptosis [3]. Thrombin plays an important role in angiogenesis and is thus involved in tumor progression and metastasis [4]. The list of physiological and pathological roles of thrombin is still expanding. The understanding of these events at a molecular level will provide new targets of therapeutic interventions in disease states.

With the advent of next generation DNA sequencing technologies [5], such as RNA-seq, a more comprehensive and accurate transcriptome analysis has become feasible and affordable. RNA-seq first sequences complementary DNA (cDNA) in short fragments and subsequent mapping of such short sequence fragments (reads) onto the reference genome. RNA-Seq enables identification of transcription initiation sites (TSSs) and new
splicing variants, and it permits a precise quantitative determination of exon and splicing isoform expression [6]. In the present study, we performed a comprehensive transcriptome analysis in human pulmonary microvascular endothelial cells (HMVEC-L) treated with or without thrombin using the Illumina RNA-Seq technique. We found significant differences in gene isoform expression levels, alternated use of promoters and transcription start sites between control and thrombin treated HMVEC-L. These data provided broader and deeper insights into thrombin’s transcriptional regulation on the vascular endothelial functions and it also provides rich reagents for further experimentations.

Results

Quality analysis of RNA-Seq data

Real-time analysis of the sequencing run, including several measurements, was performed by the Illumina HiSeq Control Software. Clusters of identical sequences were generated on the Illumina cBot and the number of those clusters was reported, along with the percentage of those clusters passing an internal quality filter. Across the 5 samples, between 599,000 and 621,000 raw clusters were detected, with a median of 603,000 clusters per lane. Between 89.5% and 92.1% of those clusters passed the filter, with a median of 91.3% of the clusters passing the filter. Each lane was aligned in real-time with the PhiX genome and between 0.6% and 0.7% of the clusters aligned. Our control lane of PhiX produced 357,000 clusters with 96.9% passing the filter and 99.1% aligning to the PhiX genome. All these values were within the recommended limits established by Illumina.

Post-run quality analysis of RNA-Seq Data was carried out as described by Twine et al. [7]. The total number of reads produced from each sample was between 97,478,180 and 105,351,222, with a median across all samples of 102,568,132 (Table 1). The difference in the number of reads between the control samples and the thrombin samples was not statistically significant (Student’s t-test, p = 0.88). To assess the quality of the reads, data was pulled from the TopHat log files as well as the output files. Between 0.31% and 0.34% of the reads were removed due to low quality before mapping to the reference genome began. Between 88.4% and 94.2% of the total reads mapped to the human genome. To ensure the uniform coverage across the genome, the data was visualized using a local copy of the Integrative Genomics Viewer. We mapped the control A reads against chromosome 1 (Figure 1). The average alignment was computed across the genome and those alignment scores were log-transformed (base 2) to better visualize the full range of the data. As expected, no reads mapped to the centromere or areas of the chromosome without genes.

Differentially expressed genes

After mapping the sequencing reads to the reference genome with TopHat, transcripts were assembled and their relative expression levels were calculated with Cufflinks in FPKM. Then, the sub-program, Cuffdiff was used to calculate the differential expression on both a gene level and a transcript level, as well as the calculation of alternative promoter usage and alternative splicing. Cufflinks calculates the differential gene expression with the ratio of the thrombin treated group to the control group for every gene and transcript along with the statistical significance of the values. Overall, there are 16,636 expressed genes in the control cells and 16,357 expressed genes in thrombin treated genes, which were aligned to the reference genome (Table 2). There are 783 genes, whose expressions were only detected in the control cells, while 504 genes whose expressions were only detected in thrombin treated cells. The differentially expressed genes in thrombin treated cells over the controls with statistically significant fold changes ranged from \(-16.32\) to \(94.23\). There are 152 upregu-

![Figure 1. A transcription profile of HMVEC cells (Control A) for chromosome 1. The RNASeq read density plotted along chromosome 1 is shown. Average alignment was computed by igvtools. The coverage values were measured along intervals of the genome. These intervals vary in size depending on how variable the read density is for a particular genomic location. Each bar represents the log, frequency of reads plotted against chromosome coordinates. doi:10.1371/journal.pone.0031229.g001](http://www.plosone.org/)

| Table 1. RNA-Seq sequence reads mapping to UCSC Human genome build 19 by TopHat v1.3.0/Bowtie v0.12.7. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 | **Control**                     | **Thrombin**                   |                                 | **Control**                     | **Thrombin**                   |                                 |
| **A**                          | **B**                           | **Average**                    | **A**                          | **A2**                          | **B**                           | **Average**                    |
| Total reads                    | 102,399,460                     | 102,568,132                    | 102,483,796                    | 105,351,222                     | 103,170,324                     | 97,478,180                     | 101,999,909                     |
| Reads removed                  | 0.32%                           | 0.31%                          | 0.31%                          | 0.33%                           | 0.32%                           | 0.34%                          | 0.33%                           |
| Read aligned to reference genome | 94.2%                           | 93.4%                          | 93.5%                          | 93.5%                           | 92.4%                           | 88.4%                          | 91.5%                           |

Total reads and the percentage of those reads removed due to low quality and aligned to hg19 by TopHat. TopHat allows two mismatches when aligning to a reference genome. doi:10.1371/journal.pone.0031229.t001
controls with statistically significant fold changes ranged from expressed novel isoforms in thrombin treated cells over the only detected in thrombin treated cells. The differentially control cells, while 424 novel isoforms whose expressions were expressed novel isoforms, whose expressions were only detected in the transcript database, there are 25,880 in the control cells and annotations are not known in the current reference gene or over the control cells (Table 2). For novel isoforms whose known isoforms in thrombin treated cells by at least 2 fold change. The fold change among top 50 up- and down- regulated genes in thrombin treated HMVEC cells are presented in Table S1, about 10% of them are not annotated.

