

# RNA-seq reveals conservation of function among the yolk sacs of human, mouse, and chicken

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**The yolk sac is phylogenetically the oldest of the extraembryonic membranes. The human embryo retains a yolk sac, which goes through primary and secondary phases of development, but its importance is controversial. Although it is known to synthesize proteins, its transport functions are widely considered vestigial. Here, we report RNA-sequencing (RNA-seq) data for the human and murine yolk sacs and compare those data with data for the chicken. We also relate the human RNA-seq data to proteomic data for the coelomic fluid bathing the yolk sac. Conservation of transcriptomes across the species indicates that the human secondary yolk sac likely performs key functions early in development, particularly uptake and processing of macro- and micronutrients, many of which are found in coelomic fluid. More generally, our findings shed light on evolutionary mechanisms that give rise to complex structures such as the placenta. We identify genetic modules that are conserved across mammals and birds, suggesting these modules are part of the core amniote genetic repertoire and are the building blocks for both oviparous and viviparous reproductive modes. We propose that although a choriovitelline placenta is never established physically in the human, the placental villi, the exocoelomic cavity, and the secondary yolk sac function together as a physiological equivalent.**

yolk sac | placenta | evolution

**T**he yolk sac is phylogenetically the oldest of the extraembryonic membranes, evolving in amniotes to absorb nutrients from their lipid-rich megalecithal eggs (1). Although the ova of eutherian mammals are microlecithal, the yolk sac has been recruited to transport maternal nutrients during earliest stages of embryonic development. In the majority of species, it makes contact with the chorion to form a transient choriovitelline placenta. This transient choriovitelline placenta functions during the critical period of organogenesis, at the end of which its functions are generally subsumed by the definitive chorioallantoic placenta. There is, however, considerable species variation, and the most elaborate development is found in rodents and lagomorphs. In these, the yolk sac continues to transport nutrients and immunoglobulins throughout gestation in parallel with the chorioallantoic placenta. For this reason, most of the experimental data on transport have been obtained in the mouse, rat, and guinea pig (2–6), and data on the human yolk sac are limited.

The human yolk sac goes through two developmental phases: A primary yolk sac develops between embryonic days 7 and 9 and is replaced by a secondary yolk sac, which is active until embryonic day 49 (7). The role of the primary yolk sac is unknown. The importance of the secondary yolk sac remains controversial. Although it is known to synthesize proteins, such as alpha fetoprotein, its transport functions are widely considered vestigial, primarily because the secondary yolk sac never makes contact with the chorion to form a choriovitelline placenta. Instead, it floats in the exocoelomic cavity, connected to the embryo by the vitelline duct. Although we, and others, have speculated that the

yolk sac plays a critical role during organogenesis (3–5, 8–10), there are limited data to support this claim. Obtaining experimental data for the human is impossible for ethical reasons, and thus we adopted an alternative strategy. Here, we report RNA sequencing (RNA-seq) data derived from human and murine yolk sacs and compare them with published data from the yolk sac of the chicken. We postulate that conservation of transcripts across these species indicates retention of key transport and synthetic functions. We support this hypothesis by comparing the human yolk sac transcriptome with the proteome of the coelomic fluid.

## Results and Discussion

We determined the transcript profile for the first-trimester human yolk sac by RNA-seq with a median sequencing depth of 39 million mapped reads per sample ( $n = 9$ ) (*SI Appendix, Table S1*). We identified 12,469 transcripts with a mean reads per kilobase per million mapped reads (RPKM)  $\geq 1$  (*Dataset S1*). Similarly, we identified 11,628 transcripts in first-trimester human placental villous samples ( $n = 11$ , median sequencing depth 30 million mapped reads) (*Dataset S2*) and 11,272 transcripts in the mouse yolk sac ( $n = 8$ , median sequencing depth 28 million mapped reads) (*Dataset S3*).

## Significance

**The human yolk sac is often considered vestigial. Here, we report RNA-sequencing analysis of the human and murine yolk sacs and compare with that of the chicken. We relate the human RNA-sequencing data to coelomic fluid proteomic data. Conservation of transcripts across the species indicates the human secondary yolk sac likely performs key functions early in development, particularly uptake and processing of macro- and micronutrients, many of which are found in coelomic fluid. More generally, our findings shed light on evolutionary mechanisms giving rise to complex structures such as the placenta. We propose that although a choriovitelline placenta is never established physically in the human, the placental villi, exocoelomic cavity, and secondary yolk sac function together as a physiological equivalent.**

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The authors declare no conflict of interest.

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Data deposition: The datasets generated during the current study are available in the European Nucleotide Archive (ENA) ([www.ebi.ac.uk/ena](http://www.ebi.ac.uk/ena)) [accession no. PRJEB18767 ([www.ebi.ac.uk/ena/data/view/PRJEB18767](http://www.ebi.ac.uk/ena/data/view/PRJEB18767))].

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In addition, we investigated the protein composition of the coelomic fluid using gel electrophoresis liquid chromatography (GELC)-MS/MS. We focused on the 165 proteins identified in any four of the five samples after immunoglobulins were excluded (Dataset S4). Proteins were mapped to unique Ensembl gene identifiers, which were used to identify overrepresented gene ontology (GO) terms (Dataset S5).

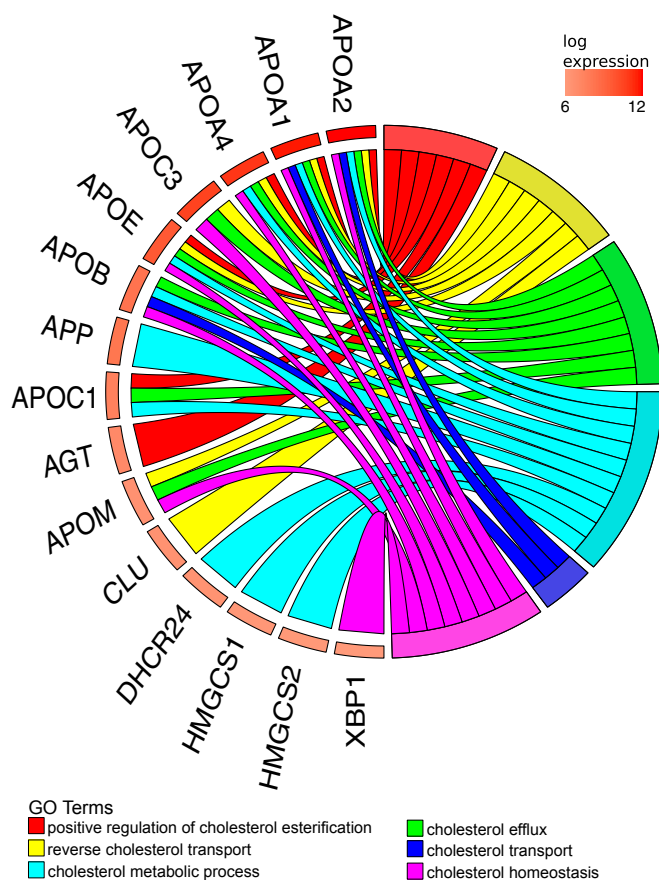
**Cholesterol.** We selected the 400 most abundant human yolk sac transcripts and identified enriched GO terms using Panther (complete reference database with Bonferroni correction) (Dataset S6). Several terms associated with lipid transport were enriched; e.g., the term “very-low-density lipoprotein particle” was enriched 23-fold ( $P = 4.5 \times 10^{-7}$ ). Indeed, the term “cholesterol” featured in many of the enriched biological process terms (Fig. 1). Cholesterol is required for development (3, 5) because it maintains the integrity of cell membranes (11), mediates metabolism through propagation of signaling pathways (12), and is the precursor for steroid hormones. In addition, activity of sonic hedgehog (SHH) proteins, which are responsible for the development of the central nervous system (13–15), is determined by covalent modification with cholesterol and other lipids (16). During organogenesis, the embryo is reliant on maternal sources of cholesterol until its liver is sufficiently mature for synthesis (4, 17). Our data show that the human yolk sac contains abundant mRNAs encoding multiple apolipoproteins, the cholesterol efflux transporter ABCA1, and lipoprotein receptors, including megalin and cubilin (18), albeit at lower levels

