The Amniotic Fluid Transcriptome as a Guide to Understanding Fetal Disease

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Numerous recent studies have shown the power of cell-free fetal RNA, obtained from amniotic fluid supernatant, to report on the development of the living fetus in real time. Examination of these transcripts on a genome-wide basis has led to new insights into the prenatal pathophysiology of multiple genetic, developmental, and environmental diseases. Each studied condition presents a unique, characteristic fetal transcriptome, which points to specific disrupted molecular pathways. These studies have also improved our knowledge of the normal development of the human fetus, revealing gestational age-related dynamic gene expression from a variety of organs. Analysis of the fetal transcriptome in normal and abnormal development has led to novel approaches for in utero prenatal treatment.

Anatomical barriers that protect the developing human fetus also limit our ability to safely and ethically access and observe the human fetus in an ongoing live pregnancy. Historically, knowledge about fetal development, normal or anomalous, was derived from investigation of either aborted fetuses, the placenta after delivery or cultured cells from amniotic fluid obtained for diagnostic purposes. These tissues have been effectively used to obtain powerful information about human development, but are nonetheless imperfect proxies for the living fetus. Terminated fetuses have been subject to trauma, and prolonged cell-culture often results in genetic changes (Sambuy et al. 2005; Li et al. 2007). Other fetal material used for diagnostic purposes includes cell-free DNA in the maternal blood, which has been applied clinically for fetal Rhesus D detection and gender deter-

mination since 2001 (Lewis et al. 2012; Clausen 2014; Hyland et al. 2014). More recently, cell-free fetal (cff) DNA in maternal plasma has been used to screen for fetal aneuploidies (Song et al. 2013; Bianchi et al. 2014). Maternal blood contains both fetal and maternal cell-free nucleic acids, but the fetal contribution to plasma cell-free DNA is a minority (Canick et al. 2013; Rava et al. 2014). To examine pure fetal nucleic acids, therefore, fetal sources such as chorionic villi, placenta or amniotic fluid (AF) must be obtained through a diagnostic procedure.

AF refers to both the cellular and noncellular components of amniotic fluid, as well as the liquid itself. It is created from maternal blood plasma in the first trimester, but later comprised primarily of fetal urine, making it a much more concentrated source of fetal material than other maternal biofluids. It is a complex biofluid,

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containing both membrane-bound fetal cells (amniocytes) and myriad cell-free components, including placenta-derived microparticles, proteins, cffDNA and cffRNA from the fetus (Fig. 1) (Larrabee et al. 2005). Amniocytes are suspended in the AF, and sometimes undergo programmed cell-death, releasing cell-free transcripts. cffRNA may also contain transcripts from fetal tissues not directly in contact with AF (Hui et al. 2012a). During the first 20 wk of pregnancy, diffusion between the fetus and the AF is bidirectional via fetal skin, which is not yet keratinized (Visscher and Narendran 2014). This permeable membrane may provide avenues for cffRNA originating in diverse tissues to contribute to the extracellular RNA found in the AF.

Amniotic fluid removed by amniocentesis can be analyzed in the laboratory setting. Centrifugation of the AF separates the amniocytes from the amniotic fluid supernatant (AFS), within which are suspended cff transcripts. The di-

verse types of cffRNA found in AFS reflect the cellular processes active at the time the cells of origin underwent apoptosis—a time capsule from a distinct moment in fetal development. This information can be used to monitor the development of the fetus while characterizing the effects of naturally occurring perturbations, whether genetic or environmental.

In our laboratory, we have examined the AF transcriptome to measure up-to-the-moment cellular processes occurring in the live human fetus. Isolation and amplification of AFS transcripts, in tandem with a DNase treatment to preclude genomic DNA contamination, results in a diverse mixture of cffRNA that can be interrogated with high-throughput genomics assays, including expression microarrays. The bioinformatic data resulting from these expression studies can be further examined using in silico functional analyses to investigate the origin of the transcripts and the larger biological

Figure 1. Cell-free fetal RNA is contributed directly by the fetus as well as from apoptotic amniocytes of fetal origin.

systems and pathways functioning within the developing fetus.

METHODS

We have capitalized on the abundance of these AFS transcripts to characterize normal fetal development (Hui et al. 2012a, 2013a) while performing discovery-driven research into the developmental underpinnings of several common genetic and nongenetic diseases, each of which will be explored here (Fig. 2, Table 1). Each of these studies made use of cffRNA in residual AF specimens collected for routine clinical indications and used similar methodological approaches (Fig. 3). The RNA was extracted, amplified, labeled and hybridized to either the Affymetrix U133A expression microarray (Larrabee et al. 2005) or the Affymetrix GeneChip Human Genome U133 Plus 2.0 expression microarray (Slonim et al. 2009; Koide et al. 2011; Hui et al. 2012a,b, 2013a,b; Edlow et al. 2014; Massingham et al. 2014). Despite the availability of more sophisticated expression microarrays, the U133 Plus 2.0 was chosen because of the need for compatibility with an essential downstream in silico tool, the Connectivity Map (Lamb et al. 2006). For each study, microarray hybridization signal adjustment, normalization, and summary (Bolstad 2004) were followed by either paired or unpaired *t*-tests, with a Benjamini–Hochberg (BH) multiple hypothesis correction (Benjamini and Hochberg 1995) to reveal significantly differentially regulated genes.