Table 2. Gene/isoform expression summary.

| Genes | Control | Thrombin |
|-------|---------|----------|
| Total Genes Expressed | 16,636 | 16,357 |
| Control Only | 783 | 504 |
| Thrombin Only | 152 | 2,190 |

| Known Isoforms | Control | Thrombin |
|----------------|---------|----------|
| Total Known Isoforms Expressed | 26,807 | 26,300 |
| Control Only | 1,492 | 985 |
| Thrombin Only | 480 | 3,574 |

| Novel Isoforms | Control | Thrombin |
|----------------|---------|----------|
| Total Novel Isoforms Expressed | 25,880 | 25,886 |
| Control Only | 418 | 424 |
| Thrombin Only | 1,775 | 12,202 |

Genes, known isoforms and novel isoforms expressed in control and thrombin-treated HMVEC cells. Expression determined by CuffDiff after Benjamini-Hochberg correction. The fold change is the ratio of thrombin FPKM to control FPKM. doi:10.1371/journal.pone.0031229.t002

The fold change among top 50 up- and down-regulated known isoforms in thrombin treated HMVEC cells are presented in the supplement (Table S2). The fold change among top 50 up- regulated known isoforms in thrombin treated HMVEC cells varies from 12 to 963. The fold change among top 50 down-regulated known isoforms in thrombin treated HMVEC cells varies from −24 to −1455. Consistent with the finding that PCDHA1 and PCDHA11 are the top regulated genes (Table 1), both genes are among the top regulated known isoforms in thrombin treated HMVEC cells (Figure 3 and Table S2). Another two known isoforms of the protocadherin gene family [protocadherin gamma gene B6 (PCDHB6) and PCDHA3] were also upregulated by 82 and 22 fold, respectively. One of the hemochromatosis (HFE) gene isoforms was the highest upregulated isoform (983 fold) by thrombin. HFE is known to be involved in iron metabolism. It may also be involved in a tissue injury and reparative process [8]. Phospholipase C-beta 4 (PLCB4) was the highest down regulated isoform (−1455 fold) by thrombin. Phospholipase C-beta hydrolyses phosphatidylinositol 4,5-bisphosphate and generates inositol 1,4,5-trisphosphate in response to activation of various G protein-coupled receptors. PLCB4 was implicated in the delayed [Ca2+]i increase in response to the stimulation by thrombin [9]. Among the top ten up- and down-regulated known gene isoforms by thrombin are three presumable transcription factors (runt-related transcription factor 1, forkhead box P2, zinc finger and BTD domain containing 20) and two ion channel gene isoforms (sodium channel and voltage-gated type IV beta, and sodium/calcium exchanger member 3). Two different isoforms of the gene ZBTB20 are listed among the top ten down-regulated known gene isoforms by thrombin. They differ in the region of their untranslated first exons.

The fold change among top 50 up- and down-regulated novel isoforms in thrombin treated HMVEC cells varies from −44 to −928. Integrin alpha FG-GA repeat containing 2 (ITGF2), a novel isoform of integrin alpha, was the highest upregulated novel isoform (431 fold) by thrombin. Integrins are heterodimeric membrane receptors consisting of α and β subunits, that play a pivotal role to
functionally integrate the extracellular matrix with the cytoskeleton across the plasma membrane [10]. Three isoforms of Zinc finger protein 518A were among the top one, three and five highest down regulated novel isoforms by thrombin (−928, 738, and 262 fold) respectively. Two upregulated novel isoforms come from two cytoskeleton proteins: non-erythrocytic 2 spectrin beta (SPTBN2) and fibrillin 2 (FBN2). More than one fourth of top 10 up- and down- regulated novel isoforms in thrombin treated HMVEC cells (6/20) are from unannotated genes (Table 3). Three different novel isoforms of the gene ZNF518A are listed among top ten downregulated unknown gene isoforms by thrombin. All these three novel isoforms are longer than any known isoforms of the gene ZNF518A.