(Fig. 1). Also present are transcripts encoding all classes of ABC transporters (A–G), which, in addition to transporting cholesterol and lipids, facilitate the excretion of toxins and confer multidrug resistance (Table 1). The high abundance (i.e., top 0.5%) of transcripts encoding apolipoproteins present in lipoprotein particles and chylomicrons (ApoB, ApoA1, ApoA2, and ApoA4) is matched by the high levels of these proteins in the coelomic fluid (Dataset S4). Indeed, most of the proteins found in coelomic fluid are highly ranked in the RNA-seq data (although some were undetectable, i.e., below the threshold of RPKM  $\geq 1$ ). Many of these proteins have functions associated with cholesterol or lipid transport and metabolism (Fig. 2).

APOB is an essential apolipoprotein found in very-low-density lipoprotein (VLDL) particles and chylomicrons. In the latter, which are assembled in enterocytes, the APOB transcript is edited by a cytidine deaminase (APOBEC1, apolipoprotein B mRNA editing enzyme catalytic subunit 1) to introduce a stop codon leading to the production of a short form of the apolipoprotein (APOB-48). The APOB transcripts in the human secondary yolk sac are all of the unedited hepatic form, and the APOBEC1 transcript was not detected. Transcripts encoding other proteins required for lipid transport (19) are also abundant; for example, the LDL receptor (LDLR) and microsomal triglyceride transfer protein, MTTP, are in the top 10% and 20%, respectively. The presence of these transcripts suggests that the secondary yolk sac plays a role in facilitating the transport of maternal lipids and cholesterol to the fetal compartments before vascularization of the placental villi is fully established.

The fluid of the exocoelom shares many proteins in common with maternal plasma, supplemented by the addition of specific decidual, trophoblastic, and yolk sac proteins. Analogous to maternal serum proteins (20), coelomic fluid proteins can be broadly categorized into common circulating proteins, coagulation and complement factors, blood transport and binding proteins, protease inhibitors, proteases and other enzymes, cytokines and hormones, channel and receptor-derived peptides, and miscellaneous (SI Appendix, Table S2) (21, 22).

**Transport.** The secondary yolk sac comprises an outer mesothelial epithelium and an inner endodermal layer, separated by dilated capillaries and a small amount of mesoderm (23). Both the mesothelial and endodermal epithelia display ultrastructural features typical of an absorptive epithelium, including numerous microvilli, coated pits, and pinocytotic vesicles. The GO term “transport” (GO:0006810) was overrepresented in the top 400 yolk sac transcripts (2.87 fold,  $P = 7.16 \times 10^{-47}$ ). Many such annotated transcripts were present in the most abundant 20% of transcripts ( $n = 87$ ) (Dataset S6). Most of these transporter genes are members of the solute carrier (SLC) family of transporters [for example: SLC38A2 (amino acids), SLC4A1 (anions), SLC20A1 (phosphate), and SLC25A37 (iron in the mitochondria)]. Transcripts encoding 259 SLC transporters were identified (the mean RPKM ranged from 134 to 1). We classified these transporters into 11 main groups on the basis of substrate category (24), i.e., amino acids, urea cycle, glucose, nucleoside sugars, metals, vitamins, neurotransmitters, inorganic ions, thyroid, organic ions, and miscellaneous, and matched them to their main substrates that we and others (21, 22, 25–35) have identified in coelomic fluid samples (Table 2). Zinc is the second most abundant trace element and is critical for embryonic development. It plays a role in numerous biological processes, including cell division, growth, and differentiation, and acts as a structural, catalytic, and regulatory component within transcription factors, enzymes, transporters, and receptors (36, 37). Absorbed zinc is mostly bound to albumin and  $\alpha 2$ -macroglobulin, both of which are abundant in coelomic fluid. In humans, zinc transport is mediated by 14 members of the ZIP family (SLC39A) and 10 members of the ZnT family (SLC30A). We detected mRNAs



**Fig. 1.** Chord plot illustrating the GO biological process terms that include “cholesterol” and that are overrepresented in the 400 most abundant yolk sac transcripts (Right) and the genes contributing to that enrichment (Left) arranged in order of their expression level.

**Table 1. Categorization of ABC transporters detected in human yolk sac tissue**

Family	Transporters detected in human yolk sac	Function
ABCA	ABCA1 <sup>*,†</sup> , ABCA2, ABCA3 <sup>*,†</sup> , ABCA5, ABCA7 <sup>*</sup>	Responsible for the transportation of cholesterol and lipids
ABCB	ABCB1 <sup>*,†</sup> , ABCB10 <sup>*,†</sup> , ABCB6 <sup>*,†</sup> , ABCB7 <sup>*,†</sup> , ABCB8 <sup>*,†</sup>	Several of the B family members are known to confer multidrug resistance in cancer cells. Some are located in the blood-brain barrier, liver, mitochondria, transports, peptides, and bile.
ABCC	ABCC1 <sup>*,†</sup> , ABCC10 <sup>*,†</sup> , ABCC2 <sup>*</sup> , ABCC3, ABCC4 <sup>*,†</sup> , ABCC5 <sup>*,†</sup> , ABCC6 <sup>*,†</sup> , ABCC6P1 <sup>*</sup> , ABCC6P2 <sup>*</sup>	Ion-channel and toxin excretion activity and reception on the cell surface; toxin excretion (fungal and bacterial toxins). Includes the CFTR protein, mutations in which cause cystic fibrosis.
ABCD	ABCD1 <sup>*,†</sup> , ABCD3 <sup>*,†</sup> , ABCD4 <sup>*,†</sup>	Are all used in peroxisomes.
ABCE/F	ABCE1 <sup>*,†</sup> , ABCF1 <sup>*,†</sup> , ABCF2 <sup>*,†</sup> , ABCF3 <sup>*,†</sup>	Not proper transporters; members contain ATP-binding domains without the transmembrane domain. Involved in regulating protein synthesis or expression.
ABCG	ABCG1 <sup>*</sup> , ABCG2 <sup>*,†</sup> , ABCG5 <sup>*,†</sup> , ABCG8 <sup>*</sup>	Transports lipids, diverse drug substrates, bile, cholesterol, and other steroids.

<sup>\*</sup>Also present in the mouse yolk sac.

