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Global Gene Expression Analysis of Term Amniotic Fluid Cell-Free Fetal RNA

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Abstract

Objective—To identify the tissue expression patterns and biological pathways enriched in term amniotic fluid (AF) cell-free fetal RNA by comparing functional genomic analyses of term and second trimester AF supernatants.

Methods—This was a prospective whole genome microarray study comparing eight AF samples collected from eight women at term who underwent prelabor cesarean delivery and eight second trimester AF samples from routine amniocenteses. A functional annotation tool was used to compare tissue expression patterns in term and second trimester samples. Pathways analysis software identified physiological systems, molecular and cellular functions and upstream regulators that were significantly overrepresented in term AF.

Results—There were 2,871 significantly differentially regulated genes. In term AF, tissue expression analysis showed enrichment of salivary gland, tracheal, and renal transcripts, as compared with brain and embryonic neural cells in second trimester. Functional analysis of genes upregulated at term revealed pathways that were highly specific for postnatal adaptation, such as immune function, digestion, respiration, carbohydrate metabolism and adipogenesis. Inflammation and prostaglandin synthesis, two key processes involved in normal labor, were also activated in term AF.

Conclusions—Transcriptomic analysis of AF cell-free fetal RNA detects fetal maturation processes activated in term pregnancy. These findings further develop the concept of AF supernatant as a real-time gene expression “summary fluid” and support its potential for future studies of fetal development.

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Introduction

In perinatal medicine, our concept of amniotic fluid (AF) has evolved from a simple electrolyte solution made up of fetal urine and sloughed cells, to a complex biological fluid that performs multiple functions. In prior work we have investigated AF cell-free fetal RNA as a source of gene expression information about normal second trimester development (1). Other studies from our group have demonstrated unique transcriptomic differences for trisomies 18 and 21, thus providing insights into their pathophysiology and generating hypotheses for further studies (2–4).

To date, the vast majority of studies on AF cell-free fetal RNA have been performed on second trimester pregnancies with clinical indications for an invasive procedure (5). During the first 20 weeks of pregnancy, there is bidirectional diffusion between the fetus and the AF across the unkeratinized fetal skin. Humans studies have shown that the majority of cells and cellular fragments in second trimester AF are derived from fetal squamous epithelial surfaces (namely skin, gastrointestinal tract, genitourinary tract), and not from the amniotic epithelium (6). More recently, AF has become recognized as a source of fetal stem cells (7). Many aspects of the biology of AF cell-free fetal RNA are unknown, but it is probable that cell-free transcripts enter AF directly from fetal organs as well as from free-floating cells and cellular fragments. In the second half of pregnancy, the composition and circulation of AF is dramatically altered by keratinization of the fetal skin, increased fetal urine output, and increased fetal swallowing and respiratory movements. While gene expression from AF supernatant has been shown to vary by gestational age (8), this has not been explored in the third trimester. Given the profound changes in fetal organ development and amniotic fluid composition between second trimester and term (9), we hypothesized that the cell-free transcripts in term AF will reflect changes associated with fetal maturation.

In this study we investigated the third trimester fetus by comparing AF cell-free fetal RNA from normal term pregnancies with second trimester controls. Specifically, we aimed to determine whether the relative representation of specific organs in term AF cell-free fetal RNA would differ from that in second trimester AF, using a bioinformatics tool for tissue expression analysis and a gene expression atlas. We also hypothesized that functional genomic analysis of upregulated transcripts in term AF would identify pathways and genes that reflect the real-time biology of the third trimester, and provide novel information about the term fetus.

Materials and Methods

This was a prospective study of women undergoing planned cesarean delivery between 37 and 40 weeks at Tufts Medical Center, Boston MA and a comparison group of women who underwent second trimester amniocentesis. The Tufts Medical Center Institutional Review Board (IRB) approved the study. For the cesarean delivery group, exclusion criteria included gestational age less than 37 or greater than 41 weeks, diagnosis of congenital anomaly, fetal growth restriction, multiple pregnancy, onset of labor, prelabor rupture of membranes, antepartum hemorrhage, preeclampsia, and preexisting maternal medical condition. One woman with gestational diabetes was included in the study.

