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A Comprehensive Analysis of the Human Placenta Transcriptome

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Abstract

As the conduit for nutrients and growth signals, the placenta is critical to establishing an environment sufficient for fetal growth and development. To better understand the mechanisms regulating placental development and gene expression, we characterized the transcriptome of term placenta from 20 healthy women with uncomplicated pregnancies using RNA-seq. To identify genes that were highly expressed and unique to the placenta we compared placental RNA-seq data to data from 7 other tissues (adipose, breast, heart, kidney, liver, lung, and smooth muscle) and identified several genes novel to placental biology (*QSOX1*, *DLG5*, and *SEMA7A*). Semi-quantitative RT-PCR confirmed the RNA-seq results and immunohistochemistry indicated these proteins were highly expressed in the placental syncytium. Additionally, we mined our RNA-seq data to map the relative expression of key developmental gene families (Fox, Sox, Gata, Tead, and Wnt) within the placenta. We identified *FOXO4*, *GATA3*, and *WNT7A* to be amongst the highest expressed members of these families. Overall, these findings provide a new reference for understanding of placental transcriptome and can aid in the identification of novel pathways regulating placenta physiology that may be dysregulated in placental disease.

Keywords

RNA-seq; development; gene expression; pregnancy; fetal tissue

INTRODUCTION

The placenta is a specialized temporary fetal organ that is requisite for fetal growth and development in eutherian mammals and is crucial for a successful pregnancy. Early in pregnancy the placenta is responsible for implantation, anchoring of the embryo into the

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DISCLOSURES

The authors have nothing to disclose.

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uterine wall, and producing hormones that initiate maternal recognition of pregnancy. Throughout gestation, placental development proceeds via an elegantly orchestrated regulation of trophoblast invasion, proliferation and differentiation, as well as, vasculogenesis and angiogenesis to ensure adequate blood supply to support placental and fetal growth. Thereafter, the placenta provides the sole conduit for transferring maternally derived nutrients and gasses to the ever-demanding fetus.

Disruptions to placental development in the first half of pregnancy and/or insufficient adaptive changes in the placenta in response to maternal environment during the latter half of pregnancy have been associated with a number of pregnancy complications and compromised fetal outcomes. In this context, defining placenta specific gene expression can contribute to the understanding of placenta development and function. Unlike microarrays, which have been the primary tool to-date to study placental gene expression [1–3] [4], RNA-seq allows digital quantitation of gene expression data and hence allows assessment of relative abundance between genes within and between samples. Here, we set out to identify genes that are specific to human placenta using deep sequencing technology in order to increase general understanding of placental function and development.

METHODS

Collection of term placental samples

Placenta were collected at term at the University of Arkansas for Medical Sciences following informed consent from mothers. The protocol was approved by the Institutional Review Board at UAMS (NCT01104454). Included in this study were non-smoking mothers without gestational diabetes, pre-eclampsia or other complications who had either vaginal or cesarean deliveries (Table 1).

Preparation, sequencing, and data analysis of RNA-seq libraries

Total RNA was isolated from 20 placenta (pooled from 6 separate locations) using RNeasy columns [5] including DNase digestion. cDNA libraries were prepared using NebNext reagents as previously described [5]. Single-read 36-bp sequencing was performed using a GAII_X (Illumina). RNA-seq data for other tissues (liver, skeletal muscle, adipose, kidney, lung breast, and heart) were acquired in FASTQ format from the Illumina BodyMap 2.0 project. Alignment to the human genome (hg19) was carried out using Bowtie [6]. All data were analyzed in Avadis-NGS and SeqMonk software packages. Uniquely aligned reads were quantified in Avadis-NGS and gene-level reads per kilobase per million mapped reads (RPKM) values were calculated. Pair-wise comparisons between placenta and individual tissues were carried out ($p < 0.05$, Audic-claverie test; and 3-fold higher expression in placenta). Corrections for multiple testing were performed using the false discovery rate method [7]. The intersection of these comparisons was used for hierarchical clustering using R-Bioconductor and for functional enrichment using TargetMine. Visualizations were performed using Circos [8].