<sup>†</sup>Also present in the chick yolk sac.

encoding 12 ZIP proteins and eight ZnT proteins in the secondary yolk sac. Immunostaining for the zinc transporter SLC39A7/ZIP7 was present in both the inner endodermal and outer mesothelial epithelia, suggesting uptake from the coelomic fluid and transport to the fetal circulation (Fig. 3). The outer mesothelial layer also expresses  $\alpha$ -tocopherol transport protein to facilitate vitamin E transport (38). Thus, our data suggest the human secondary yolk sac has a role in the transport of multiple nutrients and vitamins, including iron (seven iron-transporting SLC transcripts were identified) (Table 2) (39, 40) and vitamins A, B<sub>12</sub>, C, E, and folic acid (Table 2).

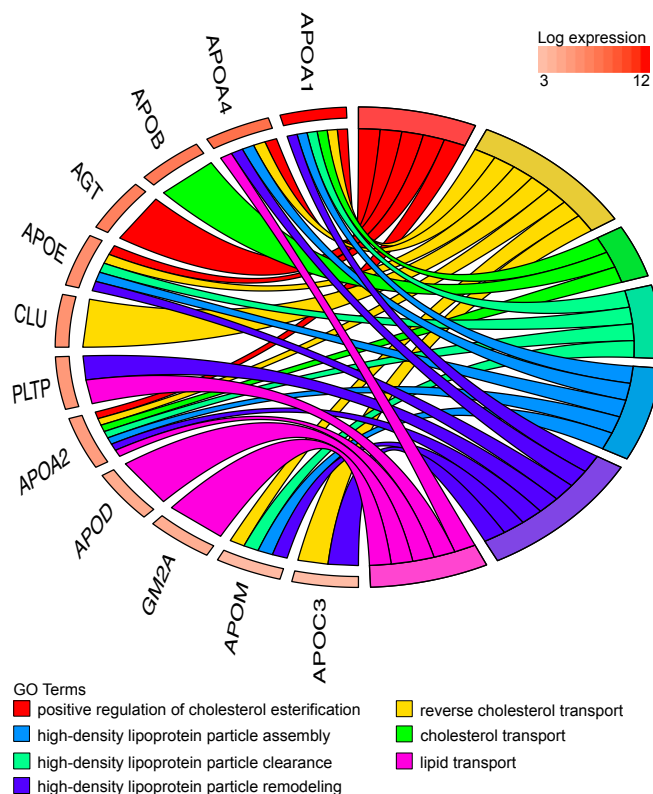
The total protein concentration is lower in coelomic fluid than in maternal plasma. However, most amino acids are at higher concentrations and must be derived from the villi and/or the yolk sac. This difference suggests that the coelomic cavity is an important route for metabolites required for embryonic development (25). The secondary yolk sac floats within this nutrient-rich milieu. It is therefore possible that uterine secretions supplemented by maternal plasma from spiral arteries are taken up by the trophoblast cells and are passed via the villous stromal channels into the exocoelomic cavity, from which they are taken up by the yolk sac and transferred to the embryonic gut and the fetal circulation via the vitelline duct (38). Thus, there appears to be free interchange between these two compartments of the human gestational sac.

The passage across the trophoblast may require lysosomal digestion of macromolecules, and indeed the GO term "lysosome" (GO:0005764) is enriched fourfold within the most abundant 400 villous transcripts (Bonferroni corrected  $P = 7.99 \times 10^{-9}$ ). The efflux amino acid transporters SLC43A2 and SLC7A8 are also highly expressed (above the 95th and 83rd percentiles, respectively). In the rat it has been estimated that ~95% of the amino acids provided to the fetus in midgestation are obtained by lysosomal digestion of endocytosed maternal proteins (2). In the mouse yolk sac, transcripts encoding five lysosomal cathepsins (Ctsl, Ctsz, Ctsb, Ctsd, and Ctsh) are among the most abundant 400 transcripts, the activity of which would allow the degradation and release of free amino acids to the developing fetus. We have previously shown that the mesothelial layer of the human yolk sac stains for glycodefin, a product of the uterine glands that is present in high concentration in the coelomic fluid (41), indicating exposure to intact maternal proteins and uptake (42). Glycodefin also colocalizes with lysosomal Cathepsin D in human first-trimester villi (43). As in the mouse, several cathepsin transcripts (*CTSB*, *CTSZ*, *CTSL*, and *CTSD*) are extremely abundant in the human secondary yolk sac. We found that the GO term "lysosome" is enriched in the list of most abundant transcripts from yolk sacs of human, mouse, and chicken (2.79 fold  $P = 1.35^{-3}$ ; 3.53 fold  $P = 9.72^{-5}$ ; and 3.6 fold  $P = 2.13^{-3}$ ,

respectively), indicating a similar capacity for digestion of endocytosed macromolecules.

These findings indicate that the exocoelomic cavity is a physiological liquid extension of the early placenta (44) and that the yolk sac is an important route of access for high molecular weight proteins to the embryonic circulation (45).

**Hematopoiesis.** In all vertebrates, primitive embryonic and definitive fetal/adult blood cells form successively within the yolk sac, fetal liver, and bone marrow (46). The human secondary yolk sac is the sole site of hematopoiesis for the first 2 wk of



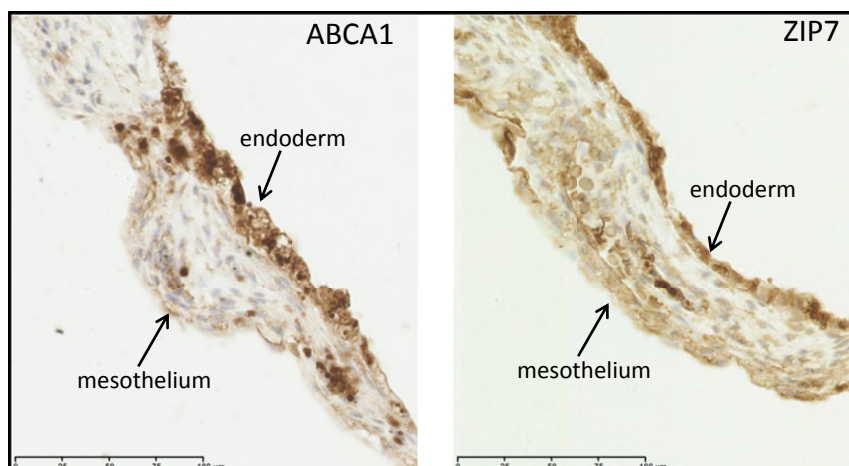
**Fig. 2.** Chord plot illustrating proteins present in the coelomic fluid that relate to GO biological process terms involving "cholesterol" and "lipid transport." The presence of these proteins is consistent with the high level of their transcripts in the human yolk sac illustrated in Fig. 1.