Amniotic fluid was collected at cesarean delivery after entry into the uterus and prior to rupture of the amniotic membranes. Approximately 10 ml of AF was aspirated through the intact membranes using a blunt plastic cannula attached to a 20 ml syringe. Specimens with gross maternal blood or meconium contamination were discarded. AF samples were centrifuged at 350*g[ACOG1]* for 10 minutes at 4°C. The supernatant samples were spun at 1600*g*[ACOG2] for 10 minutes at 4°C to remove residual vernix and then stored at −80°C.

Second trimester AF supernatant samples were obtained as discarded residual clinical samples from the Cytogenetics Laboratory at Tufts Medical Center, also under an IRB-approved protocol. Only samples from structurally normal fetuses with euploid karyotypes were included. These samples were spun at 165*g*[ACOG3] for 10 minutes at room temperature to remove amniocytes for diagnostic testing. The residual fluid and stored at −20°C for up to one week and then archived at −80°C.

All included AF samples were collected between November 2011 and June 2012. RNA was extracted from 5–10 mLs of AF supernatant within 4 months of collection according to a customized protocol (10). All samples were processed using the Qiagen Circulating Nucleic Acid kit (Qiagen Inc, Valencia, CA) with an on-column DNase digestion step to remove genomic DNA. The RNA was then purified and concentrated with the RNeasy MinElute Clean up kit (Qiagen Inc) and eluted in RNase free water. RNA was converted to cDNA and amplified using the Ovation Pico WTA kit V2 (NuGEN Inc, San Carlos, CA), then purified with the QIAquick PCR Purification kit (Qiagen, Inc). cDNA yield and purity was measured with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA). Five micrograms of cDNA from each sample were biotinylated and fragmented using the Encore Biotin Module (NuGEN Inc) and hybridized to a whole human genome expression array (Affymetrix GeneChip Human Genome U133 Plus 2.0, Affymetrix Inc, Santa Clara, CA).

Normalization was performed with the threestep command from the AffyPLM package in BioConductor, using ideal mismatch background and signal adjustment, quantile normalization, and the Tukey biweight summary method (11). This summary method included a logarithmic transformation to improve the normality of the data. We identified genes that were differentially regulated in term AF compared with second trimester AF samples using the independent *t* test, and adjusted for multiple testing using the Benjamini-Hochberg correction (12). We defined genes as significantly differentially regulated if the *P* value using the Benjamini-Hochberg correction was <.01. Our microarray datasets are publicly available in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE46286.

We used the BioGPS Gene Expression Atlas at http://biogps.org to identify upregulated genes that had tissue-specific expression patterns. This atlas of the human protein-encoding transcriptome contains the gene expression profiles of 78 normal human tissues (13). The BioGPS Gene Expression Atlas allowed us to assess which organs were specifically represented in the 10 most upregulated genes at term and second trimester. We categorized genes as highly organ-specific if they mapped to a single organ with an expression value greater than 30 multiples of the median (MoM) in accordance with a previously established stringency threshold (14).

We also used the tissue expression function in the Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify tissues that most highly expressed the genes upregulated in term and second trimester fetuses, respectively (http://david.abcc.ncifcrf.gov/) (15). DAVID integrates tissue expression databases from publicly available sources to allow the identification of the most enriched gene expression patterns across hundreds of normal and diseased tissues for any given gene list. For each resource, the expression values for a given gene in all tissues are ranked from greatest to least. All tissues with an expression value in the top quartile are associated with that gene and an enrichment *P* value called the EASE score (a modified Fisher exact *P* value) is calculated for that tissue (16). DAVID also calculates a more conservative *P* value using the Benjamini-Hochberg correction to control the false discovery rate of family-wise enriched terms. We considered results significant if the tissue term was enriched by twofold or more with a

Benjamini-Hochberg corrected $P<.05$. We report the results derived from the UniProt database (www.uniprot.org).

Functional analysis was performed using IPA Version 9.0 software (release date 11 Aug 2012) (Ingenuity Systems, www.ingenuity.com). This pathways analysis tool is a manually-curated database that identifies overrepresented biological processes in a given data set and calculates a significance score for each result using the right tailed Fisher exact test. The Affymetrix probe set identifiers for the significantly differentially-regulated genes were uploaded to the pathways analysis software with their corresponding median fold change and Benjamini-Hochberg-corrected *P* values.