Quantitative RT-PCR

Total RNA was isolated from placenta as described above while RNA for other tissues was obtained commercially from Clontech (#636643, #636576, and #636558, Mountain View, CA). Total RNA (1 μ g) was reverse transcribed using iScript cDNA synthesis kit, and subsequent real-time PCR analysis was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Gene specific primers were designed using Primer Express Software (Applied Biosystems) for *QSOX1* (forward: 5'-GACTGTGCTGAGGAGACCAACA-3', reverse: 5'-CCCAGCCGTTCTTGGTAA-3'), *DLG5* (forward: 5'-AAGCGGAACGCATTAATAATC-3', reverse: 5'-

TGCATTGAGAATGTGACACTGAAC-4') and *SEMA7A* (forward: 5'-TCATGTCCCGAGACCCCTACT-3', reverse: 5'-GTGTGGCTCGGCTGGATTAAT-3'). The relative amounts of mRNA were quantified using the Δ CT method and normalized to the expression of *cyclophilin A* mRNA [5].

Immunohistochemistry

Placenta tissues were fixed over-night in 3% paraformaldehyde, dehydrated through 5–15% sucrose, and frozen in OCT. Five micron sections were air dried and subjected to antigen retrieval (#45080-9K, Biogenex, San Ramon, CA). Immunolocalization was carried out using VectaStain Elite reagents (Vector labs). Briefly, sections were blocked with either rabbit or goat serum, followed by incubation with primary antibodies: QSOX1 #HPA042127 (Sigma, St. Louis, MO); DLG5 #ab56492 (Abcam, Cambridge, MA); SEMA7A #sc-374432 (Santa Cruz Biotechnology, Dallas, TX); FoxO4 #9472 (Cell Signaling, Danvers, MA); GATA-3 #sc-9009 (Santa Cruz Bio.); Wnt-7a #HPA015719 (Sigma). Primary antibodies were used at 1:100 concentrations. Controls excluding primary antibodies were used for all staining procedures. Incubations were carried out for 2 h at room temperature. Visualization of staining was performed using diaminobenzidine (Dako).

RESULTS and DISCUSSION

This dataset included ~200 million reads covering 20 biological replicates. Quantitation of reads over genes revealed that ~80% of all annotated UCSC genes had at least one read and ~54% showed RPKM values > 1. We ranked genes based on transcript abundance in the placenta (top 100 shown in track 2 of Figure 1A). High-resolution images are provided in supplementary material. Consistent with a previous study where microarray analysis was used to find placental specific genes [2], many of the top expressed placental genes have been shown to regulate placental and fetal growth and have been identified as markers for placental diseases. For example *H19* and *IGF2*, both imprinted genes that produce potent growth factors [9], had a RPKM of > 1000. *HTRA1* was also highly expressed with a RPKM of ~300. *HTRA1* encodes a peptidase that may play a role in the regulation of IGF bioavailability by cleaving IGF-binding proteins [10] and is dysregulated in trophoblastic diseases [11]. Additionally, GDF15 is a transforming growth factor-beta (TGF- β) cytokine that has implications in cardiovascular disease and has been identified to be dysregulated in pre-eclamptic and diabetic pregnancies [12]. *GDF15* also had a RPKM of >1000 and was within the top 20 expressed genes in the placenta.

Previous reports have suggested that placenta-specific transcripts in maternal circulation might serve as biomarkers for placental dysfunction [13, 14]. Hence, we utilized our data to identify genes uniquely enriched in placenta (>3-fold higher) relative to 7 other tissues. This analysis identified 285 genes of which top 50 are presented in Table 2 and tracks 2 and 5 of Figure 1A. Hierarchical clustering of these genes is presented in Figure 1B. TargetMine analysis showed that placenta-enriched genes functionally represented adaptive immunity, immune response, interferon signaling, focal adhesion and cell cycle, among other processes, classically associated with placental function (Track 6, Figure 1). Several genes novel to placental biology were identified: *IFI6* (interferon α -inducible protein 6), *QSOX1* (quiescin sulfhydryl oxidase-1), *DLG5* (Discs large homologue 5), and *SEMA7A* (semaphoring-7a), (Table 2).