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Each study identified candidate genes of interest through application of specific thresholds for significance based on corrected *p*-values, consistency among samples, magnitude of fold-change gene expression, and manual literature searches. Identification of misregulated gene expression pathways and tissue-specific expression was undertaken with a variety of powerful and well established in silico functional genomic analysis tools (Box 1).

STUDIES IN THE DEVELOPING FETUS

The majority of amniocenteses are performed in the second trimester of pregnancy, between 15 and 21 weeks' gestational age (GA). Common indications for this diagnostic procedure include advanced maternal age and soft markers for aneuploidies, such as elevated levels of alpha-fetoprotein in the maternal serum or an ultrasonographic observation of an enlarged nuchal fold or an echogenic focus in the fetus. In many cases, karyotyping confirms that the fetus is in fact euploid, and a healthy pregnancy follows. cffRNA from these samples can serve as a control for comparison against cffRNA from fetuses with diagnosed diseases.

Preliminary Studies: Disease, Gestational Age and Gender

The first in vivo whole-transcriptome microarray study of the living human fetus was a proof-of-principle experiment designed to consider

Figure 2. Cell-free fetal RNA in amniotic fluid supernatant provides an opportunity to study fetal gene expression in real-time.

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Table 1. Major features of recent studies in fetal biology using amniotic fluid supernatant (AFS) cell-free fetal (cff) RNA

Reference	Biological focus	Samples	Major findings	Number of differentially regulated genes	GEO accession number
Larrabee et al. 2005	Gestational age, sex, fetal polyhydramnios	10	<i>AQP1</i> , a water transport gene, was up-regulated in fetuses with TTTS. Surfactant proteins, mucins, and keratins varied with GA. Placental gene expression was not observed.	3342–6462 probe sets	N/A
Slonim et al. 2009	Down syndrome (T21)	14	Misregulation of ion transport and G protein signaling was observed, suggesting a link to the pathological symptoms in both neural and cardiac tissues later seen in DS.	414 probe sets	GSE 16176
Koide et al. 2011	Edwards syndrome (T18)	11	Misregulated genes and pathways of interest included <i>ROCK1</i> , ion transport, MHCII/T-cell mediated immunity, and adrenal development, among others. <3% of differentially regulated genes were on chromosome 18, suggesting that the majority of misregulation is caused by downstream effects.	251 genes (352 probe sets)	N/A
Hui et al. 2012a	Euploid fetal development	12	Multiple fetal tissues, including the central nervous system tissues, lung, placenta, and tongue contribute to AFS cffRNA.	N/A	GSE 33168
Hui et al. 2013b	Twin–twin transfusion syndrome (TTTS)	16	Differential expression of genes included those with known functions in fluid homeostasis, blood pressure regulation and angiogenesis. Enrichment was seen in pathways belonging to nervous, cardiovascular and hematological systems.	801 genes (872 probe sets)	GSE 47394
		10	Differentially regulated genes and pathways include <i>APLN</i> , and cardiovascular system development and function pathways.	611 genes (780 probe sets)	GSE 47394

Continued

Table 1. Continued

Reference	Biological focus	Samples	Major findings	Number of differentially regulated genes	GEO accession number
Edlow et al. 2014	Maternal obesity	16	Misregulated genes and pathways include <i>APOD</i> , <i>FOS</i> , <i>STAT3</i> and apoptotic cell-death. Patterns of gene misregulation suggest that maternal obesity affects fetal neurodevelopmental and metabolic gene regulation.	205 genes (205 probe sets)	GSE 48521
Massingham et al. 2014	Turner syndrome	10	Misregulated genes point to involvement of perivascular tissue, cholesterol homeostasis, and hematologic/immune system.	470 genes (807 probe sets)	GSE 58435

questions about the effects of sex, GA, and disease on the transcripts present in AFS (Larrabee et al. 2005). AF was obtained from four pregnancies in which each woman underwent therapeutic amnioreduction for polyhydramnios caused by twin–twin transfusion syndrome

(TTTS) or fetal hydrops. GA ranged from 20 to 32 wk for the three male and one female fetal cases. cffRNA from the pooled AFS of six unaffected euploid male fetuses (average GA = 17 wk) was used as a control. Between 15% and 29% of probe sets showed differential ex-

Figure 3. Experimental flow from preparation of cell-free fetal RNA to testing candidate drugs in a mouse model of disease.

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BOX 1. RESOURCES FOR IN SILICO FUNCTIONAL ANALYSES

The Online Mendelian Inheritance in Man Database (OMIM) (see <http://www.omim.org>) is an online catalog of human genes and genetic disorders. This comprehensive database provides references to relevant molecular and clinical research.

BioGPS (see <https://biogps.org>, Wu et al. 2009) is a centralized aggregation of protein-coding gene expression data for 78 noncancerous human tissues, including four tissues of fetal origin, and the placenta. Organ specificity was defined by a single organ expression value greater than 30 multiples of the median with the next highest organ expression at no more than one-third the maximum level. A limitation of BioGPS is that it lacks expression profiles for two fetal tissues of relevance to amniotic fluid—kidney and skin.

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov>, Dennis et al. 2003) synthesizes data from publicly available gene expression atlases into gene enrichment profiles for hundreds of normal and diseased human tissues. Given a list of expressed genes, DAVID can suggest the most likely tissues of origin and identify misregulated pathways.