We used the pathways analysis software to identify any physiological systems or molecular and cellular functions that were enriched in term fetuses by focusing on the genes that were significantly upregulated by at least four-fold in the term group. To reduce false positive results, we applied the Benjamini-Hochberg correction for multiple comparisons within the pathways analysis as we were examining all potentially significant biological categories. We considered pathways significant if they had a corrected $P<.05$.

We also performed an upstream regulator analysis with the pathways analysis software using the list of all significantly upregulated and downregulated genes with a fold change value of 1.5 or more or −1.5 or less. This analysis uses prior published knowledge of expected effects between upstream regulators and their target genes to identify which upstream regulators are predicted to be activated or inhibited based on the direction of gene expression in the dataset. The activation z-score is used to infer likely activation states of upstream regulators based on comparison with a model that assigns random regulation directions. We reported the bias-corrected activation z scores for upstream regulators that corrects for any skews in the direction of gene regulation in the dataset or in the direction of molecular network relationships for a particular upstream regulator. We considered results with an activation z score greater than 2 or less than −2 as statistically significant according to thresholds recommended by the pathways analysis software. An overview of our analytical methods is provided in Figure 1.

Results

We obtained AF samples from eight term and eight second trimester pregnancies. The median gestational ages were 38 weeks (range 37–39) and 17 weeks (range 16–19) in the term and second trimester groups, respectively. The most common indication for second trimester amniocentesis was increased risk of trisomy 21 (Appendix 1 available at http://links.lww.com/xxx). The maternal age of the second trimester group was significantly higher than the term group (mean age 37 years compared with 33 years, $P=.04$, independent *t* test). There were equal proportions of male to female fetuses in each group.

The average microarray hybridization rate for all samples was 41% (range 33.0–50.1%). There were a total of 2,871 genes that were significantly differentially regulated in term compared with second trimester AF; 1,307 genes were upregulated and 1564 were downregulated (Appendix 2 available at http://links.lww.com/xxx).

The 10 most upregulated genes by fold change value in the term group were dominated by lung and saliva-specific transcripts (Table 1). All five pulmonary surfactant protein genes (*SFTPA1, SFTPA2, SFTPB, SFTPC, SFTPD*) were significantly upregulated in term AF. In contrast, the top 10 genes with known functions that were most upregulated in second trimester AF were associated with the lower gastrointestinal tract (Appendix 3 available at http://links.lww.com/xxx).

The DAVID tissue expression analysis of the genes upregulated in term AF showed statistically significant enrichment of transcripts highly expressed by saliva glands, trachea, and kidney. In contrast, AF transcripts upregulated in second trimester were highly enriched for fetal brain and Cajal-Retzius (embryonic neuronal) cells (Table 2).

There were 609 well-annotated genes that were upregulated by at least fourfold in term compared with second trimester AF. Core pathways analysis of these genes showed enrichment of multiple physiological systems involved in newborn functions, such as immune defense, eating, and breathing (Table 3 and Appendix 4 available at http://links.lww.com/xxx).

The three most statistically significant molecular and cellular functions upregulated in term fetuses were carbohydrate metabolism (41 genes), cellular movement (139 genes) and lipid metabolism (93 genes) (Appendix 5 available at http://links.lww.com/xxx). Within the lipid metabolism category were endocrine processes known to be involved in parturition, such as phospholipid concentration and prostaglandin synthesis. Individual upregulated genes included the key enzyme in inducible prostaglandin synthesis, prostaglandin-endoperoxidase synthase 2 (also known as cyclo-oxygenase 2), phospholipase A2, and the genes for prostaglandin E receptors type 3 and 4, (*PTGER3, PTGER4*).

We identified 17 upstream regulators in the pathways analysis that were significantly upregulated in term infants and predicted to be activated based on the differential regulation of target genes within our dataset (Appendix 6 available at http://links.lww.com/xxx). Of interest were several genes involved in pro-inflammatory pathways and immune activation such as nuclear factor-kappaB (*NFkB*), interleukin 8 (*IL8*), interleukin-1 beta (*IL1B*) and the interleukin 6 receptor (*IL6R*). There were six upstream regulators that were statistically significantly predicted to be activated in second trimester. These included genes involved in thyroid function, oxidative stress and liver development (Appendix 7 available at http://links.lww.com/xxx).

Discussion

The results of our analysis of term compared with second trimester AF demonstrate upregulation of fetal maturation processes that normally occur at term, supporting our hypothesis that AF cell-free fetal RNA reflects real-time developmental fetal physiology.

