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

Tereza Cindrova-Davies[a], Eric Jauniaux[b], Michael G. Elliot[a,c], Sungsam Gong[d,e], Graham J. Burton[a,*], D. Stephen Charnock-Jones[a,d,e,*]

[a] Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3EG, UK. [b] EGA Institute for Women's Health, Faculty of Population Health Sciences. University College London, London, WC1E 6BT, UK. [c] St John's College, University of Cambridge, Cambridge, CB2 1TP [d] Department of Obstetrics and Gynaecology, University of Cambridge, The Rosie Hospital, Cambridge, CB2 0SW, UK [e] National Institute for Health Research, Cambridge Comprehensive Biomedical Research Centre.

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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 known to synthesize proteins, transport functions are widely considered vestigial. Here, we report RNA-Seq data for the human and murine yolk sacs, and compare with data for the chicken. We also relate the human RNASeq 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 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

Introduction

The yolk sac is phylogenetically the oldest of the extraembryonic membranes, evolving in anamniotes 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 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 which develops between embryonic days 7 and 9 and is replaced by a secondary yolk sac which is active until 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, this is 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-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 12469 transcripts with a mean RPKM (Read Per Kilobase Per Million Mapped Reads) ≥1 (Dataset S1). Similarly, we identified 11628 transcripts in first trimester human placental villous samples (n=11, median sequencing depth 30 million mapped reads, Dataset S2) and 11272 transcripts in the mouse yolk sac (n=8, median sequencing depth 28 million mapped reads, Dataset S3).

In addition, we investigated the protein composition of the coelomic fluid using GELC-MS/MS. We focused on the 165 proteins identified in any 4 of the 5 samples having excluded immunoglobulins (Dataset S4). Proteins were mapped to unique

Significance

The human yolk sac is often considered vestigial. Here, we report RNA-Seq analysis of the human and murine yolk sacs, and compare with that of the chicken. We relate the human RNA-Seq 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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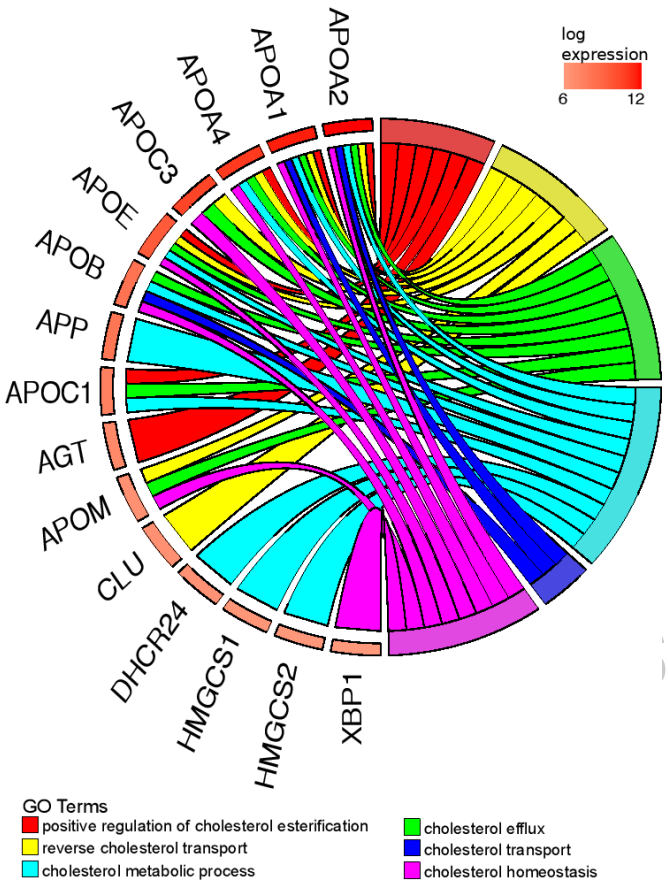

Fig. 1. Chord plot illustrating the GO biological process terms that include "cholesterol" and that are overrepresented in the 400 most abundant yolk sac transcripts on the right, and the genes contributing to that enrichment on the left arranged in order of their expression level.

Ensembl gene identifiers, which were used to identify over-represented GO terms (Dataset S5).