Network and pathway analysis of differentially expressed genes

The differentially expressed gene lists of a 2-fold or greater change after the thrombin treatment were submitted to Ingenuity Pathway Analysis (IPA) v9.0-3211 (Ingenuity Systems, Inc., Redwood City, CA). Networks of Network Eligible Molecules, as described in the Method section, were then algorithmically generated based on their connectivity. Networks affected by upregulated genes/isolforms after thrombin treatment of HMVEC cells are listed in Table 6. Networks affected by downregulated genes/isolforms after thrombin treatment of HMVEC cells are listed in Table 7. Notably, inflammatory response is among the top networks of both upregulated genes and isoforms by thrombin (Table 4). Some of genes involved in anti-inflammatory response were downregulated by thrombin (Table 5). This could provide a molecular insight into the known notion that thrombin plays a significant role in inflammatory diseases [11]. Other top affected networks are Cell-To-Cell Signaling and Interaction, Tissue Morphology, Cellular Movement (Table 4 and 5). These observations are consistent with known functions of thrombin that can cause dissociation of cell-cell junctions between endothelial cells as well as cytoskeleton contraction, leading to a widened intercellular space that facilitates transendothelial flux [1].

Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Genes with a 2-fold or greater change in expression after thrombin treatment and that were associated with a canonical pathway in Ingenuity’s Knowledge Base were considered for the analysis. Top canonical pathways affected by upregulated genes/isolforms after thrombin treatment of HMVEC cells are listed in Table 6. Top canonical pathways affected by downregulated genes/isolforms after thrombin treatment of HMVEC cells are listed in Table 7. In concordance with the above observation that top affected networks by thrombin is inflammatory response, nearly all top canonical pathways affected by upregulated genes/isolforms after thrombin treatment are related to inflammatory response. IL17A is a proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide (NO). High levels of this cytokine are associated with several chronic inflammatory diseases including rheumatoid arthritis, psoriasis and multiple sclerosis [12]. TREM-1 is a

Figure 2. Top 10 up-/down-regulated genes in thrombin treated HMVEC. The differentially expressed genes in thrombin treated HMVEC vs those in control cells were determined by CuffDiff, after Benjamini-Hochberg correction. The fold change is the ratio of FPKM of those genes in thrombin treated HMVEC to FPKM of those genes in control cells. The differentially expressed genes were ranked on their fold change and the 10 with the highest or lowest fold changes are shown here. The full name of each gene symbol is provided in text and Table S1.

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positive regulator of inflammatory responses [13]. CD40 and CD40L are considered as neo-inflammatory molecules in vascular diseases [14]. In addition, Enhanced triggering receptor expressed on myeloid cells 1 (TREM1) Signaling may provide a new signal transduction pathway on the effect of Thrombin stimulated calcium influx into the cells since a novel TREM1 member, TREM-like transcript-1, can enhance calcium signaling via SHP-2 [15]. DNA Double-Strand Break Repair and BRCA1 in DNA Damage Response are top canonical pathways affected by down-regulated genes/isoforms after thrombin treatment of HMVEC cells (Table 7). Dysfunction of DNA repair pathways is among the major causes of tumorogenesis [16]. Mutated BRCA1 or 2, essential components of a repair pathway for repairing DNA double-strand breaks, underlie the pathogenesis of breast or ovarian cancers [16]. Thrombin is recognized as a potent mitogen in cancer and tumor metastasis [17]. The observation provided in Table 7 may shed a new light into the molecular mechanisms underlying thrombin in the tumor pathogenesis.

We also examined differentially expressed genes in traditional thrombin signaling pathway (Figure 4 and Table S4). Notably, 6 h thrombin treatment significantly upregulated thrombin receptor Par4 and down-regulated thrombin receptor Par 3 while there was no change in the expression of thrombin receptor Par 1. Expression of NFkB1, NF κB2 and Src was also significantly upregulated. Some of Rho family genes (Rho B, C, F and G) were upregulated while others (Rho J, Q, T1, U and V) were down regulated (Table S4). Myosin light chain gene 9 (MYL9) was upregulated while MYL12B was downregulated. Expression of other genes was either down-regulated or not affected.

### Detailed analysis of the differentially regulated protocadherin gene by Thrombin

Protocadherin gene is of particular interest due to its multiple isoforms, which varied from upregulation to downregulation after thrombin treatment of HMVEC cells. The mapping of reads for all protocadherin alpha isoforms except PCDHA2 and PCDHA5, which are expressed in control cells but not thrombin-treated cells, to the reference genome shows differences in averaged expression levels for protocadherin alpha isoform exons between normal controls and thrombin-treated group (Fig. 5A). A histogram shows the fold changes between thrombin-treated HMVEC cells and control cells, as calculated by CuffDiff (Fig. 5B). Different protocadherin alpha isoforms from PCDHA1 to PCDHA13 except PCDHA2 and PCDHA5 responded to the thrombin treatment differently from the downregulation by ~6.46 folds (PCDHA12) to the upregulation by 94.23 fold (PCDHA1). This result suggests that one PCDH isoform could function differently from another isoform. Gene isoforms are often produced by alternative splicing. The alternative spliced forms of PCDHA1, 11, 3, 10, 12, 6 were among the top ten up- and down- regulated genes showing alternative splicing in thrombin treated HMVEC cells (Table S5). Four protocadherin alpha family members, protocadherin alpha 10, 3, 11 and 1, were among the top 50 up- and down- regulated genes with alternative promoter usage in thrombin treated HMVEC cells (Table S6). Focusing on Protocadherin gene expression in the...
Validation of three differentially regulated genes by qRT-PCR

To validate the RNA-seq results in this study using an alternative approach, we performed a qRT-PCR experiment to assay three different genes (Figure 6). In RNA-seq data, TFN receptor-associated factor 1 (TRAF1) was upregulated by 7.96 fold; CUGBP, Elav-like family member 1 (CELF1) was down-regulated by 1.16 fold; and Fanconi anemia, complementation group D2 (FANCD2) was down-regulated by 1.70 fold. In qRT-PCR data, these corresponding results of these three genes assayed by RNA-seq and qRT-PCR are in good agreement, which corroborates our RNA-seq results.