**Table 2. Categorization of SLC transporters detected in human yolk sac tissue**

Substrate category	SLC transporters detected in human yolk sac	Known substrates	Substrates detected in human coelomic fluid
Amino acids	SLC38A2 <sup>*,†</sup> , SLC7A7 <sup>*</sup> , SLC7A2, SLC1A5 <sup>*</sup> , SLC38A3, SLC3A2 <sup>*</sup> , SLC3A1 <sup>†</sup> , SLC25A38 <sup>*</sup> , SLC38A4 <sup>*</sup> , SLC38A10 <sup>*</sup> , SLC25A13 <sup>*,†</sup> , SLC7A8 <sup>*</sup> , SLC7A6 <sup>*,†</sup> , SLC43A1 <sup>*</sup> , SLC7A4, SLC7A9 <sup>*</sup> , SLC1A4 <sup>*</sup> , SLC38A7 <sup>*,†</sup> , SLC7A1 <sup>*</sup> , SLC15A4 <sup>*,†</sup> , SLC38A5 <sup>*</sup> , SLC7A6O5 <sup>*,†</sup> , SLC43A3 <sup>*,†</sup> , SLC38A9 <sup>*,†</sup> , SLC43A2 <sup>*,†</sup> , SLC36A4 <sup>*</sup> , SLC38A6 <sup>*,†</sup> , SLC38A11, SLC6A17, SLC7A10	Ala, Asn, Cys, Gln, Gly, His, Met, Pro, Ser, Glu, Trp, Asp	Thr, His, Arg, Val, Met, Ile, Leu, Phe, Lys, Ser, Gln, Glu, Tau, Ala, Pro, Tyr, Orn, Trp (21, 22, 25–27)
Urea cycle	SLC25A15 <sup>*,†</sup> , SLC2A9 <sup>†</sup>	Lys, Orn, Cit, Asp, Glu, uric acid	Urea (28, 29)
Glucose and sugars	SLC2A1 <sup>*,†</sup> , SLC2A3 <sup>*,†</sup> , SLC2A4RG, SLC50A1 <sup>*</sup> , SLC2A2 <sup>*,†</sup> , SLC1A1 <sup>†</sup> , SLC2A10 <sup>†</sup> , SLC2A14, SLC5A9 <sup>*</sup> , SLC45A4 <sup>*</sup> , SLC2A8 <sup>*</sup> , SLC2A12, SLC2A6, SLC2A13 <sup>†</sup> , SLC37A1, SLC2A5 <sup>†</sup> , SLC37A2 <sup>†</sup> , SLC2A7 <sup>*</sup> , SLC45A1, SLC2A11, SLC2A1-AS1	Glucose, galactose, fructose, mannose, glucosamine	Glucose, galactose, fructose, mannose, glucosamine, galactosamine, erythritol, ribitol, mannitol, inositol, glycerol, sorbitol (22, 30)
Nucleoside sugars	SLC35D2 <sup>*</sup> , SLC17A9 <sup>*</sup> , SLC35E2B <sup>†</sup> , SLC29A1 <sup>*</sup> , SLC35C2 <sup>*,†</sup> , SLC35A4 <sup>*,†</sup> , SLC35A2 <sup>*</sup> , SLC35E1 <sup>*,†</sup> , SLC25A36 <sup>*,†</sup> , SLC35D1 <sup>*,†</sup> , SLC35A5 <sup>*,†</sup> , SLC35F5 <sup>*,†</sup> , SLC37A4 <sup>*,†</sup> , SLC35F6 <sup>*</sup> , SLC35A3 <sup>*,†</sup> , SLC37A3 <sup>*,†</sup> , SLC35B1 <sup>*,†</sup> , SLC35C1 <sup>*</sup> , SLC52A2 <sup>*</sup> , SLC29A2 <sup>*</sup> , SLC29A3 <sup>*</sup> , SLC35B4 <sup>*,†</sup> , SLC35G1 <sup>*</sup> , SLC35E3 <sup>*,†</sup> , SLC29A4, SLC35F2 <sup>*,†</sup> , SLC35F3, SLC25A19 <sup>*</sup> , SLC35G2 <sup>*</sup> , SLC28A1 <sup>*</sup>	UDP-glucuronic acid, UDP-galactose, UDP-N-acetyl-galactosamine, GDP-fructose	
Metals	SLC30A5 <sup>*,†</sup> , SLC30A9 <sup>*,†</sup> , SLC40A1 <sup>*,†</sup> , SLC39A7, SLC39A14 <sup>*,†</sup> , SLC25A37 <sup>*,†</sup> , SLC39A6 <sup>*</sup> , SLC39A5 <sup>*</sup> , SLC39A1 <sup>*</sup> , SLC30A1 <sup>*,†</sup> , SLC31A1 <sup>*,†</sup> , SLC25A28 <sup>*,†</sup> , SLC39A13 <sup>*,†</sup> , SLC39A9 <sup>*,†</sup> , SLC39A8 <sup>*,†</sup> , SLC11A2 <sup>*,†</sup> , SLC30A6 <sup>*,†</sup> , SLC30A7 <sup>*</sup> , SLC41A1 <sup>*</sup> , SLC41A3 <sup>*</sup> , SLC39A11 <sup>*,†</sup> , SLC30A10 <sup>*,†</sup> , SLC31A2 <sup>*</sup> , SLC39A10 <sup>*</sup> , SLC41A2 <sup>†</sup> , SLC30A2 <sup>*</sup> , SLC39A4 <sup>*</sup> , SLC11A1, SLC39A3 <sup>*</sup> , SLC30A4 <sup>*,†</sup>	Zinc, iron, magnesium, copper, cadmium, cobalt, manganese, nickel, lead, barium, strontium	Zinc, iron, cadmium, magnesium, copper, manganese, lead, selenium (28, 31, 32)
Vitamins	SLC19A2 <sup>*</sup> , SLC46A3 <sup>*,†</sup> , SLC25A32 <sup>*,†</sup> , SLC23A2 <sup>*</sup> , SLC5A6 <sup>*</sup> , SLC46A1 <sup>*,†</sup> , SLC23A1 <sup>*</sup> , SLC19A3 <sup>*</sup> , SLC19A1 <sup>*</sup> , SLC23A3 <sup>*</sup>	Thiamine (vitamin B <sub>1</sub> ), folate, ascorbic acid, biotin, lipolate, pantothenate, thiamine	Vitamins A, E, B <sub>12</sub> , folate, cobalamin, retinol-binding protein 4, vitamin D-binding protein (21, 27, 29, 33–35)
Neurotransmitters	SLC44A2 <sup>*</sup> , SLC44A4 <sup>*</sup> , SLC6A6 <sup>*,†</sup> , SLC44A1 <sup>*,†</sup> , SLC36A1 <sup>*,†</sup> , SLC6A9 <sup>*,†</sup> , SLC1A3 <sup>*</sup> , SLC6A13 <sup>*</sup> , SLC44A3 <sup>†</sup> , SLC17A2, SLC25A22 <sup>*,†</sup> , SLC25A18, SLC25A12 <sup>*,†</sup> , SLC17A4, SLC6A12	Noradrenaline, serotonin, dopamine, glutamate, glycine, aspartate, choline	Glutamine, glutamic acid (21, 25)
Inorganic ions	SLC25A3 <sup>*,†</sup> , SLC9A3R1 <sup>*</sup> , SLC4A1 <sup>*,†</sup> , SLC20A1 <sup>*</sup> , SLC12A7 <sup>*,†</sup> , SLC4A2 <sup>*,†</sup> , SLC9A3R2 <sup>*</sup> , SLC34A2 <sup>*,†</sup> , SLC12A4 <sup>*,†</sup> , SLC26A6 <sup>*</sup> , SLC9A1 <sup>*</sup> , SLC20A2 <sup>*</sup> , SLC9A6 <sup>*,†</sup> , SLC4A1AP <sup>*</sup> , SLC9A9 <sup>†</sup> , SLC26A11 <sup>*,†</sup> , SLC9B2, SLC26A2 <sup>*</sup> , SLC24A3, SLC9A8 <sup>*</sup> , SLC12A8, SLC8B1 <sup>*</sup> , SLC12A2 <sup>*,†</sup> , SLC24A1, SLC4A7 <sup>*</sup> , SLC12A6 <sup>*</sup> , SLC4A3, SLC26A10, SLC26A1 <sup>*</sup> , SLC5A5, SLC9A7P1, SLC9A5 <sup>*</sup> , SLC8A1, SLC4A4 <sup>*</sup>	Na <sup>+</sup> , K <sup>+</sup> , Cl <sup>-</sup> , HCO <sub>3</sub> <sup>-</sup> , Ca <sup>2+</sup> , phosphate	Na <sup>+</sup> , K <sup>+</sup> , Cl <sup>-</sup> , HCO <sub>3</sub> <sup>-</sup> , Ca <sup>2+</sup> , phosphate (28, 29)
Thyroid Organic ions	SLC16A2 <sup>†</sup> , SLC7A5 <sup>*,†</sup> , SLC16A10 <sup>*,†</sup> , SLCO2B1 <sup>†</sup> , SLC22A7, SLC10A1, SLC10A3 <sup>*</sup> , SLC22A23 <sup>*,†</sup> , SLC51A <sup>*</sup> , SLC22A9, SLC22A17 <sup>*</sup> , SLCO4C1 <sup>*</sup> , SLCO2A1 <sup>*</sup> , SLC22A31, SLC22A18 <sup>*,†</sup> , SLC22A4 <sup>*</sup> , SLC22A5 <sup>*,†</sup> , SLC10A7 <sup>*,†</sup> , SLC51B, SLCO1B3, SLC22A15, SLC22A18A5, SLCO1B1, SLCO3A1 <sup>*,†</sup> , SLC17A3, SLC22A3 <sup>*</sup>	Iodide, iodothyronines Estradiol, glucuronide, bilirubin	Tyrosine, thyroxine (26) Bilirubin
Miscellaneous	SLC25A5 <sup>*</sup> , SLC5A12, SLC25A39 <sup>*,†</sup> , SLC25A1 <sup>*,†</sup> , SLC16A3 <sup>*</sup> , SLC35B2 <sup>*</sup> , SLC25A20 <sup>*</sup> , SLC17A5 <sup>*,†</sup> , SLC16A1 <sup>*,†</sup> , SLC16A4 <sup>†</sup> , SLC13A5, SLC35B3 <sup>*</sup> , SLC6A8 <sup>*</sup> , SLC27A2 <sup>*,†</sup> , SLC25A24 <sup>*,†</sup> , SLC25A23 <sup>*</sup> , SLC25A11 <sup>*</sup> , SLC25A44 <sup>*</sup> , SLC25A25 <sup>*</sup> , SLC45A3 <sup>*</sup> , SLC15A1 <sup>*</sup> , SLC25A46 <sup>*</sup> , SLC25A27, SLC25A42 <sup>*</sup> , SLC27A3 <sup>*</sup> , SLC25A17 <sup>*,†</sup> , SLC25A43 <sup>*,†</sup> , SLC35A1 <sup>*,†</sup> , SLC12A9 <sup>*,†</sup> , SLC16A5, SLC25A29 <sup>†</sup> , SLC18B1 <sup>*</sup> , SLC16A13 <sup>*,†</sup> , SLC25A30 <sup>*,†</sup> , SLC27A4 <sup>*,†</sup> , SLC16A9 <sup>*,†</sup> , SLC48A1 <sup>*,†</sup> , SLC33A1 <sup>*,†</sup> , SLC25A4 <sup>*</sup> , SLC25A14 <sup>*,†</sup> , SLC25A33 <sup>*</sup> , SLC25A16 <sup>*,†</sup> , SLC25A40 <sup>*</sup> , SLC25A34 <sup>*</sup> , SLC27A1 <sup>*,†</sup> , SLC25A26 <sup>*</sup> , SLC47A1 <sup>*,†</sup> , SLC15A3, SLC16A12 <sup>*,†</sup> , SLC25A45 <sup>*</sup> , SLC5A11 <sup>*</sup> , SLC25A51 <sup>*</sup> , SLC13A3 <sup>*,†</sup> , SLC16A14, SLC25A21-AS1, SLC16A7 <sup>†</sup> , SLC16A6 <sup>*</sup> , SLC15A2, SLC27A5 <sup>*</sup> , SLC17A1	ATP-ADP, carnitine, creatinine, acetyl-CoA, sialic acid, pyruvate, lactate, ketone bodies, bile acids, oxoglutarate, succinate, citrate, ketoglutarate, ornithine, acylcarnitine, melanin, prostaglandin, long-chain and very long-chain fatty acids, haem, ammonia, adenosine, taurocholic acid	Lactate, creatinine (29, 30)