The major strength of this functional analysis of term AF cell-free fetal RNA is the detailed biological interpretation in the context of normal fetal maturity. High dimensional biology techniques such as functional genomics and proteomics have been applied to study abnormal pregnancy using a variety of maternal and fetal samples (17–18). In contrast, there are very few “-omic” studies of AF that aim to characterize normal third trimester fetal physiology. One proteomic study specifically examining the effect of gestational age on AF failed to yield any significant information on the third trimester (19). A metabolomic study on second and third trimester AF samples identified metabolites associated with increasing gestational age (20). Neither of these studies attempted to provide any detailed biological interpretation of the differentially-expressed proteins in third compared with second trimester.

Gene expression atlas mapping and DAVID tissue expression analysis of genes upregulated at term showed a dominance of lung, upper gastrointestinal tract and renal transcripts. It is not surprising that the organs that actively secrete or excrete into the amniotic cavity appear to be the major sources of AF cell-free RNA at term. The DAVID tissue expression analysis also demonstrated that the relative abundance of cell-free transcripts associated with the fetal nervous system varies according to gestational age, with more nervous system-related transcripts present at second trimester compared with term.

Many of the physiological systems upregulated at term were highly specific for adaptations required after birth, including the digestive system and respiratory function. Preparation for postnatal energy requirements and thermal regulation was also suggested by the upregulation of carbohydrate metabolism and adipogenesis. Free radical scavenging was one of the enriched cellular and molecular functions at term, which is consistent with the positive linear association between gestational age and antioxidant capacity (21).

One of the striking features of the term AF results was the evidence of preparation for labor in the term pregnancies, through significant upregulation of known inflammatory and endocrine pathways. Normal term labor is associated with acute inflammation of fetal membranes in the absence of overt infection (22). One of the fetal signals believed to initiate normal term labor is the increased production of surfactant protein A, which leads to macrophage activation and migration and subsequent NFkB and IL8 production in mice (23). We report here significant results for both immune cell trafficking function and *SFTPA1* and *SFTPA2* upregulation in term AF, which fits this proposed fetal signaling model. The downstream effects analysis also identified increased activity of three pro-inflammatory genes with key roles in labor, *NFkB, IL1B* and *IL8*.

Of the endocrine processes involved in the onset of labor, lipid metabolism and prostaglandin synthesis were significantly upregulated functions in term AF. The increase in prostaglandin production prior to labor is due largely to increased activity of *PTGS2*, which was upregulated 15 fold in term AF. Taken in total, our findings are very consistent with current models of the immune and endocrine influences on parturition (24).

One of the challenges of studying AF cell-free RNA is ascertaining the tissue(s) of origin of transcripts. There is currently no model that allows us to observe transcripts trafficking from specific fetal organs into the amniotic cavity in vivo. We therefore adopted an “in silico” approach utilizing bioinformatics tools to obtain information on the tissue expression profiles in AF cell-free fetal RNA. We took care to avoid maternal cellular contamination during sample collection so that we could be confident that the RNA was only fetal in origin. While maternal microchimerism (the presence of maternal cells in the fetus) is a recognized phenomenon, we feel that any potential contribution of these rare maternal cells to our functional analysis of AF would be negligible. We also cannot exclude the theoretical possibility of cell-free transcripts in maternal plasma entering AF through unknown routes. However, as we limited our core pathways analysis to those transcripts upregulated by at least fourfold at term, it is unlikely that any such maternal transcripts would have a major impact on results.

Our cross-sectional study design was a result of logistic constraints related to patient recruitment and sample collection. Ideally, we would have used a longitudinal design with paired second trimester and term AF samples from the same pregnancies, but this was impractical to implement in our single center study. Another limitation of our study is the sample size. Our target of eight samples per group was based on findings that near-maximal levels of statistical stability are obtained with 8–15 biological replicates (25). We acknowledge that, in general, the more replicates, the stronger the inferences that can be made from the data. However, even at our minimum target sample size, we were able to demonstrate significant differential gene expression in 7.44% of the total probe sets at a very stringent false discovery rate (Benjamini-Hochberg $P<.01$).

Determining the reproducibility of our findings in a larger independent dataset would be an important step to validate our approach to studying fetal gene expression in AF.