QIAGEN's Ingenuity Pathway Analysis (IPA) (see <http://www.ingenuity.com/products/ipa>) is a commercially available platform to identify gene expression pathways enriched within a given data set. IPA will analyze a list of differentially expressed genes to suggest misregulated pathways and cellular functions. IPA has also been used with lists of expressed genes to identify active functions in a given population of fetuses.

Gene Set Enrichment Analysis (GSEA) (see <http://www.broadinstitute.org/gsea/index.jsp>, Subramanian et al. 2005) can identify sets of genes that are consistently differentially regulated as a group, even when the differential expression of individual genes lacks statistical significance. The Developmental Functional Annotation project at Tufts (DFLAT) (see <http://dflat.cs.tufts.edu/data.htm>, Wick et al. 2014) is gene ontology annotation specific to the developing human fetus. This database can be used with GSEA to identify developmental processes and functions associated with the observed differential gene expression.

The Connectivity Map (CMap) (see <https://www.broadinstitute.org/cmap/>, Lamb et al. 2006) is a database of gene expression signatures resulting from the treatment of human cell lines with known FDA-approved drugs. Associated software uses these signatures to suggest drugs that are expected to reverse the user-provided disease-associated gene expression patterns.

The Library of Integrated Network-Based Cellular Signatures (LINCS) (see <http://www.lincsproject.org>, Duan et al. 2014) shows the observed and predicted gene expression profile effects of treating 20 cell types with 4000 small molecule drugs. LINCS is an extension of CMap that shares the same goal of identifying drugs with a strong therapeutic potential to correct gene expression misregulation.

Developed by the Reproductive Toxicology Center, Reprotox (see <http://www.reprotox.org>) is a database summarizing the effects of various chemicals, infections and environmental toxins on reproductive health, pregnancy, and fetal development.

pression when compared between the TTTS cases and the pooled male control. Genes of particular relevance to the pathophysiology of hydrops or TTTS were examined, with significantly differential expression of aquaporin 1 (*AQP1*). These findings served as the basis for a subsequent, more thorough, examination of the effects of TTTS on fetal development (Hui et al. 2013b). Additionally, gene families known to be involved in fetal lung, skin and gastroin-

testinal development (surfactant, keratin, and mucin) showed differential expression between the control and cases. This suggested that the population of cffRNA present in AFS is affected by the sex and GA of the fetus. Outside the scope of this review, additional work has since been undertaken, which reveals marked differences between gene expression in the second and third trimesters of euploid fetal life (Hui et al. 2013a).

Absent from these data was evidence of several genes known to be expressed specifically in the placenta, including corticotropin-releasing hormone (*CRH*), chorionic somatomammotropin hormone 1 (placental lactogen), and the beta subunit of chorionic gonadotropin (β -*hCG*). This led to the conclusion that the placenta did not contribute to AFS cffRNA. More recent experiments, however, suggest that this conclusion may have been incomplete. Subsequent to this early work, changes in the RNA extraction technique to a superior column-based protocol, the improved chemistry of the amplification system, and an increased number of samples studied, may all contribute to the fact that more recent evidence shows that AFS does show expression of genes that are specific to the placenta (Hui et al. 2012a).

Our laboratory recently conducted a study comparing transcriptomes obtained from the AFS cffRNA and the first-passage amniocytes of eight fetuses (see section Applications to Fetal Therapy, below). In this study, we found that 40 unique placenta-specific genes are present in either the amniocytes or AF of at least seven of the eight individuals (Table 2). These analyses suggest that either the placenta does in fact contribute cffRNA to the AFS and cells to the population of amniocytes, or that these genes are not in fact specific to the placenta, but rather are expressed from other fetal tissues.

The Second Trimester Euploid Fetus

In 2012, Hui et al. performed a meta-analysis of previously published euploid fetal transcriptomes, considering only those genes expressed by all 12 of the subjects (six male, six female, GA = 16–21 wk) (Hui et al. 2012a). This “amniotic fluid core transcriptome” comprised 476 well-annotated genes, including 23 transcripts that were previously known to be expressed in a tissue-specific manner (including those from the liver, lung, and fetal brain). Additionally, functional analysis indicated that the 476 genes reported on six distinct physiologic functions, including musculoskeletal and nervous system development and function. This study detailed the biological richness of amniotic fluid and the

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Table 2. Placenta-specific gene expression

Gene symbol	Probe sets	Tissue
<i>ABP1</i>	1	AFS
<i>ADAM12</i>	2	AC
<i>ADM</i>	1	Both
<i>AIM1L</i>	1	AFS
<i>BMP1</i>	2	Both
<i>CAPN6</i>	2	AFS
<i>CDKN1C</i>	5	Both
<i>COBLL1</i>	1	Both
<i>CREB3L2</i>	1	Both
<i>CRIM1</i>	2	Both
<i>DAB2</i>	4	Both
<i>DAPK1</i>	1	Both
<i>DLG5</i>	1	Both
<i>EFHD1</i>	1	Both
<i>EGFR</i>	2	Both
<i>EPS8L1</i>	3	Both
<i>EVA1B</i>	1	Both
<i>EXPH5</i>	1	Both
<i>FAM46A</i>	1	Both
<i>FBN2</i>	1	AFS
<i>GDF15</i>	1	AC
<i>GPR126</i>	1	Both
<i>MAFF</i>	1	Both
<i>PAPPA</i>	2	AC
<i>PSG3</i>	1	AFS
<i>RASA1</i>	1	Both
<i>RHOBTB1</i>	1	Both
<i>RHOBTB3</i>	2	Both
<i>S100P</i>	1	AFS
<i>SDC1</i>	1	Both
<i>SLC5A6</i>	1	Both
<i>TAC3</i>	1	AFS
<i>TFAP2A</i>	1	AFS
<i>TGM2</i>	2	Both
<i>TIMP2</i>	1	Both
<i>TIMP3</i>	2	AFS
<i>TMEM2</i>	1	Both
<i>TPPP3</i>	1	AFS
<i>TUSC3</i>	1	Both
<i>VGLL1</i>	2	AFS