\*Also present in the mouse yolk sac.

†Also present in the chick yolk sac.



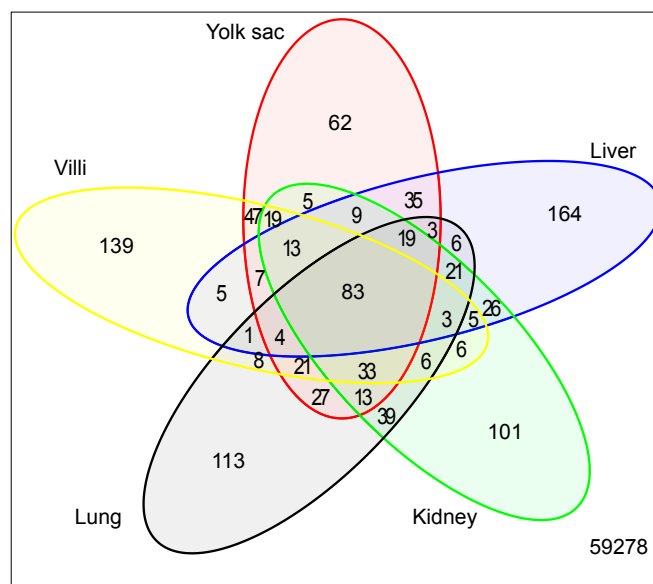
**Fig. 3.** Immunolocalization of ABCA1 and SLC39A7/ZIP7 transporter proteins in the human yolk sac at gestational age 11 wk. Sections were immunostained with anti-ABCA1 or anti-ZIP7 antibodies. In both cases, staining was present in the inner endodermal and outer mesothelial layers, although it was stronger in the former.

pregnancy, and the fetal liver commences blood cell production at week 6 of gestation (47, 48). The term “hemoglobin complex” was significantly enriched among abundant yolk sac transcripts (33 fold,  $P = 2.95 \times 10^{-6}$ ). The yolk sac produces predominantly nucleated erythrocytes, which synthesize embryonic hemoglobin (HBZ). There is morphological evidence of the first blood islands in the secondary yolk sac at about day 18 of gestation (49). Yolk sac-derived primitive erythrocytes have been detected in the cardiac cavity as early as the three-somite stage (21 d), indicative of an established functional network between the yolk sac and embryo (50). The human yolk sac, like that of the mouse, also produces macrophage and multipotential hematopoietic progenitors (48).