Gene expression studies of the human fetus are frequently limited by practical and ethical considerations. Most genomic studies of the human fetus have relied on tissue specimens

obtained after spontaneous abortions, terminations of pregnancy, or cultured extra-embryonic cells. In contrast, the results presented here demonstrate that AF cell-free RNA is a source of biologically meaningful gene expression data obtainable from the live human fetus. This has clinical relevance for future human studies where AF may provide a feasible alternative to direct tissue biopsy, for example, in investigations of fetal renal function at term. The findings of this study thus advance the concept of AF as a real-time gene expression summary fluid and support its potential for future studies of abnormal fetal development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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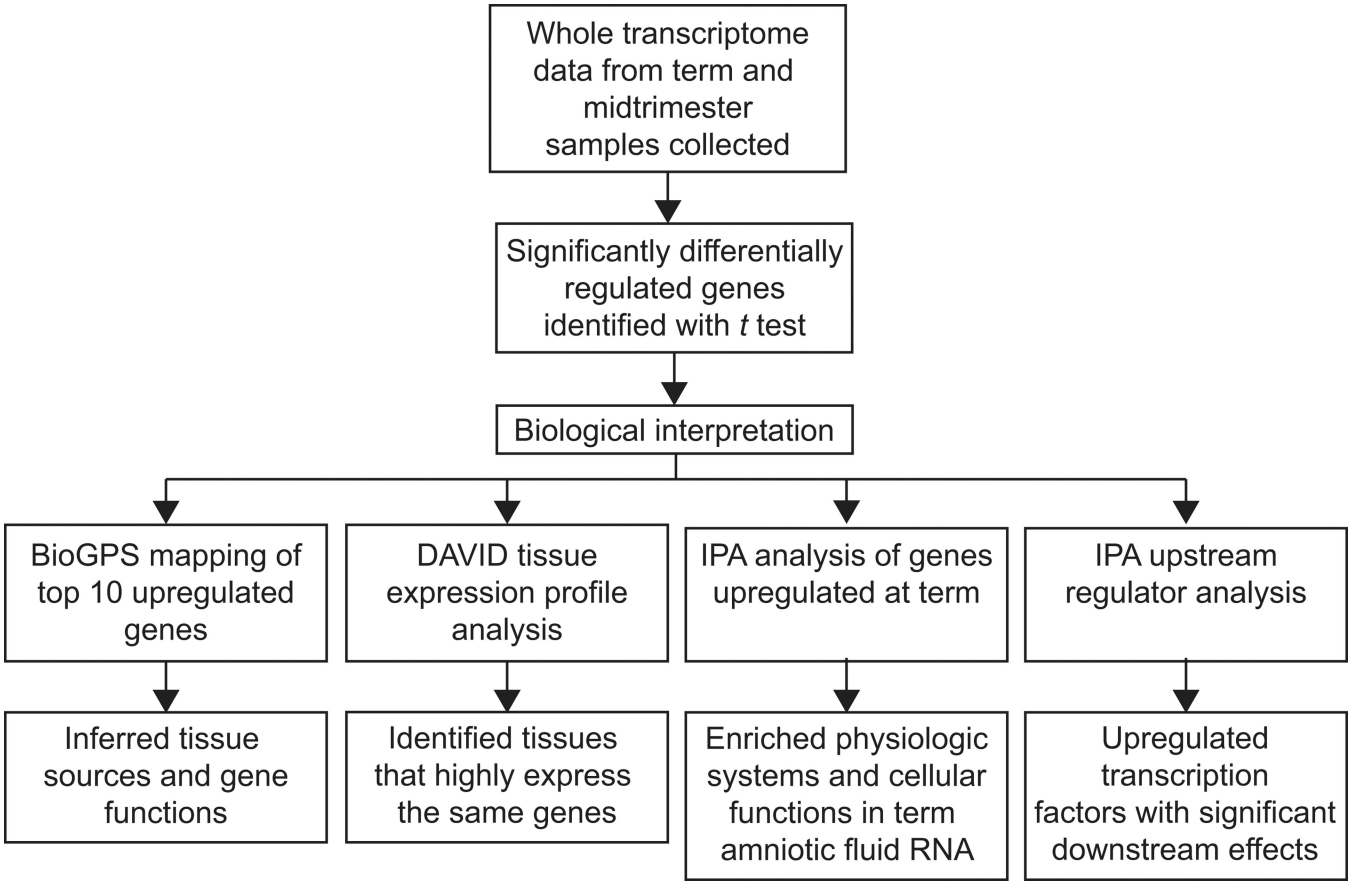

Figure 1.
Analytic methods used in the study. BioGPS, BioGPS Gene Expression Atlas; DAVID, Database for Annotation, Visualization and Integrated Discovery; IPA, pathways analysis software.