Genes for which probe set(s) known to have placenta-specific expression are observed as present in more than seven of the eight samples of either amniotic fluid supernatant (AFS), amniocytes (AC) or both.

contribution of many organ systems to AFS cffRNA, including those not in direct physical contact with amniotic fluid at the time of amniocentesis. An important implication of this work is the suitability of using cffRNA for the

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study of developmental disorders affecting a variety of fetal tissues, including those involving the central nervous system.

this was the first study to identify the misregulation of intermediate mechanisms, such as G protein signaling and ion transport.

Genetic Disorders: Down Syndrome

Down syndrome (DS) is estimated to occur in 1:691 live births, making it the most common genetic cause of intellectual disability (Parker et al. 2010). The disease is caused by a partial or complete trisomy of chromosome 21 (Lejeune et al. 1959) and results in a variety of physical differences, including short stature, cardiac defects, intellectual disability, and early-onset dementia. In the United States, prenatal screening for DS is offered for all pregnancies, but in the case of a positive diagnosis, there is no fetal treatment available.

In 2009, work from Slonim et al. began to compare the development of second trimester fetuses with DS (caused by a full trisomy 21, T21) to that of healthy euploid fetuses (Slonim et al. 2009). Expression microarray studies revealed a set of genes significantly differentially regulated between the seven age- and sex-matched pairs of fetuses. Of the 414 probe sets found to be differentially regulated, only five were located on chromosome 21. Using GSEA, a second set of genes, located on chr21q22, was found to be significantly up-regulated as a group, although the individual genes did not show significant signals of dysregulation. Importantly, the level of up-regulation for all 515 genes represented on the array from chromosome 21 was not simply the 1.5-fold that might be expected because of the presence of a third copy of chromosome 21. Rather, expression differences ranged from fivefold down-regulation to 16-fold up-regulation, suggesting that T21 results in complex intragenomic transcription dysregulation in the fetus. Using DAVID, the list of differentially expressed genes was examined for signatures of disrupted cellular processes. Slonim et al. concluded that the excess of reactive oxygen species in fetal cells disrupts ion transport and signal transduction, leading to pathological symptoms in both neural and cardiac tissues. Oxidative stress had previously been identified in DS (Esposito et al. 2008), but

Genetic Disorders: Edwards Syndrome

Edwards syndrome, or Trisomy 18 (T18), is the second-most common trisomy, occurring in 1:3762 live births (Parker et al. 2010). Because of high levels of prenatal mortality (75%) and low rates of postnatal survival (5%–10%) (Baty et al. 1994; Rasmussen et al. 2003), T18 is poorly studied by comparison with DS. Mortality is most often linked to cardiac and renal malformations or to postnatal feeding difficulties resulting from central nervous system abnormalities. A 2011 study by Koide et al. (2011) sought to compare the genome wide transcriptome of five female fetuses with T18 to six female euploid fetuses. Two hundred and fifty-one (251) genes showed statistically significant differential expression, seven of which were located on chromosome 18, and six of which overlapped with the 419 genes differentially regulated in DS (Table 3, Fig. 4). Specific genes of interest included *ROCK1* (Rho-associated kinase 1), known to be involved in valvuloseptal and endocardial formation and *ACTH* (adrenocorticotrophic hormone), which is required for normal morphological and functional development. Many of these genes were different from those first noted as differentially expressed by prior work in first trimester chorionic villi and cultured amniocytes (Altug-Teber et al. 2007). Pathways analysis using IPA and DAVID implicated dysregulation of ion transport functioning, MHCII/T-Cell mediated immunity, adrenal development, and cardiovascular and respiratory system functions. These findings are reminiscent of many clinical observations in T18, including the adrenal cortical zone gross and microscopic hypoplasia.

Genetic Disorders: Neurological Transcripts in DS and T18

Although trisomies 18 and 21 are the most common live-born autosomal aneuploidies, little was known about the dysregulated biological

Table 3. Relatively few genes are found to be differentially regulated in more than one study