Discussion

Our study provides the first comprehensive insight into the transcriptome of human pulmonary microvascular endothelial cells treated with thrombin using RNA-seq, a powerful next-generation DNA sequencing platform, since a literature search for the PubMed database with the combination of key words of thrombin and RNA-seq retrieves zero records as of Sept. 16, 2011. For RNA-seq, we used the Illumina HiScanSQ instrument to perform a 2×101 paired end run for all of our samples. The advantage of a paired end run is that both reads contain long range positional information, allowing for highly precise alignment of reads. We calculated the number of differentially expressed genes between thrombin treated cells and controls based on mean values of two duplicated samples in each group. We obtained a mean value of 102,568,132 reads per sample, which is more than enough to deliver sufficient sequence coverage for transcriptome profiling according to a previous report by Sultan et al. [18]. Our mean rate of 91.3% total reads that map to the reference genome meets quality standards of the RNA-seq technique [19]. A breadth of the RNA sequencing reads covering chromosome 1 indicates quality RNA-seq coverage [Fig. 1]. Hence, we are confident that our mRNA-Seq data provide a good quality and objective profile of transcriptome in human pulmonary microvascular endothelial cells treated with thrombin. In addition, a qRT-PCR experiment
Table 4. Networks affected by up-regulated genes/isoforms after thrombin treatment of HMVEC cells.

| Up regulated genes | Networks                                                                 | Score | Genes |
|--------------------|--------------------------------------------------------------------------|-------|-------|
|                    | Inflammatory Response, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function | 41    | 34    |
|                    | Inflammatory Response, Inflammatory Disease, Skeletal and Muscular Disorders | 38    | 32    |
|                    | Cellular Growth and Proliferation, Tissue Morphology, Cellular Movement   | 24    | 24    |

| Up regulated isoforms | Networks                                                                 | Score | Genes |
|-----------------------|--------------------------------------------------------------------------|-------|-------|
|                       | Hematological System Development and Function, Tissue Morphology, Cellular Growth and Proliferation | 102   | 70    |
|                       | Cell Death, Cell Cycle, Cellular Growth and Proliferation                | 45    | 45    |
|                       | Cellular Growth and Proliferation, Cellular Movement, Cell Death         | 23    | 30    |
|                       | Inflammatory Disease, Connective Tissue Disorders, Skeletal and Muscular Disorders | 20    | 28    |

Networks significantly down-regulated by thrombin treatment as determined by Ingenuity Pathway Analysis. The score is based on the p-value calculation. Networks with a score of 15 or greater were defined as significant. doi:10.1371/journal.pone.0031229.t004

Table 5. Top networks affected by down-regulated genes/isoforms after thrombin treatment of HMVEC cells.

| Down regulated genes | Networks                                                                 | Score | Genes |
|----------------------|--------------------------------------------------------------------------|-------|-------|
|                      | DNA Replication, Recombination, and Repair, Molecular Transport, Cellular Growth and Proliferation | 27    | 33    |
|                      | Cell Cycle, Cancer, Cellular Growth and Proliferation                    | 22    | 33    |
|                      | Cancer, Cellular Development, Organisinal Injury and Abnormalities       | 16    | 33    |
|                      | Cellular Growth and Proliferation, Cancer, Cell Cycle                    | 16    | 33    |
|                      | Cell Death, Cellular Movement, Cellular Growth and Proliferation         | 16    | 33    |
|                      | Inflammatory Response, Antimicrobial Response, Cell-To-Cell Signaling and Interaction | 15    | 32    |

| Down regulated isoforms | Networks                                                                 | Score | Genes |
|-------------------------|--------------------------------------------------------------------------|-------|-------|
|                         | Cancer, Cell Cycle, Cell Death                                           | 60    | 70    |
|                         | Connective Tissue Disorders, Genetic Disorder, Ophthalmic Disease         | 25    | 33    |
|                         | Cell Cycle, Cellular Growth and Proliferation, Cardiovascular System Development and Function | 21    | 33    |
|                         | Cell Death, Cell Cycle, Post-Translational Modification                  | 18    | 42    |
|                         | Cell Death, Cellular Growth and Proliferation, Cancer                    | 16    | 40    |
|                         | Cell Death, Organismal Survival, Cellular Development                    | 15    | 39    |