QSOX1 encodes a protein belonging to the family of enzymes that catalyze disulfide bond formation and are important for cell growth [15] and was 3x higher than lung and almost 9x higher than adipose, breast, heart, kidney liver and smooth muscle (Table 2). Similar results were confirmed for *QSOX1* expression using qRT-PCR, with the exception of placenta vs. lung expression (Figure 2A). These proteins have been shown to be protective against

oxidative stress-induced cell death [16] suggesting that, in the complex redox state of placental development *QSOX1* may be essential for trophoblast survival. Accordingly, expression of *QSOX1* in the placenta was localized to the syncytium, as well as to the fetal endothelial cells (Figure 2B). Additionally, *QSOX1* is a secreted enzyme that plays a role in extracellular matrix formation [17], consistent with the immunolabeling of *QSOX1* in the villous stroma (Figure 2B). Another potential regulator of placental growth is *IFI6*, which appears to have an anti-apoptotic function in cancer [18, 19].

DLG5 plays an important role in lung branching morphogenesis and progenitor differentiation [20] and is a regulator of apical polarity complexes [20] and epithelial-to-mesenchymal transition (EMT) [21]. Cytotrophoblast fusion requires loss of cell-cell junctions [22], not dissimilar to EMT, suggesting that *DLG5* may be important for the regulation syncytialization. Quantitative RT-PCR confirmed that *DLG5* was most highly expressed in placental tissue (Figure 2C) and was localized to the syncytium (Figure 2D). Similarly, semaphoring-7a, (*SEMA7A*) has also been implicated in regulating TGF- β induced EMT [23]. *SEMA7A* is a signaling molecule that is transcriptionally regulated by Ets2-receptor factor and HIF-1 α and has been involved in the regulation of cell migration in the immune and nervous systems, as well as in cancer (recently reviewed, [24]). Corresponding with the RNA-seq results, qRT-PCR confirmed that *SEMA7A* was highly expressed in the placenta compared to the 7 other tissues (Figure 2E). Additionally, immunohistochemical analysis of *SEMA7A* indicated high expression in the placenta that was not cell-specific (Figure 2F), consistent with its role in a number of cell types.

Several developmentally regulated gene families have been shown to play a role in placental development and function. Hence, we mined our RNA-seq data to map the relative expression of key gene families within the placenta (Figure 3, Table S1). High-resolution images are provided in supplementary material. To date, 50 forkhead box (FOX) gene family members have been identified in humans [25] and of 45 FOX genes examined, we found *FOXF1*, *FOXJ3*, *FOXK2*, and *FOXO4* were amongst the highest expressed FOX genes (Figure 3A, Table S1). Additionally, *FOXO4* expression in the placenta was >3-fold higher when compared to other metabolic tissues (Table 2) suggesting it may have a specific role in placental physiology. FOXO proteins have been previously localized in placental tissue and fetal membranes [26] and transcriptionally regulate cell cycle progression, apoptosis, angiogenesis, inflammation, and anti-oxidant production [27], all of which are important pathways for placenta development and function. *FOXO4* was localized to the syncytium and showed both nuclear and cytoplasmic expression (Figure 3D), consistent with its role as a transcriptional regulator. The SOX family of transcription factors regulate many developmental processes such as sex differentiation, neural development [28], cellular differentiation, [29] and blood vessel development [30]. *SOX7*, *13*, *17* and *18*, with known angiogenic and cardiovascular developmental functions, were among the highest expressed in placenta, consistent with the vascular nature of the placenta (Figure 3B, Table S1). Along with the many other roles of GATA-binding transcriptions factors in development, they have been identified as important for many reproductive developmental processes [31]. RNA-seq analysis showed that *GATA2* and *3* were both highly and uniquely, enriched in placenta compared to other tissues (Table 2). *GATA3* was localized to the cytoplasm of the syncytium, villous stroma, and showed nuclear and cytoplasmic staining in fetal endothelial cells (Figure 3E). We also identified high expression of the Hippo-kinase pathway, *LATS1* and *2*, and *YAP1* and *TEAD1* and *3* (Figure 3B, Table S2). This pathway has been recently shown to be critical in early trophectoderm lineage commitment [32]; however, it is yet to be characterized in term human placenta. Likewise, we also identified high expression of specific Wnt family members *WNT2*, *3a* and *7a*, *FZD1* and the canonical effector β -catenin in the placenta (Figure 3C). *Wnt2* expression is affected by pre-eclampsia [33] and *WNT7A* plays a critical role in uterine smooth muscle patterning and maintenance of adult uterine