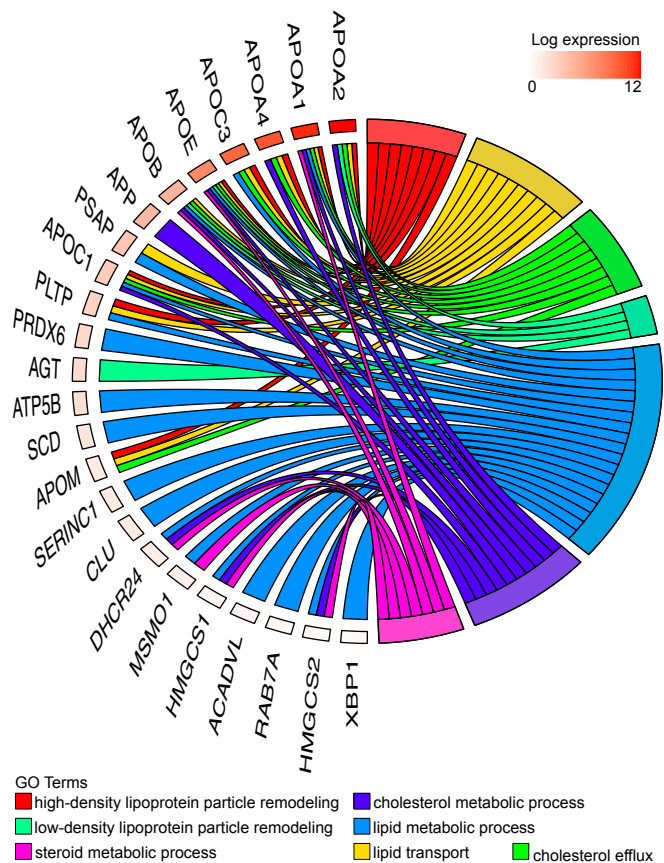
**Transcription Factors.** Within the most abundant 400 transcripts in the human yolk sac, 19 genes are annotated as “regulation of transcription, DNA-templated” (GO:0006355), including several transcription factors (ATF4, FOS, JUN, JUNB, and JUND.) In the mouse dataset, eight genes are similarly annotated (*SI Appendix, Table S3*). Candidate motifs recognized by these transcription factors (where known) were identified in the regions 1 kb and 5 kb upstream of the transcription start site (TSS) of genes that were highly correlated with the transcription factor transcripts (*Datasets S7–S10*). The FOS and JUN families and ATF4 are closely related, interact functionally, have multiple target genes, and are widely expressed. There are numerous candidate binding sites in the highly expressed human yolk sac genes (181 genes with sites for the FOS and JUN families and ATF4) (*Datasets S7 and S8*) and 18 genes in the mouse with candidate Atf4-binding sites (*Datasets S9 and S10*). The evidence used for the assignment of GO terms varies and for two genes (*IGF2* and *BHLHE40*) depends on a Nontraceable Author Statement (NAS) (*SI Appendix, Table S3*). Furthermore, binding motifs are not available for all candidate factors even in the most recent JASPAR database.

**Yolk Sac and Villi Compared with Adult Lung, Liver, and Kidney.** The inaccessibility of the human yolk sac severely constrains any functional investigation of these candidate transcription binding sites. We therefore compared the transcript profile of the human yolk sac with tissues whose function has been better defined experimentally. The placental villi serve functions similar to those of the adult lung, liver, and kidney, and we therefore compared the overlap among the 400 most abundant transcripts from these tissues and the yolk sac (Fig. 4). The transcripts that

are unique to each tissue and those shared among these tissues are listed in *Dataset S11*. As expected, the transcripts shared by all five tissues encoded abundant housekeeping proteins, such as ribosomal proteins, and those involved in mitochondrial energy generation, with GO terms such as “cytosolic small ribosome subunit,” “cytosolic large ribosome subunit,” and “mitochondrial respiratory chain” being significantly overrepresented ( $P < 8.5 \times 10^{-8}$  after Bonferroni correction) (*Dataset S12*). The enriched GO terms associated with the 35 transcripts shared only by the yolk sac and liver include “high-density lipoprotein particle receptor binding,” “cholesterol transporter activity,” and “lipid transporter



**Fig. 4.** Venn diagram comparing the most abundant 400 transcripts in the human yolk sac with first-trimester placental villi and adult liver, lung, and kidney. Transcripts shared by all five tissues ( $n = 83$ ) principally encoded housekeeping proteins, whereas those shared uniquely with liver ( $n = 35$ ) encoded proteins involved in cholesterol and lipid metabolism, suggesting that the yolk sac may perform these functions while the fetal liver develops. By contrast, there are few transcripts shared uniquely with the kidney ( $n = 5$ ), suggesting that the yolk sac plays little role in excretion.



**Fig. 5.** Chord plot connecting GO biological process terms associated with “lipid metabolism” and genes encoding transcripts that are shared by the human yolk sac and adult liver. The overlap suggests the yolk sac may perform hepatic functions while the fetal liver differentiates.

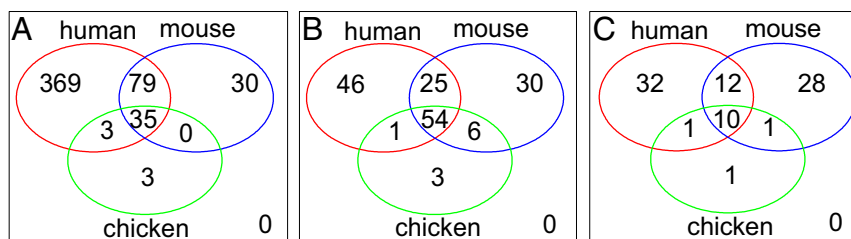
activity” (all greater than 28-fold enrichment and  $P < 2 \times 10^{-4}$  after Bonferroni correction) (Dataset S13).

We also examined the overlap among overrepresented GO terms in the most abundant transcripts in these five tissues (Datasets S6 and S14–S17). For example, the biological process terms “high-density lipoprotein particle remodeling,” “lipid transport,” “cholesterol efflux,” and “cholesterol metabolic process” are all overrepresented and shared between the yolk sac and liver (Fig. 5 and Datasets S18–S20). It is striking that among the shared transcripts many (18 of 35) encode proteins with transport capacity. Besides the genes associated with lipid and cholesterol transport (*APOA1*, *APOA2*, *APOA4*, *APOB*, *APOC1*, *APOC3*, and *APOH*), the major serum carriers of corticosteroids and progesterone (*SERPINA6*) are present. In addition, the thyroxine (T<sub>4</sub>) hormone carrier

*SERPINA7* (also known as “TBG” and “TTR”) was detected. Maternal thyroxine is essential for fetal brain development before the commencement of fetal thyroid function at the end of the first trimester (26). Furthermore, transcripts encoding several metal-binding or -transporting proteins are present (MT1E, MT1H, TF, HPX, and SEPP1). SEPP1 is a selenoprotein which contains 10 selenocysteine residues per molecule and is postulated to function as a carrier of selenium (51). These data indicate that the yolk sac shares many abundant transcripts with other metabolically active fetal and adult tissues and that it contains transcripts that encode several liver specific functions, including those encoding several essential hormone transporters.