Cholesterol

We selected the 400 most abundant human yolk sac transcripts and identified enriched gene ontology (GO) terms using Panther (complete reference database with Bonferroni correction, Dataset S6). Several terms associated with lipid transport were enriched, for example "very-low-density lipoprotein particle" (23 fold, $P=4.5x10^{-7}$). Indeed, "cholesterol" featured in many of the enriched "biological process" terms (Figure 1). Cholesterol is required for development (3, 5) as it maintains 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, as well as lipoprotein receptors, including megalin, cubilin (18), albeit at lower levels (Figure 1). Also present are transcripts encoding all classes of ABC transportors (A to G), which, in addition to transporting cholesterol and lipids, facilitate 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

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

Family	Transporters detected in human YS	Function
ABCA	ABCA1*#, ABCA2, ABCA3*#, ABCA5, ABCA7*	Responsible for the transportation of cholesterol and lipids.
ABCB	ABCB1*#, ABCB10*#, ABCB6*#, ABCB7*#, ABCB8*#	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*#, ABCC10*#, ABCC2*, ABCC3, ABCC4*#, ABCC5*#, ABCC6*#, ABCC6P1*, ABCC6P2*	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*#, ABCD3*#, ABCD4*#	Are all used in peroxisomes.
ABCE/F	ABCE1*#, ABCF1*#, ABCF2*#, ABCF3*#	Not proper transporters; members contain ATP-binding domains without the transmembrane domain. Involved in regulating protein synthesis or expression.
ABCG	ABCG1*, ABCG2*#, ABCG5*#, ABCG8*	Transports lipids, diverse drug substrates, bile, cholesterol, and other steroids.

* also present in the mouse yolk sac; # also present in the chick yolk sac

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 RPKM ≥ 1 threshold). Many of these proteins have functions associated with cholesterol or lipid transport and metabolism (Figure 2).

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 (87) (Dataset S5). Most of these transporter genes are members of the solute carrier (SLC) family of transporters (for example: SLC38A2 (amino acids), SLC4A1 (anions), SLC20A1

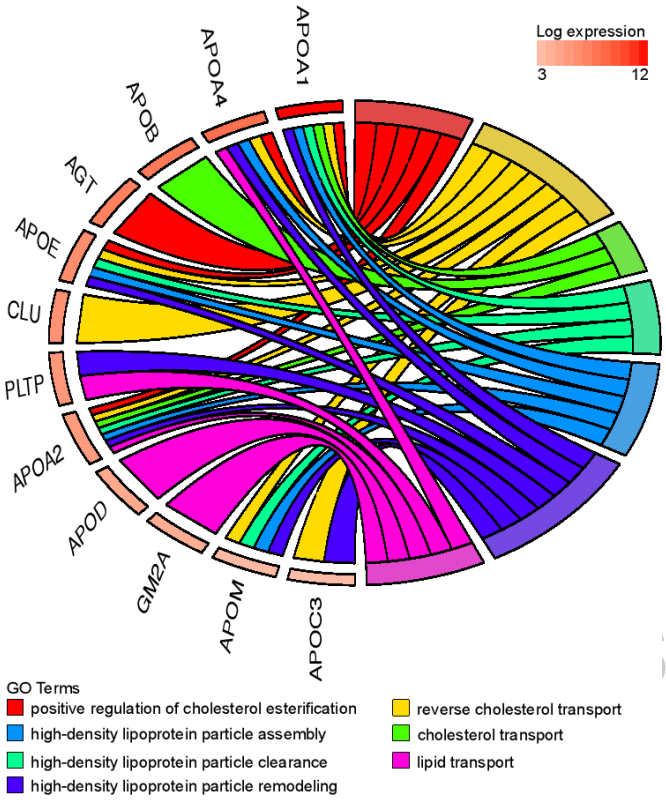

Fig. 2. Chord plot illustrating proteins present in the coelomic fluid that relate to GO biological process terms involving "cholesterol" and "lipid transport". The presence of these proteins is consistent with the high level of their transcripts in the human yolk sac illustrated in Figure 1.

(phosphate) and SLC25A37 (iron in the mitochondria)). Transcripts encoding 259 SLC transporters were identified (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 α2-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 encoding 12 ZIP proteins and 8 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 (Figure 3). The outer mesothelial layer also expresses α-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 identified, Table 2) (39, 40), vitamins A, B12, C, E, and folic acid (Table 2).