function [34]. However, its role in term placenta has been not been recognized. WNT7A showed strong immunolocalization to the cytoplasm and basal plasma membrane of the syncytium (Figure 3F), suggesting that WNT7A is produced in syncytiotrophoblast cells and may be released towards fetal circulation. Accordingly, WNT7A was also localized to the endothelium and showed some staining in villous stromal cells (possibly placental macrophages).

The present study has many strengths, including the use of deep sequencing technology to identify novel genes expressed in placental tissue and immunohistochemistry to identify cell specific localization. It also has some limitations. First, the wide range of BMI indicates that a heterogeneous population in regards to maternal adiposity was used; however we feel that this diversity provides a more translatable data set. Next, the cohort was composed mostly of male placenta, which could skew the expression of specific genes that show sex dependence, as previously reported [2]. Additionally, we did not perform our analysis on the various placental compartments (decidua, villous, chorion), which have previously been shown to have distinct gene expression signatures [2, 35]. Finally, because RNA-seq data for the 7 other tissues was obtained from the Illumina BodyMap 2.0 project, we were unable to confirm gene expression in the exact same samples, which may explain some of the discrepancies between the RNA-seq analysis and qRT-PCR results.

CONCLUSION

In conclusion, we present a genome-wide transcriptomic view of placental tissue and have identified novel genes highly expressed in term placenta that may be involved in both normal and pathogenic placental physiology. These findings may provide a starting point for future explorations of the potential roles for *QSOX1*, *DLG5*, *SEMA7A*, and other novel genes in placental development and disease. Additionally, taking advantage of the quantitative power of RNA-seq, we were able to identify the most abundant developmentally regulated gene family members within each sample, a comparison that is not possible with other gene expression methods. Overall, these findings provide a new reference for understanding of placental transcriptome and can aid in the identification of novel pathways regulating placenta physiology that may be dysregulated in placental disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. RNA-seq of human term placenta (n=20)

A) Circos diagram depicting whole-genome RNA-seq data. *Track 1:* cytoband, chromosomes are depicted qter to pter. *Track 2:* Genomic location of top 100 highly expressed genes in placenta based on average RPKM values. Gene names in red represent genes specifically enriched in placenta. *Track 3:* Average RPKM values summarized over 6 MB regions showing regions of high gene expression. *Track 4:* Locations of genes related to placenta in the OMIM database. *Track 5:* Genes specifically enriched in placenta (3-fold over 7 other tissues); *Track 6:* Biological functions enriched among placenta-enriched genes. High-resolution images are provided in supplementary material. **B)** Hierarchical clustering of the 288 genes that were at least 3-fold higher and had an RPKM >1 in the placenta compared to liver, heart, smooth muscle, adipose, breast, kidney, and lung. High expression is represented in red and low expression in blue.

Figure 2. Genes novel to placental biology

Gene expression analysis via qRT-PCR of **A) *QSOX1***, **C) *DLG5***, and **E) *SEMA7A*** in placenta and 7 other tissues. Values are expressed as the fold change relative to placenta $\pm$ SD. Representative 40x images of term placenta from 3 subjects were immunostained with **B) *QSOX1***, **D) *DLG5***, and **F) *SEMA7A***. Images (40x) of secondary-only negative controls for rabbit (left) and mouse (right) are depicted at the bottom of the figure.