Study	Probe set	Gene symbol	Gene name
DS & T18	1565537_at	<i>NKX1-1</i>	NK1 homeobox 1
	213250_at	<i>CCDC85B</i>	Coiled-coil domain containing 85B
	214420_s_at	<i>CYP2C9</i>	Cytochrome P450, family 2, subfamily C, polypeptide 9
	216251_s_at	<i>TTLL12</i>	Tubulin tyrosine ligase-like family, member 12
	223296_at	<i>SLC25A33</i>	Solute carrier family 25 (pyrimidine nucleotide carrier), member 33
TTTS & T18	230239_at	<i>ROCK1</i>	Rho-associated, coiled-coil containing protein kinase 1
	210835_s_at	<i>CTBP2</i>	Carboxy-terminal binding protein 2
	212745_s_at	<i>BBS4</i>	Bardet–Biedl syndrome 4
	213417_at	<i>TBX2</i>	T-box 2
	221306_at	<i>GPR27</i>	G protein-coupled receptor 27
TTTS & Ob	223007_s_at	<i>TMEM245</i>	Transmembrane protein 245
	202630_at	<i>APPBP2</i>	Amyloid beta precursor protein (cytoplasmic tail) binding protein 2
	205628_at	<i>PRIM2</i>	Primase, DNA, polypeptide 2 (58 kDa)
	210457_x_at	<i>HMGA1</i>	High mobility group AT-hook 1
	213615_at	<i>LPCAT3</i>	Lysophosphatidylcholine acyltransferase 3
	214429_at	<i>MTMR6</i>	Myotubularin related protein 6
	219503_s_at	<i>TMEM40</i>	Transmembrane protein 40
	219654_at	<i>PTPLA</i>	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A
	206286_s_at	<i>TDGF1</i>	Teratocarcinoma-derived growth factor 1
	208255_s_at	<i>FKBP8</i>	FK506 binding protein 8, 38 kDa
TTTS & DS	213753_x_at	<i>EIF5A</i>	Eukaryotic translation initiation factor 5A
	221766_s_at	<i>FAM46A</i>	Family with sequence similarity 46, member A
	222389_s_at	<i>WAC</i>	WW domain containing adaptor with coiled-coil
	223122_s_at	<i>SFRP2</i>	Secreted frizzled-related protein 2
	1554239_s_at	<i>ZADH2</i>	Zinc binding alcohol dehydrogenase domain containing 2
TTTS & Turner	202124_s_at	<i>TRAK2</i>	Trafficking protein, kinesin binding 2
	204191_at	<i>IFNAR1</i>	Interferon (alpha, beta and omega) receptor 1
	208767_s_at	<i>LAPTM4B</i>	Lysosomal protein transmembrane 4 beta
	211678_s_at	<i>RNF114</i>	Ring finger protein 114
	212415_at	<i>SEPT6</i>	Septin 6
Turner & T18	212530_at	<i>NEK7</i>	NIMA-related kinase 7
	226235_at	<i>LINC00667</i>	Long intergenic nonprotein coding RNA 667
	236219_at	<i>SLC35G1</i>	Solute carrier family 35, member G1
	223792_at	<i>ZNF2</i>	Zinc finger protein 2
	201111_at	<i>CSE1L</i>	CSE1 chromosome segregation 1-like (yeast)
Turner & Ob	207723_s_at	<i>KLRC3</i>	Killer cell lectin-like receptor subfamily C, member 3
	209176_at	<i>SEC23IP</i>	SEC23 interacting protein
	211741_x_at	<i>PSG3</i>	Pregnancy-specific beta-1-glycoprotein 3
	223054_at	<i>DNAJB11</i>	DnaJ (Hsp40) homolog, subfamily B, member 11

DS, Down syndrome (Slonim et al. 2009); Ob, obesity (Edlow et al. 2014); T18, Edwards syndrome (Koide et al. 2011); TTTS, Twin–twin transfusion syndrome (Hui et al. 2013b); Turner, Turner syndrome (Massingham et al. 2014).

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Figure 4. Examined transcriptomes share few misregulated genes. Numbers in parentheses show total numbers of misregulated genes for a given study (vs. GA- and sex-matched controls). Numbers in white squares represent common genes that are dysregulated in both overlapping conditions.

pathways that resulted in well-characterized fetal morphological anomalies. In the studies of both DS and T18, differentially regulated genes included those that are thought to be expressed specifically from the central nervous system. This finding motivated a 2012 meta-analysis to mine previously published data (Slonim et al. 2009; Koide et al. 2011; Hui et al. 2012a) with a specific focus on neural transcripts (Hui et al. 2012b). Hui et al. identified the 536 genes universally present in the cffRNA of 12 of 12 euploid samples, 746 genes universally present in the cffRNA of five of five fetuses with T18, and the 1184 genes universally present in the cffRNA of seven of seven fetuses with DS. Using the functional analytics available through DAVID, IPA, and BioGPS, Hui et al. identified enrichment of the nervous system among differentially regulated genes in the aneuploid fetuses. This meta-analysis also reexamined genes previously identified as being differentially expressed in aneuploid fetuses and discovered that several are known to be expressed specifically from the central nervous system, including ones known to be critical for nervous

system development. These genes included *NEUROD2* (neuronal differentiation 2, down-regulated in T18), which induces neurogenic differentiation and *SOX11* (SRY-related HMG-box Gene 11, down-regulated in DS), a transcription factor that is essential for pan-neuronal protein expression and axonal growth of sensory neurons.

Genetic Disorders: Turner Syndrome

Turner syndrome (TS) is a sex chromosome aneuploidy (45, X female) characterized by anatomical differences, including short stature, a webbed neck, dysfunction of the kidneys, heart and ovaries, and an increased risk of autoimmune disorders. Comorbidities include obesity and glucose intolerance, scoliosis, atherosclerosis, hyperlipidemia, and juvenile rheumatoid arthritis. In a 2014 study, Massingham et al. (2014) compared five second trimester female fetuses, monosomic for the X chromosome, to five GA- and sex-matched euploid control fetuses. Four hundred and seventy (470) differentially regulated genes were identified (BHp <0.015).