Networks significantly down-regulated by thrombin treatment as determined by Ingenuity Pathway Analysis. The score is based on the p-value calculation. Networks with a score of 15 or greater were defined as significant. doi:10.1371/journal.pone.0031229.t005
These results corroborate the role of thrombin in vascular inflammation, but more importantly, they have provided a complete list of differentially expressed genes after thrombin treatment, as well as, full components in the top affected network and canonical pathway of inflammatory response. By examining differentially expressed genes in a traditional thrombin signaling pathway (Figure 4), we found that thrombin receptor, Par 4 not Par 1, was up-regulated at the 6 h thrombin treatment. This makes sense since Par 1 is involved in an early response and Par 4 is involved in an later response of Thrombin. PARs contribute to the proinflammatory phenotype observed in endothelial dysfunction [20]. Along the same line, expression of NF-κB was significantly up-regulated at the 6 h thrombin treatment. NF-κB has been documented as the key regulator of inflammation [21]. The up-regulated Par4- and NF-κB-mediated effects may in part contribute to the thrombin mediated inflammatory response as a major affected transcriptional network or pathway in this study although other genes in this traditionally annotated thrombin pathway were either down-regulated or not affected (Table S4). Several protocadherin alpha members such as PCDHA1, PCDHA3 and PCDHA11 were among top upregulated genes by thrombin (Figure 2 and Table S2). Figure 5 displays a full spectrum of differential regulations of protocadherin alpha 1 to 13 by thrombin. Protocadherins are an important subfamily of the

**Table 6.** Top canonical pathways affected by up-regulated genes/isoforms after thrombin treatment of HMVEC cells.

| Up regulated genes | Canonical Pathway                              | p value | Genes |
|-------------------|-----------------------------------------------|---------|-------|
|                   | Role of IL-17A in Psoriasis                    | 0.000   | 23%   |
|                   | Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F | 0.000   | 22%   |
|                   | TREM1 Signaling                                | 0.000   | 19%   |
|                   | Role of Cytokines in Mediating Communication between Immune Cells | 0.000   | 13%   |
|                   | Role of IL-17F in Allergic Inflammatory Airway Diseases | 0.000   | 13%   |

| Up regulated isoforms | Canonical Pathway                              | p value | Genes |
|-----------------------|-----------------------------------------------|---------|-------|
|                       | Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F | 0.000   | 28%   |
|                       | TREM1 Signaling                                | 0.000   | 23%   |
|                       | TNFR2 Signaling                                | 0.000   | 21%   |
|                       | Lymphotxin β Receptor Signaling                | 0.000   | 15%   |
|                       | Acute Myeloid Leukemia Signaling               | 0.000   | 13%   |
|                       | CD40 Signaling                                 | 0.000   | 13%   |

Top canonical pathways significantly up-regulated by thrombin treatment as determined by Ingenuity Pathway Analysis. Pathways with a p-value less than 0.05 defined as significant.
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**Table 7.** Top canonical pathways affected by down-regulated genes/isoforms after thrombin treatment of HMVEC cells.

| Down regulated genes | Canonical Pathway                              | p value | Genes |
|----------------------|-----------------------------------------------|---------|-------|
|                      | DNA Double-Strand Break Repair by Homologous Recombination | 0.000   | 64%   |
|                      | DNA Double-Strand Break Repair by Non-Homologous End Joining | 0.000   | 57%   |
|                      | Role of BRCA1 in DNA Damage Response           | 0.000   | 36%   |
|                      | Aldosterone Signaling in Epithelial Cells      | 0.001   | 19%   |
|                      | Role of CHK Proteins in Cell Cycle Checkpoint Control | 0.004   | 29%   |

| Down regulated isoforms | Canonical Pathway                              | p value | Genes |
|-------------------------|-----------------------------------------------|---------|-------|
|                         | DNA Double-Strand Break Repair by Homologous Recombination | 0.000   | 71%   |
|                         | DNA Double-Strand Break Repair by Non-Homologous End Joining | 0.000   | 64%   |
|                         | Role of BRCA1 in DNA Damage Response           | 0.000   | 45%   |
|                         | Role of CHK Proteins in Cell Cycle Checkpoint Control | 0.000   | 42%   |
|                         | RAN Signaling                                  | 0.011   | 39%   |

Top canonical pathways significantly down-regulated by thrombin treatment as determined by Ingenuity Pathway Analysis. Pathways with a p-value less than 0.05 defined as significant.
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Figure 4. Display in the Thrombin Signaling pathway of those differentially expressed genes from thrombin treated HMVEC. Thrombin Signaling pathway was constructed by Ingenuity. Those with 1.3 fold up- or down-regulated genes in our data were uploaded and displayed into this pathway. Red color indicates up-regulation of gene expression and green down-regulated genes while white indicate no change of gene expression. The detailed fold changes and full name of each gene or isoform were presented in Table S4.