Figure 3. Developmentally regulated gene families in term placenta

Heat maps depicting the expression of A) *FOX*, B) *SOX*, *GATA*, and *TEAD* and C) *WNT* and *FZD* genes in placental tissue (n=20). Heat map colors red, white, and blue represent high, moderate, and low expression of transcripts, respectively. Details of average gene expression (RPKM) values are provided in supplementary table S1. High-resolution images are provided in supplementary material. Immunostaining of D) FoxO4, E) Gata3, and F) Wnt7a in human placenta. Representative images from 3 subjects were taken at 10x (top) and 40x (bottom) to show cell specific and intercellular localization. A 10x (left) and 40x (right) image of a secondary-only negative control is depicted on the bottom of the figure.

Table 1

Maternal Characteristics

	Mean $\pm$ SE	[Min, Max]
<i>n</i>	20	---
Age (yrs.)	29.2 $\pm$ 1.4	[18, 43]
Weight (lb.)	179.7 $\pm$ 11.5	[110, 277]
Height (in.)	65.6 $\pm$ 0.7	[60, 72]
BMI (kg/m ²)	29.3 $\pm$ 1.8	[18.3, 43.4]
Parity (n)	2.1 $\pm$ 0.3	[0, 5]
Placenta Weight (g)	659 $\pm$ 29.1	[459, 951]
C-Section (n)	11	---
Fetal sex	7-F, 13-M	---

n = number, F = female, M = male

Table 2

Top 50 genes uniquely enriched in placenta

Gene ID	Gene Symbol	Placenta	* Adipose	* Breast	* Heart	* Kidney	* Liver	* Lung	* Smooth Muscle
283120	<i>H19</i>	4354.5	270.6	245.0	65.7	52.3	28.6	27.2	660.0
3481	<i>IGF2</i>	1527.7	89.0	63.1	16.3	15.1	76.0	11.7	18.4
9518	<i>GDF15</i>	1337.5	13.4	0.5	0.3	24.7	0.4	25.7	2.6
2192	<i>FBLN1</i>	606.8	66.3	36.8	18.3	13.5	1.2	128.3	9.4
3048	<i>HBG2</i>	586.8	0.5	44.8	0.4	0.0	0.0	47.3	0.0
6513	<i>SLC2A1</i>	463.2	4.9	2.1	4.7	9.1	0.3	7.9	1.0
191585	<i>PLAC4</i>	458.3	0.2	0.2	0.1	0.1	0.0	0.1	0.0
6382	<i>SDC1</i>	446.0	1.1	5.7	0.1	85.9	100.8	25.4	0.0
2537	<i>IFI6</i>	367.1	15.1	27.0	18.3	21.7	17.2	97.5	4.7
3880	<i>KRT19</i>	339.1	1.6	38.4	0.2	53.3	0.8	56.0	0.1
5654	<i>HTRA1</i>	307.1	50.3	97.2	13.4	39.5	99.6	21.4	8.8
3856	<i>KRT8</i>	283.0	1.4	16.1	8.7	59.1	50.2	58.3	0.1
10272	<i>FSTL3</i>	256.4	15.6	28.6	7.7	11.1	1.5	37.1	0.6
4320	<i>MMP11</i>	249.4	0.7	0.4	1.4	0.3	0.0	0.3	0.2
1441	<i>CSF3R</i>	246.5	4.2	0.7	0.4	0.4	0.8	16.8	0.3
5228	<i>PGF</i>	243.4	6.0	1.8	1.5	1.1	1.3	1.9	0.7
2896	<i>GRN</i>	239.6	66.3	50.8	18.2	37.3	21.7	73.3	14.0
124935	<i>SLC43A2</i>	236.8	7.1	5.6	0.6	10.3	0.5	5.5	9.6
1028	<i>CDKN1C</i>	229.8	37.6	22.4	12.4	53.4	2.1	13.1	15.7
649	<i>BMP1</i>	203.3	5.5	3.7	0.8	3.4	2.5	1.8	0.7
5270	<i>SERPINE2</i>	201.6	16.9	3.3	4.5	18.2	0.9	7.4	0.2
9022	<i>CLIC3</i>	190.4	0.2	0.6	0.6	0.4	0.1	10.1	0.2
3875	<i>KRT18</i>	185.0	2.7	11.8	1.2	45.0	57.5	65.6	0.8
9506	<i>PAGE4</i>	178.4	0.0	0.0	0.0	0.3	0.0	0.0	0.0
23089	<i>PEG10</i>	172.7	1.0	5.6	0.3	3.1	0.5	5.7	0.8
7975	<i>MAFK</i>	171.3	17.4	7.3	7.0	4.4	1.6	25.5	17.5
10912	<i>GADD45G</i>	158.9	2.1	1.2	4.4	5.3	11.5	3.2	6.3
64855	<i>FAM129B</i>	158.1	10.7	10.6	5.0	16.4	2.5	50.7	2.1