Genes showing the most extreme differential expression (fold-change ≥ 5) were submitted to IPA, and tissue-specific genes belonging to the top five most common organ systems were subjected to manual curation using Online Mendelian Inheritance in Man (OMIM). These in silico analyses revealed that among differentially regulated genes, there was enrichment for genes and pathways related to known symptoms of TS. This includes *NEATC3*, which is involved in perivascular tissue remodeling (of interest given that coarctation of the aorta is a feature of TS) and *LDLR*, which is involved in cholesterol homeostasis (of interest given that hyperlipidemia is a feature of TS). This enrichment also encompassed genes related to the hematologic/immune pathway, which may relate to the autoimmune disorders commonly seen in women with TS (Table 1). Additionally, by identifying sets of differentially regulated genes, this study revealed several candidate genes not previously known to be associated with the condition, which are suitable for follow-up study. A “Turner syndrome transcriptome” was also created, comprised of genes for which each of the five TS samples consistently yielded a “present” signal on the microarray. The TS transcriptome was compared against other fetal transcriptomes (e.g., euploid [Hui et al. 2012a], trisomies 18 and 21 [Hui et al. 2012b]) and was subjected to BioGPS analysis to identify tissue-specific transcripts enriched specifically in amniotic fluid from fetuses with TS.

The four studies presented above all examined the genome-wide changes to fetal gene expression that result from a single chromosome aneuploidy. As a whole, they suggest that the pathophysiology of these disorders does not arise from simple gene dosage effects of the aneuploid chromosome. Rather, an intragenomic web of expression misregulation contributes to create a distinct molecular and clinical phenotype that is characteristic of each disease, and shares few similarities with the others (Table 3, Fig. 4). Studies of nongenetic complications of pregnancy, such as maternal obesity and further work on TTTS, have also yielded compelling evidence of disease-specific fetal molecular phenotypes, and are examined below.

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Developmental Disorders: Twin–Twin Transfusion Syndrome

TTTS is a complication of monochorionic diamniotic twin pregnancy associated with very high perinatal mortality rates (Shah and Chaffin 1989; Gonsoulin et al. 1990; Saunders et al. 1992). Shared placental vascular anastomoses allow a net transfer of blood from one twin (the donor) to the other (the recipient). This disrupts regulation of fluid balance and fetal growth, leading to cardiovascular dysfunction and major neurodevelopmental irregularities in the fetus, as well as low birth-weight and premature delivery. TTTS diagnoses are further divided into stages; fetuses with stages II and III TTTS were the focus in this study. Stages II and III both show discordant AF volumes relative to a healthy fetus, but a stage III diagnosis involves a critically abnormal Doppler study for either the donor twin, the recipient twin, or both.

The typical treatment for TTTS is amnioreduction of the recipient twin combined with prenatal laser ablation of the shared placental anastomoses, which results in significantly improved survival of one or both fetuses (Senat et al. 2004; Roberts et al. 2008). Long-term health complications persist, however, including abnormal neurological function and increased risk of cardiac structural dysmorphism and long-term diastolic dysfunction. A long-standing question within the field has been whether these morbidities arise from the disease itself, or are complications resulting from the laser ablation treatment. As discussed above, preliminary examination of fetal gene expression in the context of TTTS suggested differential expression of the water-transport gene *AQP1* in the recipient twin (Larrabee et al. 2005). In 2013, Hui et al. revisited this disease, making a focused study of transcriptome-wide changes to fetal gene expression of the recipient twin in the second trimester, comparing gene expression between unaffected fetuses and fetuses with stage III TTTS, and comparing expression for fetuses with stages II and III TTTS.

In this study, eight recipient twins were each paired (for sex and GA) with eight healthy sin-

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gleton controls. Eight hundred and one (801) genes showed statistically significant differential gene expression between TTTS and unaffected fetuses. This list included genes with known functions in fluid homeostasis, blood pressure regulation and angiogenesis. Additionally, functional analyses of the 801 genes identified their enrichment in physiological systems consistent with known long-term complications of TTTS in the cardiovascular and nervous systems (Hui et al. 2013b).

Real-time quantitative PCR validation was performed for six of these genes, selected for clinical significance and magnitude of gene expression fold-change. The microarray results were confirmed for four of the six, including *NRXN3*, *NTRK3*, *FLT1*, and *AVPR1A*, which are involved in synapse formation, nerve growth and formation of cardiac structures, angiogenesis and vasoconstriction, respectively. The amniotic fluid used in these studies was collected just before the initiation of laser ablation treatment. This allowed the investigators to conclude that the neurological and cardiovascular anomalies suggested by the differential gene expression analysis are intrinsic to TTTS itself, and are not a side effect of the laser ablation treatment.

In a second experiment from this same study, five stage II and five stage III samples were compared as groups in an unpaired analysis. Six hundred and eleven (611) genes showed statistically significant differential expression between these two groups. Functional analysis using IPA showed enrichment of genes associated with cardiovascular system development and function.