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cadherin superfamily proteins. They are calcium-dependent cell adhesion molecules. They are also involved in many biological processes, including cell recognition and cell signaling [22]. Network analysis of the top differentially regulated genes indicate that Cell-To-Cell Signaling and Interaction, Tissue Morphology, and Cellular Movement are among the top affected networks by the thrombin treatment (Table 4 and 5). These observations provide a transcriptional level support and insight into known roles of thrombin that can regulate cell-cell junctions between endothelial cells as well as cytoskeleton contraction. Thrombin is also recognized as a potent mitogen in cancer and tumor metastasis [17]. We found that DNA Double-Strand Break Repair and BRCA1 in DNA Damage Response were the top canonical pathways of downregulated genes/isoforms after thrombin treatment of HMVEC cells (Table 9). Dysfunction of the DNA repair pathway is among the major causes of tumorigenesis [16]. Our observation may provide new molecular mechanisms underlying thrombin in the tumor pathogenesis.

Previously, DNA microarray analysis has been performed to profile gene expression pattern in human vascular endothelial cells treated with thrombin. By comparing the RNA-seq data from this study to those by Uzonyi et al. [23] in which gene expression profile of 6 h thrombin treated HUVEC was assayed by Affymetrix Human Genome U133A Array, we found that 38 genes were consistently up-regulated and 395 genes were consistently down-regulated in both datasets (Table S7). Many of the changes we observed in gene expression between control and thrombin group were similar to those reported. For example, the upregulation trend of several inflammatory response genes (CX3CL1, 6.05 fold; VCAM1, 4.75 fold; ICAM1, 3.29 fold; IL6, 2.27 fold; NFkB2, 2.02 fold) in thrombin treated HMVEC-L cells revealed by RNA-seq in this study is the same as that detected using DNA microarray array (CX3CL1, 1.39 fold; VCAM1, 4.16 fold; ICAM1, 3.14 fold; IL6, 1.34 fold; NFkB2, 1.41 fold) by Uzonyi et al. [23]. Generally, RNA-seq is much more sensitive than DNA microarray to detect the magnitude of difference in differentially expressed genes after the thrombin treatment. Some highly expressed genes or isoforms such as AS3MT (6.96 fold) and PCDHA11 (58.5 fold) as well as lowly expressed genes and isoforms in this study were not detected in DNA microarray [23]. Although the slightly different cell type and old DNA microarray chips may be contributing factors to some of the disconcordance between our RNA-seq transcriptome data set and the result of DNA microarray, these differences most likely stem from inherent limitations in microarray systems. We also compared the data from this study to those by Viemann et al. [24] in which gene expression profile of 5 h TNFα treated HMVEC was assayed by Affymetrix Human Genome U133A Array. We found that 49 genes were consistently up-regulated and 264 genes were consistently down-regulated in both datasets (Table S8). TNFα
plays an important pathogenic role in inflammatory disorders [25]. Several upregulated genes known to be involved in the inflammatory response such as NFkB2, VCAM1, ICAM1, IL6, IL8, TNFRSF9 were similarly detected in 5 h TNFz treated HMVEC and 6 h thrombin treated HMVEC in our study though the reported magnitude of increased expressions were different. These cross comparisons between our RNA-seq data and those of DNA microarray analysis of either 6 h thrombin treated HUVEC or 5 h TNFz treated HMVEC have provided a significant overlapping list of differentially expressed genes, supporting the robust utility of our dataset. One notable advantage of RNA-seq is that it can measure the less abundant transcripts that are inaccurate or not detected by microarray. RNA-seq also enabled the detection of unknown RNAs, alternative spliced transcripts and alternative promoter usages which are not feasible by microarray [6,26]. Indeed, our RNA-Seq study reveals nearly 1/3 of the differentially expressed RNA transcripts are unannotated, while about 26,000 novel isoforms are detected. Among those novel isoforms, 418 were only detected in the control cells, while 424 were only detected in thrombin treated cells. The differentially expressed novel isoforms in thrombin treated cells over the controls with statistically significant fold changes ranged from −927.74 to 431.33. There are 1,775 upregulated- and 12,202 downregulated- novel isoforms in thrombin treated cells by at least a 2 fold change over the control cells (Table 2). This study has provided rich reagents for further experimentation.

It should be pointed out that one limitation of our study is the one time point, 6 h, of the thrombin treatment. The reason we selected 6 h of thrombin treatment was based on at least the following three references: 1) Simoncini et al. showed that thrombin mediated upregulation of TNFSF10 (TRAIL), a TNF-z superfamily inflammatory cytokine that displays multiple effects on endothelial cells, started to peak at 6 h time point in both human microvascular endothelial cells and HUVEC as quantified by real-time PCR [27]. 2) Moser et al. showed that a 6 h thrombin treatment significantly enhanced the adhesive interaction of neutrophils to HUVEC as IL-1, TNF and LPS did compared to those of the 5 min thrombin treatment [28]. A transendothelial neutrophil passage is one of hallmark features in the inflammatory process. 3) Liu et al. employed a 6 h thrombin treatment to investigate thrombin mediated myocyte-specific enhancer factor-2 (MEF2)-dependent gene transcription of endothelial cells in angiogenesis [29]. Our data from this study have provided a valuable global picture on the thrombin mediated up-regulation and down-regulation of multiple genes in the endothelium at the 6 h thrombin treatment as discussed above. Nevertheless, previous studies have showed that of the genes that were induced by thrombin, some peaked at 1 hour, some at 4 hours, and some at 18 hours [30]. Our study with 6 h thrombin treatment failed to detect early and late thrombin response genes. It is certainly warranted that a series of time course experiments of thrombin treatment should be carried out to systematically survey the transcriptional regulation of thrombin on endothelial cells to provide more complete and dynamic datasets using the new and powerful RNA-seq technology. A better understanding of the temporal dynamics of these thrombin regulated transcriptional networks may help to decipher the link between thrombin signaling in the endothelium and multiple phenotypic changes.