Gene ID	Gene Symbol	Placenta	* Adipose	* Breast	* Heart	* Kidney	* Liver	* Lung	* Smooth Muscle
54507	<i>ADAMTSL4</i>	156.8	10.5	5.2	4.5	1.5	2.0	9.3	5.1
23764	<i>MAFF</i>	143.8	48.9	9.7	2.8	1.9	1.5	50.3	25.2
827	<i>CAPN6</i>	142.5	0.1	1.4	0.0	3.4	0.1	0.3	0.2
7262	<i>PHLDA2</i>	141.3	1.1	1.7	0.2	0.9	0.5	3.5	0.4
58190	<i>CTDSP1</i>	138.7	37.9	29.9	13.1	29.7	22.6	19.5	18.8
5768	<i>QSOX1</i>	133.7	15.8	11.0	16.3	14.0	9.8	49.6	8.2
3074	<i>HEXB</i>	127.9	37.0	31.2	29.4	28.5	27.8	43.1	16.9
1082	<i>CGB</i>	121.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6625	<i>SNRNP70</i>	118.3	24.6	21.4	9.3	22.1	10.3	37.4	12.1
79586	<i>CHPF</i>	102.7	3.9	5.2	10.4	7.8	15.2	10.1	5.6
9231	<i>DLC5</i>	100.0	4.8	4.1	1.4	3.2	0.5	3.1	6.0
8482	<i>SEMA7A</i>	97.8	0.4	0.6	0.1	0.3	0.1	9.5	0.2
55194	<i>FAM176B</i>	96.7	9.9	6.9	1.5	6.7	1.1	11.2	1.3
4303	<i>FOXO4</i>	94.6	16.2	14.0	8.4	5.4	2.1	9.6	25.5
5590	<i>PRKCZ</i>	91.0	0.3	1.0	2.2	5.9	1.4	13.0	0.2
85360	<i>SYDE1</i>	89.5	6.6	5.0	2.3	4.3	0.7	4.1	0.8
114336	<i>CGB2</i>	84.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1
6484	<i>ST3GAL4</i>	83.7	6.6	5.5	7.0	12.3	11.4	13.7	0.9
64787	<i>EPS8L2</i>	77.6	0.9	3.0	0.1	14.6	8.6	7.1	0.1
9123	<i>SLC16A3</i>	76.4	12.3	2.3	1.4	6.0	0.9	14.7	1.8
2548	<i>GAA</i>	75.6	10.6	11.3	4.9	9.0	8.0	13.1	13.9
22836	<i>RHOBTB3</i>	74.5	18.7	25.3	11.7	22.4	8.2	11.9	10.7

Values expressed as mean RPKM.

* RPKM values were calculated following alignment of fastq files from the Illumina BodyMap 2.0 project