Environmental Exposures: Maternal Obesity

Maternal obesity is present in about one third of pregnancies in the United States (Flegal et al. 2012). Maternal obesity during pregnancy has been associated with neurodevelopmental and metabolic conditions in the offspring, but the developmental underpinnings are poorly studied. In 2014, Edlow et al. conducted a study to examine whole-transcriptome differences in fetal gene expression from pregnancies of eight

obese (BMI ≥ 30) and eight lean women (BMI < 25). Two hundred and five (205) genes were found to be significantly differentially regulated between each of the eight pairs of fetuses, which were matched for sex and GA. At ninefold up-regulation, apolipoprotein D (*APOD*) was the most up-regulated gene in fetuses of obese women. *APOD* is highly expressed in tissues of the central nervous system and is essential to proper lipid regulation. Functional analysis of the differentially regulated genes using IPA suggested down-regulation of apoptotic cell death, particularly in nervous system pathways involving the cerebral cortex. IPA analysis also allowed the prediction of activation of transcript regulators in fetuses of obese women, identifying three genes involved in estrogen and cytokine signaling, *ESR1/2*, *FOS*, and *STAT3*. Together, these findings suggest that maternal obesity creates a proestrogenic and proinflammatory intrauterine environment.

APPLICATIONS TO FETAL THERAPY

The initial goal of these studies was to assess fetal development in several organ systems at a discrete time point of fetal life in response to a known abnormality (genetic, hemodynamic, or environmental). The detail and complexity revealed by these investigations has allowed for progression toward a second goal—the development of in utero treatments that can be offered at the time of disease diagnosis. Treatment at these critical early stages of development could mitigate the life-long health problems that originate in altered fetal development.

Work in our laboratory is already underway to develop prenatal treatments for oxidative stress in human fetuses with DS (Guedj and Bianchi 2013a; Guedj et al. 2014). Compounds identified using the Connectivity Map database (see <http://www.broadinstitute.org/cmap/>), including the antioxidant drug apigenin, are being tested through in vitro studies on amniocytes from human AF and in vivo studies in the Ts1Cje mouse models of Down syndrome (Guedj et al. 2013b). A preliminary stage of candidate drug testing is assessing the drug's toxicity and effects on cell proliferation in vitro through treatment

of cells in culture. Secondly, the efficacy of the drug in reducing oxidative stress is objectively measured by the COMET assay that quantifies the number of double stranded DNA breaks per cell (Tice et al. 2000). In vivo studies include embryonic brain gene expression analysis, as well as neonatal and adult behavioral testing in apigenin-treated and untreated Ts1Cje mice and their littermate controls (Fig. 3). The in vitro experiments require mitotically active cells, so fresh amniocytes are used in lieu of cffRNA.

Comparing AFS Cell-Free and Cellular RNA

The cffRNA found in AFS is a complex assortment of transcripts from many organs (Hui et al. 2012a). With this in mind, we assessed the suitability of amniocytes as a proxy for cffRNA, by comparing gene expression in cffRNA and first passage amniocytes. In a previously unpublished study, whole amniotic fluid was collected from eight second-trimester fetuses (six singletons and one twin-pair, five male and three female) free from known structural and cytogenetic anomalies. The median GA was 17 wk (range 16⁵/₇–22⁴/₇ wk). For each sample, the

amniocytes were separated from the AFS by centrifugation and the cells were cultured for one passage. Thus, only mitotically active cells remained. Cellular RNA was then extracted from the amniocytes and cffRNA was extracted from the AFS of the same sample. Microarray expression analysis of this RNA resulted in surprising conclusions, including the fact that the cffRNA comprised a subset of the transcripts observed in the amniocytes (Fig. 5). Analysis by BioGPS and DAVID of the genes that were significantly differentially expressed between the two data sets showed that relative to the AFS cffRNA, amniocytes were enriched for transcripts originating from endothelial, fibroblast, and aortic cells. Compared with amniocytes, cffRNA showed enrichment in transcripts originating from the tongue and intestine.

The higher quality of cellular RNA compared with cffRNA likely contributes to the finding that amniocytes contained transcripts for a greater number of genes (10,825 vs. 7,531) (Fig. 5). Cellular RNA is better protected from degradation, and so more consistently observed in the samples. Indeed, 52% of the genes observed in any one amniocyte RNA sample are observed consistently in all eight samples; this number drops to 36% for AFS cffRNA. Addi-

Figure 5. The majority of genes detected as present in amniotic fluid supernatant cell-free fetal RNA (AFS cffRNA) are also detected as present in amniocyte RNA.

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tionally, it may be that although a diversity of fetal tissues contribute cells to AF, not all of these tissues or cells go on to contribute to the cffRNA found in AFS, resulting in a decreased number of transcripts in cffRNA relative to those seen in amniocyte cellular RNA.