In summary, our first complete transcriptome analysis of human pulmonary microvascular endothelial cells treated with thrombin using the RNA-seq has provided important insights into the transcriptional regulation of gene expression in HMVEC-L cells by thrombin. Further in-depth follow-up analysis of the transcriptional regulation reported in this study may shed light on molecular mechanisms underlying thrombin mediated endothelial dysfunction in the pathogenesis of inflammatory conditions, cancer, diabetes, coronary heart disease and provide new leads of therapeutic targets to those diseases.

**Materials and Methods**

**Cell culture and RNA isolation**

Human Lung Microvascular Endothelial Cells (HMVEC, Lonza catalog # CC-2815) were cultured as described previously [31]. Briefly, cells were grown to 90–100% confluence on the 7th passage in T-75 flasks in EGM-2 medium with 5% FBS, growth factors and antibiotics (Lonza, Bullet Kit catalog # CC-3202). Media was changed to a starvation media (0% FBS) 30 minutes prior to treatment. The cells were treated with 0.05 U/ml thrombin (Sigma-Aldrich, catalog # T4393, Lot 087K7575) or left untreated for 6 hours at 37°C. Total RNA was isolated from the treated and control cells using the MirVana kit (Ambion, catalog # AM1560) according to manufacturer’s instructions.

**Library preparation and sequencing**

Concentration of the RNA samples was determined using the Quant-iT RiboGreen Kit (Invitrogen catalog # R11490) according to manufacturer’s instructions. Each sample was assayed 3 times and the results averaged. The quality of the RNA was checked using the RNA Std Sense Chip on the Bio-Rad Experion system (catalog # 700-7103). The RNA was prepared for sequencing using the TruSeq RNA Sample Prep Kit, Set A (Illumina catalog # FC-122-1001) according to manufacturer’s instructions. Each sample was prepared in duplicate and 2 μg total RNA was used. The resulting cDNA libraries were quantified using the Epoch system and checked for quality and size using the Experion DNA 1K chip (catalog # 700-7107). A portion of each library was diluted to 10 nM and stored at −20°C. 2 μL of the 10 nM libraries were diluted and denatured, according to instructions from Illumina. The resulting cDNA was diluted to 5 pM and 1% PhiX control (Illumina catalog # FC-110-3001) was spiked in each sample. A 6 PM dilution of PhiX Control was also prepared. The samples were loaded on to the cBot (Illumina) for clustering on a flow cell according to manufacturer’s instructions.

The flow cell was then sequenced using a HiScanSQ (Illumina) according to manufacturer’s instructions. A paired-end (2x101) run was performed using the SBS Kit (Illumina catalog # FC-401-1001). Real-time analysis and basecalling was performed using the HiSeq Control Software Version 1.3.8 (Illumina).

**Primary processing of Illumina RNA-Seq reads**

The number of clusters detected,percentge of those clusters passing the software’s internal filter and the alignment to the PhiX genome were all within acceptable limits. The resulting .bcl files were converted first to .qseq files using Illumina’s Off-Line Basecaller (OLB) and the .qseq files were subsequently converted to FASTQ files. The sequence data have been submitted to the NCBI Short Read Archive with accession number SRA046349.2.

**Mapping of RNA-Seq reads and transcript assembly and abundance estimation using Tuxedo Suite**

The sequences were aligned to the UCSC Homo sapiens reference genome hg19 using TopHat v1.3.0 [32], which is integrated with Bowtie v0.12.7 [33]. TopHat removes a small number of reads based on quality scoring accompanying each read and then maps the reads to a provided reference genome. The pre-built UCSC H. sapiens hg19 bowtie index was downloaded from the Illumina FTP
site (ftp://ftp.illumina.com/Homo_sapiens/UCSC/hg19/Homo_sapiens_UCSC_hg19.tar.gz). TopHat’s default settings were used: 40 alignments per read were allowed, with up to 2 mismatches per alignment. Each contiguously-mapping read is considered a “coverage island,” which are often spliced together with neighboring “islands.” These “islands” can be considered probable exons. TopHat uses this initial mapping to build a database of potential splice junctions and verifies them by comparing the previously unmapped reads against the junction database.