**Mouse Yolk Sac RNA-Seq Data.** On a comparative basis, the yolk sac provides an important pathway for nutrient uptake in many species during early pregnancy (52, 53), and its role has been well documented in experimental rodents, such as the rat and mouse (54). At the ultrastructural level, the endodermal layers of the human and rodent yolk sacs display many similar morphological characteristics typical of an absorptive epithelium (10, 55). However, their orientation is different, because the human yolk sac floats in the exocoelom with the endodermal cell layer lining the interior of the sac, whereas the rodent yolk sac is inverted, with the endodermal layer facing outwards following degeneration of the parietal layer (56, 57).

**Cross-Species Comparison.** To compare the most abundant transcripts in the human, mouse, and chicken yolk sacs, we identified the homologous genes by mapping the human Ensembl gene identifiers to the corresponding mouse and chicken Ensembl gene IDs using BioMart (release ENSEMBL Genes 85) (Dataset S21). The raw and processed chicken yolk sac data were obtained directly from the authors of ref. 58. GO analysis was carried out as described above, using the 400 most abundant chicken yolk sac transcripts observed at E17 (Dataset S22). The intersection between the overrepresented GO terms in the human, mouse, and chicken yolk sacs was determined as described above (SI Appendix, Table S4). Venn diagrams showing the overlaps are shown in Fig. 6. The  $P$  values for all the observed intersections are highly significant ( $P < 1.70 \times 10^{-22}$ ) and are summarized in Dataset S23. The depth of the GO annotation varies among species and is more limited in the chicken, thereby precluding the detection of a high degree of overlap with the human genes. Nonetheless, shared biological process terms include “translation,” “ribosome biogenesis,” “oxidation-reduction process,” and “small molecule metabolic process,” reflecting cellular processes associated with metabolically active tissue. Transcripts reflecting more specialized cellular function, such as lipid and cholesterol transport, which are shared by the human yolk sac and liver (*APOA1*, *APOA4*, *APOB*, *RBP4*, *SEPP1*, and *TTR*) are also present among the 400 most abundant mouse and chicken yolk sac transcripts ( $P = 2.3 \times 10^{-13}$ ).



**Fig. 6.** Venn diagrams illustrating the overlap among overrepresented GO terms associated with the 400 most abundant transcripts in each of the human, mouse, and chicken yolk sacs. (A) Biological process terms. (B) Cellular component terms. (C) Molecular function terms. The considerable overlap among the species in all three categories suggests conservation of functions.

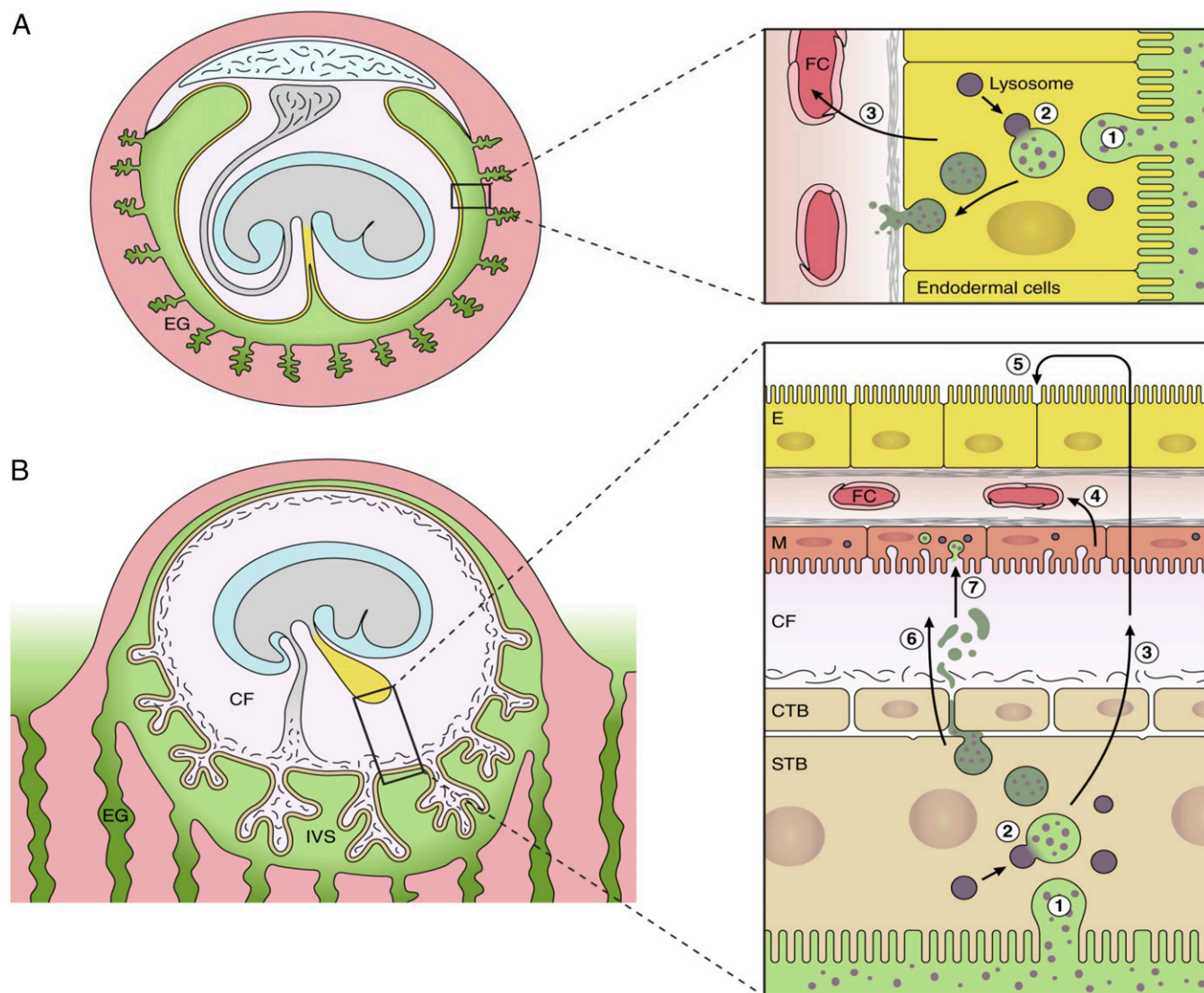
## Conclusion

Overall, our data show that the human secondary yolk sac appears to be far from vestigial with little or no biological role and that it may perform many key functions in the early weeks of development. In particular, it contains abundant transcripts encoding an array of transporter proteins involved in the uptake of macro- and micronutrients. Our data showing the presence of transporter proteins in both yolk sac epithelia and of their substrates in coelomic fluid support this concept and are consistent with the morphological appearances of absorptive surfaces (23, 59, 60). Our data also confirm significant synthetic activity, especially of apolipoproteins, a function most likely performed in the endodermal cells, given their high content of endoplasmic reticulum and Golgi cisternae (23, 59, 60). The handling of cholesterol, which

is essential for synthesis of cell and organelle membranes as well as being a cofactor in signaling pathways involved in axis determination and other fundamental developmental events, appears to be of particular significance. Furthermore, the high level of conservation of transcripts compared with the mouse and the chicken, in which the yolk sac is known to be essential for development, suggests maintenance of function.