Considering the presence of some of the genes of interest from the Hui et al. (2012a) “transcriptome” study shows that nearly all

the genes that are observed in the AFS cffRNA are also observed in amniocyte RNA, including transcripts specific to the central nervous system (Table 4). There are some differences between the two AFS cffRNA studies, perhaps caused by the degraded nature of cffRNA resulting in inconsistent microarray signal calling, or to variations in the sex and GA of the studied fetuses. However, these data show that amniocytes are

Table 4. Tissue-specific gene expression

Probe Set	Gene symbol	Gene name	Tissue	AFS cffRNA	AC
207608_x_at	<i>CYP1A2</i>	Cytochrome P450, family 1, subfamily A, polypeptide 2	Adult liver	8	8
207300_s_at	<i>F7</i>	Coagulation factor VII (serum prothrombin conversion accelerator)	Adult liver	8	7
213940_s_at	<i>FBNP1</i>	Formin binding protein 1	B lymphoblasts	8	8
221923_s_at	<i>NPM1</i>	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	B lymphoblasts	8	8
214354_x_at	<i>SFTPB</i>	Surfactant protein B	Adult lung	7	6
215454_x_at	<i>SFTPC</i>	Surfactant protein C	Adult lung	2	1
33322_i_at	<i>SFN</i>	Stratifin	Bronchial epithelial cells	8	8
33323_r_at	<i>SFN</i>			8	8
218309_at	<i>CAMK2N1</i>	Calcium/calmodulin-dependent protein kinase II inhibitor 1	Prefrontal cortex	8	8
201853_s_at	<i>CDC25B</i>	Cell division cycle 25 homolog B (<i>Schizosaccharomyces pombe</i>)	CD4 ⁺ T cells	8	8
217878_s_at	<i>CDC27</i>	Cell division cycle 27 homolog (<i>Saccharomyces cerevisiae</i>)	CD71 ⁺ early erythroid	8	8
207030_s_at	<i>CSRP2</i>	Cysteine and glycine-rich protein 2	Fetal brain	8	8
204971_at	<i>CSTA</i>	Cystatin A (stefin A)	Tongue	8	1
209570_s_at	<i>D4S234E</i>	DNA segment on chromosome 4 (unique) 234 expressed sequence	Fetal brain	8	7
201278_at	<i>DAB2</i>	Disabled homolog 2, mitogen-responsive phosphoprotein (<i>Drosophila</i>)	Placenta	7	8
201681_s_at	<i>DLG5</i>	Discs, large homolog 5 (<i>Drosophila</i>)	Placenta	8	8
214395_x_at	<i>EEF1D</i>	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	Fetal thyroid	8	8
215017_s_at	<i>FBNP1L</i>	Formin binding protein 1-like	Fetal brain	8	8
218330_s_at	<i>NAV2</i>	Neuron navigator 2	Fetal brain	8	8
201928_at	<i>PKP4</i>	Plakophilin 4	Spinal cord	8	8
216470_x_at	<i>PRSS1/2/3</i>	Potase, serine, 1 (trypsin 1) / protease, serine, 2 (trypsin 2) / protease, serine, 3	Pancreas	8	8
205064_at	<i>SPRR1B</i>	Small proline-rich protein 1B	Tongue	8	2
208539_x_at	<i>SPRR2B</i>	Small proline-rich protein 2B	Tongue	8	0
212774_at	<i>ZNF238</i>	Zinc finger protein 238	Cerebellar peduncles	8	8

Genes for which probe set(s) known to have tissue-specific expression and are present in 12 of 12 amniotic fluid supernatant (AFS) cell-free fetal (cff) RNA samples contributing to the amniotic fluid core transcriptome (Hui et al. 2012a). Their presence in n of 8 AFS cffRNA or amniocyte (AC) RNA samples is indicated.

reasonably equivalent to AFS, and can be used as a proxy for the fetus when testing the effects of different therapies.

FUTURE DIRECTIONS

RNA-Sequencing

With the increasing affordability and availability of RNA-Sequencing (RNA-Seq), many microarray-based laboratory and clinical applications are transitioning to this newer, more efficient platform. Pilot experiments from our laboratory comparing the suitability of RNA-Seq and expression microarrays for studying AFS cffRNA have shown that the former is currently more susceptible to the technical challenges of working with cffRNA (Zwemer et al. 2014). cffRNA is by nature degraded and dilute, necessitating the development of a custom protocol for library creation. Work on this goal is currently underway in our laboratory, and will eventually allow our knowledge of fetal gene expression to expand to include many more genes than those represented on the HGU133 Plus 2.0 microarray.

The power of a whole-transcriptome approach lies in the ability to generate novel hypotheses for experimental approaches. In addition to creating new knowledge about relatively unknown aspects of fetal disease, these studies result in the identification of genes that are misregulated at the very time in development when essential neurological and physiological patterns are being established. Correcting gene misregulation through the use of pharmacological in utero treatments could minimize or prevent altered physiology. The work that began in DS in 2009 by examining cffRNA has resulted in preliminary experiments treating a mouse model of DS during pregnancy (Fig. 3). The ultimate goal, however, is to translate the work to human pregnancies.

CONCLUDING REMARKS

Taken together, this set of disease studies has shown the unique power of cffRNA to identify novel aspects of the prenatal development of multiple fetal organs in both healthy and dis-

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eased states, allowing identification of candidate disease-related genes. These studies also support the hypothesis that the developmental underpinnings of a variety of genetic and environmental diseases can be observed as early as the second trimester of pregnancy. Furthermore, each of these studies supports the view that altered gene expression in these affected fetuses is a genome-wide phenomenon, extending beyond the causal aneuploidy, to produce a unique and characteristic pattern of fetal gene expression. Finally, several studies have shown that the source of cffRNA can be traced to a variety of fetal organs as well as the placenta, and that fetal transcriptomes between first-passage amniocytes and AFS cffRNA share many similarities, making both useful resources in the efforts to develop prenatal therapies.

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