The resulting aligned reads were analyzed further by Cufflinks v1.0.3 [34] in several ways. Cufflinks assembled the aligned reads into transcripts, either with or without a reference genome and reported the expression of those transcripts in Fragments Per Kilobase of exon per Million fragments mapped (FPKM). FPKM is an expression of the relative abundance of transcripts. To determine differential expression of known isoforms between the treatment and control groups, Cuffdiff analyses were performed using the reference genome comparing the thrombin treatment samples to the control samples. The biologic replicates were pooled for those analyses. To detect novel isoforms, Cufflinks was run without a reference genome. This method allows the construction of the minimum number of transcripts that described the data without a bias towards already discovered transcripts. The sample transcript files were then compared to the reference genome using Cuffcompare, effectively filtering out those already-discovered transcripts.

To test the differential expression of these novel isoforms, Cuffdiff analyses were performed using the combined transcript files as the reference genome. Cuffdiff analyses were performed two ways: comparing the thrombin transcripts to the control transcripts, using the thrombin transcripts as the reference genome; and comparing the thrombin transcripts to the control transcripts, using the control transcripts as the reference genome.

Visualization of mapped reads
Aligned reads were visualized using a local copy of the Integrative Genomics Viewer (www.broadinstitute.org/igv/). The output files generated from TopHat were converted into files viewable in IGV by BEDTools [35] and then further processed by the “count” function in igvtools (included with the IGV software) to create an average alignment track viewable as a bar chart. The log2 of the frequency of the reads was plotted to better visualize the extensive range of the read coverage. Individual gene views were created by first merging the TopHat output files from the control and thrombin-treatment samples into two files using SAMTools [36]. These merged files were processed in the same way as above with the “count” function in igvtools. The raw frequency of the reads was visualized in this case.

Functional analysis of differentially expressed gene lists using Ingenuity Pathway Analysis
The differentially expressed gene lists were submitted to Ingenuity Pathway Analysis (IPA) v9.0-3211 (Ingenuity Systems, Inc., Redwood City, CA). Genes with a 2-fold or greater change in expression after thrombin treatment were uploaded to the application. Each identifier was mapped to its corresponding object in Ingenuity’s Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity’s Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. The following settings were used: Ingenuity Knowledge Base; Endogenous Chemicals not included; Direct and Indirect relationships; molecules per pathway: 70; and networks per analysis: 25.

Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Genes with a 2-fold or greater change in expression after thrombin treatment and that were associated with a canonical pathway in Ingenuity’s Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) the ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway, and 2) Fisher’s exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Computer hardware specifications
TopHat, Bowtie and Cufflinks were installed and run on a CentOS 5.5 Advanced Clustering Pinnacle 1BX5501 machine with 20 nodes, totaling 240 cores and 960 GB RAM.

qRT-PCR Validation of RNA Seq Results
The following genes were selected for validation and TaqMan Gene Expression Assays were ordered from Applied Biosystems (cat. #4331102): CELF1 (Hs01090669_m1), FANDCD2 (Hs00276999_m1), TRAF1 (Hs01090170_m1), and ACTB, as a control (Hs99999903_m1). All genes were significantly differentially expressed in our RNA Seq data as determined by Cufflinks. cDNA was made from RNA previously isolated from control HMVEC and thrombin-treated HMVEC. Gene expression assays were run according to manufacturer’s instructions (www.appliedbiosystems.com) on a Viaa7 qPCR instrument. Replicates (n=4) of each sample were run. The results were loaded into a local copy of DataAssist (www. appliedbiosystems.com/dataassist) and normalized to ACTB Ct values. The fold change and range of the fold change was calculated for each gene.

Statistical Analysis
Besides those statistical tools embedded in Illumina HiScanSQ instrument or other softwares, additional statistical analyses were performed using Sigmaplot, a scientific data analysis and graphing software (ver 12, Systat Software, Inc., San Jose, CA). Thrombin treated groups were compared with the control groups by unpaired Student’s t-test. p<0.05 was considered statistically significant.

Supporting Information
Table S1 Top 50 up- and down-regulated genes in thrombin treated HMVEC cells. Significantly differentially expressed genes were determined by CuffDiff, after Benjamini-Hochberg correction. The fold change is the ratio of thrombin FPKM to control FPKM. The genes were ranked on their fold change and the 50 with the highest or lowest fold changes are listed here.

Table S2 Top 50 up- and down-regulated known isoforms in thrombin treated HMVEC cells. Significantly differentially expressed isoforms were determined by CuffDiff, after Benjamini-Hochberg correction. The fold change is the ratio of thrombin FPKM to control FPKM. The isoforms were ranked on their fold change and the 50 with the highest or lowest fold changes are listed here.

Table S3 Top 50 up- and down-regulated novel isoforms in thrombin treated HMVEC cells. Significantly differential-
ly determined novel isoforms, determined by CuffDiff, were identified in the experiments: DC MG. Analyzed the data: DPH MG SQY. Conceived and designed the experiments: LQZ SD DF SQY. Performed technical consultant teams for their quick responses and helpful suggestions on the smooth running of the next generation DNA sequencing instrument, HiScanSQ, and data quality analysis.

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**Author Contributions**

Conceived and designed the experiments: LQZ SD DF SQY. Performed technical consultant teams for their quick responses and helpful suggestions on the smooth running of the next generation DNA sequencing instrument, HiScanSQ, and data quality analysis.

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