The secondary yolk sac thus is likely to be essential for the survival of the embryo during the first weeks of development. Morphological abnormalities of the secondary yolk sac have been reported in 70% of first trimester human miscarriages (61), but separating cause from effect is impossible.

More generally, our findings shed light on the evolutionary mechanisms that give rise to complex structures such as the placenta. The placenta has evolved repeatedly from an oviparous



**Fig. 7.** Diagrammatic comparison of the nutrient pathway during early pregnancy in the mouse (A) and the speculated pathway in the human (B). In the mouse, histotrophic secretions (green) released from the endometrial glands are phagocytosed (step 1) by the endodermal cells of the visceral layer of the inverted yolk sac. Following fusion with lysosomes (step 2), digestion of maternal proteins leads to release of amino acids that are transported (step 3) to the fetal circulation (FC). In the human, histotrophic secretions are released from the endometrial glands through the developing basal plate of the placenta into the intervillous space (IVS) and are phagocytosed (step 1) by the syncytiotrophoblast (STB) (42). We speculate that following digestion by lysosomal enzymes (step 2), free amino acids are transported (step 3) by efflux transporters to the coelomic fluid (CF), where they accumulate. Nutrients in the CF may be taken up by the mesothelial cells (M) of the yolk sac and transported (step 4) into the fetal circulation (FC). Alternatively, they may diffuse into the cavity of the yolk sac and be taken up by the endodermal cells (step 5). Some intact maternal proteins may also be released into the CF by exocytosis of residual bodies (step 6) and may be engulfed by the mesothelial cells (step 7). CTB, cytotrophoblast cells.

background in mammals, reptiles, and fish, sometimes over very short timescales (62, 63). Development of a yolk sac for direct maternal provisioning of the developing fetus is a common feature of such evolutionary transitions to the extent that the yolk sac may be regarded as a “fundamental vertebrate fetal nutritional system” (64). The yolk sacs studied in this paper constitute a broad, albeit incomplete, sampling of the variation present in the vertebrates. The inverted yolk sac placenta of the mouse is found in several rodents and lagomorphs (1) and also in the distantly related nine-banded armadillo (65). The free-floating secondary yolk sac of the human is found in the other haplorhine primates (*Macaca mulatta*) (66) but also, surprisingly, in distantly related Afrotherian species. The yolk sac floats freely in the exocoelom in at least one tenrec species, the Nimba otter shrew (*Micropotamogale lamottei*) (67), in another Afrotherian insectivore, the eastern rock elephant shrew (*Elephantulus myurus*) (68), and in two species of bat [*Myotis lucifugus* (little brown bat) (69) and *Tadarida brasiliensis* (70)]. These observations suggest that mouse and human yolk sacs reflect convergent evolution toward similar forms found elsewhere in the mammalian phylogenetic tree. The yolk sac of the chicken, of course, is characteristic of oviparous species, i.e., all birds and the majority of reptiles.

Under a classic Darwinian model of macroevolution, small genetic mutations gradually occur and are accompanied by correspondingly small phenotypic changes until, over time, a high degree of morphological and functional diversification accumulates between species that are distantly related. The results presented here suggest, however, that the genetic systems underlying the function of the yolk sac are robust. We identify a number of genetic modules, including those involved in cholesterol processing, lipid transport, redox processes, and nutrient delivery, which presumably were reorganized and redeployed during evolutionary change while being internally conserved. These findings are in line with an extended evolutionary–developmental model in which it is the gene regulatory networks and underlying transcriptional control elements that change (71). The repeated convergent evolution of yolk sac placentas in all major groups of vertebrates other than birds is characteristic of what has come to be known as “deep homology,” which describes the origin of complex structures through modification and reorganization of preexisting genetic systems (72). Given that the modular conservation of systems active in the yolk sac is shown to extend across mammals and birds, the common ancestor of which was a reptile, it is possible that genetic modules of the yolk sac are part of the core amniote genetic repertoire. That is, conserved genetic systems of yolk sac function, for example cholesterol and lipid metabolism, form part of the common heritage shared by all mammals, reptiles, and birds and are the building blocks for both oviparous and viviparous reproductive modes.

Our findings indicate that extensive high-level morphological diversification of the extraembryonic membranes masks a surprising degree of functional conservation at the molecular genetic level. Evolutionary conservation at the level of nucleotide sequence, gene regulation, and modularity of gene expression is widely regarded as evidence of functional significance in both healthy development and

in disease (73–75). Therefore, we propose that, although a choriovitelline placenta is never established physically in the human, the early placental villi, the exocoelomic cavity, and the secondary yolk sac combine to function as a physiological equivalent (Fig. 7).

## Methods

**Human Tissue Collection.** Tissue and fluid samples were collected with informed written patient consent and approval of the Joint University College London/University College London Hospital Committees on the Ethics of Human Research (05/Q0505/82) from 7- to 12-wk uncomplicated pregnancies. Gestational age was confirmed by ultrasound measurement of the crown rump length of the embryo. All samples were collected from patients undergoing surgical pregnancy termination under general anesthesia for psycho-social reasons. Coelomic fluid samples were obtained by transvaginal puncture under sonographic guidance as previously described (27). Villous samples were obtained under transabdominal ultrasound guidance from the central region of the placenta using a chorionic villus sampling (CVS) technique. Intact secondary yolk sacs were obtained by gentle aspiration guided by ultrasound. All samples were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

**Mouse Tissue Collection.** Yolk sacs were collected from time-mated virgin C57BL/6J mice. Experiments were carried out in accordance with the United Kingdom Animals Scientific Procedures Act 1986 which mandates ethical review. A single randomly selected yolk sac was collected from each pregnant female at E9.5 (day of plug = E0.5). Tissue was dissected free from decidua and amnion, snap-frozen, and stored at  $-80^{\circ}\text{C}$  until processing.

**RNA Extraction and RNA-Seq.** RNA was extracted from human and mouse yolk sacs and human first-trimester placental villi using the RNeasy Plus Universal Mini Kit (catalog no. 73404; Qiagen). Libraries were made using the Illumina TruSeq Stranded mRNA Library Kit according to the manufacturer's instructions. Libraries were quantified (kappa qPCR), and equimolar pools were sequenced (single end 50 base reads, SE50) in several lanes of the Illumina HiSeq2500. Additional details are provided in the *SI Appendix*.

**Data Availability.** The datasets generated during the current study are available in the European Nucleotide Archive (ENA [www.ebi.ac.uk/ena](http://www.ebi.ac.uk/ena)) under the accession number PRJEB18767 ([www.ebi.ac.uk/ena/data/view/PRJEB18767](http://www.ebi.ac.uk/ena/data/view/PRJEB18767)).

**Proteomic Analysis of Coelomic Fluid Samples.** Coelomic fluid samples were run on 1D gels, enzymatically digested, and analyzed using an LC-MS/MS (Dionex Ultimate 3000 RSLC nanoUPLC; Thermo Fisher Scientific, Inc.) system and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.). Detailed methods are supplied in *SI Appendix, Methods*.

**Immunohistochemistry.** Immunohistochemistry was performed as previously described (76) using the following primary antibodies: anti-5LC39A7 (ZIP7; ab117560; Abcam) and anti-ABCA1 (ab7360; Abcam).

**Supporting Information.** Additional methods are given in *SI Appendix, Methods* online.

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