Table 1

Top 10 Upregulated Transcripts in Term Amniotic Fluid

Symbol	Gene Name	Gene Function	Tissue Specificity*	Fold Change	Benjamini-Hochberg *P*
MUC7	mucin 7, secreted	May function in a protective capacity by promoting the clearance of bacteria in the oral cavity and aiding in mastication, speech, and swallowing	Salivary gland > 30 MoM, trachea >10 MoM	1019.92	$< 1\times10^{-15}$
SFTPC	surfactant protein C	Pulmonary surfactant associated proteins promote alveolar stability by lowering the surface tension at the air-liquid interface in the peripheral air spaces	Lung > 30 Mom, fetal lung > 10 MoM	424.49	$< 1\times10^{-15}$
STATH	statherin	Salivary protein that stabilizes saliva supersaturated with calcium salts	Salivary gland > 30 MoM, trachea >10 MoM	416.19	$< 1\times10^{-15}$
RPTN	repetin	Involved in the cornified cell envelope formation. Multifunctional epidermal matrix protein.	NA	288.74	8.21×10^{-12}
SLC34A2	solute carrier family 34 (sodium phosphate), member 2	May have a role in the synthesis of surfactant in lungs' alveoli	Lung > 30 MoM	251.31	$< 1\times10^{-15}$
HTN3	histatin 3	Salivary proteins that are considered to be major precursors of the protective proteinaceous structure on tooth surfaces (enamel pellicle); exhibit antibacterial and antifungal activities	Salivary gland > 30 MoM	238.93	$< 1\times10^{-15}$
GKN1	gastrokine 1	Has mitogenic activity and may be involved in maintaining the integrity of the gastric mucosal epithelium	Not organ specific	161.50	$< 1\times10^{-15}$
SFTPA2	surfactant protein A2	Binds to surfactant phospholipids and is essential for normal respiration	Lung > 30 MoM	152.73	6.86×10^{-14}
MSMB	microseminoprotein, beta-	Member of the immunoglobulin binding factor family. This protein has inhibin-like activity.	Prostate > 30 MoM, trachea > 10 MoM	130.93	$< 1\times10^{-15}$
SFTPD	surfactant protein D	Contributes to pulmonary defense against inhaled microorganisms. May participate in the extracellular reorganization or turnover of pulmonary surfactant.	Lung > 30 MoM	99.05	$< 1\times10^{-15}$

MoM, multiples of the median; NA, not applicable.

Gene functions obtained from public databases (UniProtKB available at www.uniprot.org and Entrez Gene available at www.ncbi.nlm.nih.gov/gene). The information has been modified from the source due to space limitations.

*Tissue specificity as mapped in the BioGPS gene expression atlas (http://biogps.gnf.org).

Table 2

Database for Annotation, Visualization and Integrated Discovery Tissue Expression Analysis

Tissue Term	Fold Enrichment	Benjamini-Hochberg *P*
Term amniotic fluid		
Saliva	5.95	< 0.001
Salivary gland	2.50	0.031
Trachea	2.14	< 0.001
Fetal kidney	2.09	0.030
Second trimester amniotic fluid		
Fetal brain cortex	2.28	< 0.001
Cajal-Retzius cell	2.11	0.031

Table 3

Physiological Systems Significantly Enriched in Term Amniotic Fluid Cell-Free Fetal RNA

Category	Number of Genes
Immune cell trafficking	72
Digestive system development and function	51
Hematopoiesis	23
Cardiovascular system development and function	39
Hematological system development and function	23
Tissue development	72
Hair and skin development and function	23
Embryonic development	7
Organismal development	87
Renal and urological system development and function	35
Organ morphology	34
Reproductive system development and function	43
Connective tissue development and function	26
Skeletal and muscular system development and function	56
Nervous system development and function	24
Tissue morphology	112
Lymphoid tissue structure and development	20
Respiratory system development and function	16

Listed in descending order of statistical significance. Detailed results are provided in Appendix 4, available online at http://links.lww.com/xxx.