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 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, 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 4-fold 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 centiles, respectively). In the rat it has been estimated that ~95% of the amino acids provided to the fetus in mid-gestation are obtained by lysosomal digestion of endocytosed maternal proteins (2). In the mouse yolk sac, transcripts encoding 5 lysosomal cathepsins (Ctsl, Ctsz, Ctsb, Ctsd and Ctsh) are among the most abundant 400 transcripts, the activity of which would allow for 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 glycodelin, a product of the uterine glands and present in high concentration in the coelomic fluid (41), indicating exposure to intact maternal proteins and uptake (42). Glycodelin 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 two weeks of 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 3-somite stage (21 days), 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 data set, 8 genes are similarly annotated (SI Appendix Table S3). Candidate motifs recognized by these transcription factors (where known) were identified in the 1kb and 5kb upstream of the TSS of genes which were the highly correlated with the transcription factor transcripts (Datasets S7-10). The FOS and JUN families and AFT4 are closely related, functionally interact, have multiple target genes and are widely expressed. There

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

Substrate Category	SLC transporters detected in human YS	Known substrates	Substrates detected in human coelomic fluid
Amino acids	SLC38A2*#, SLC7A7*, SLC7A2, SLC1A5*, SLC38A3, SLC3A2*, SLC3A1#, SLC25A38*, SLC38A4*, SLC38A10*, SLC25A13*#, SLC7A8*, SLC7A6*#, SLC43A1*, SLC7A4, SLC7A9*, SLC1A4*, SLC38A7*#, SLC7A1*, SLC15A4*#, SLC38A5*, SLC7A6OS*#, SLC43A3*#, SLC38A9*#, SLC43A2*#, SLC36A4*, SLC38A6*#, 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*#, SLC2A9#	Lys, Orn, Cit, Asp, Glu, uric acid	Urea (28-29)
Glucose and sugars	SLC2A1*#, SLC2A3*#, SLC2A4RG, SLC50A1*, SLC2A2*#, SLC1A1#, SLC2A10#, SLC2A14, SLC5A9*, SLC45A4*, SLC2A8*, SLC2A12, SLC2A6, SLC2A13#, SLC37A1, SLC2A5#, SLC37A2#, SLC2A7*, 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*, SLC17A9*, SLC35E2B#, SLC29A1*, SLC35C2*#, SLC35A4*#, SLC35A2*, SLC35E1*#, SLC25A36*#, SLC35D1*#, SLC35A5*#, SLC35F5*#, SLC37A4*#, SLC35F6*, SLC35A3*#, SLC37A3*#, SLC35B1*#, SLC35C1*, SLC52A2*, SLC29A2*, SLC29A3*, SLC35B4*#, SLC35G1*, SLC35E3*#, SLC29A4, SLC35F2*#, SLC35F3, SLC25A19*, SLC35G2*, SLC28A1*	UDP-glucuronic acid, UDP-galactose, UDP-N-acetyl-galactosamine, GDP-fructose	
Metals	SLC30A5*#, SLC30A9*#, SLC40A1*#, SLC39A7, SLC39A14*#, SLC25A37*#, SLC39A6*, SLC39A5*, SLC39A1*, SLC30A1*#, SLC31A1*#, SLC25A28*#, SLC39A13*#, SLC39A9*#, SLC39A8*#, SLC11A2*#, SLC30A6*#, SLC30A7*, SLC41A1*, SLC41A3*, SLC39A11*#, SLC30A10*#, SLC31A2*, SLC39A10*, SLC41A2#, SLC30A2*, SLC39A4*, SLC11A1, SLC39A3*, SLC30A4*#	Zinc, iron, magnesium, copper, cadmium, cobalt, manganese, nickel, lead, barium, strontium	Zinc, iron, cadmium, magnesium, copper, manganese, lead, selenium (28, 31, 32)
Vitamins	SLC19A2*, SLC46A3*#, SLC25A32*#, SLC23A2*, SLC5A6*, SLC46A1*#, SLC23A1*, SLC19A3*, SLC19A1*, SLC23A3*	Thiamine (vitamin B1), folate, ascorbic acid, biotin, lipoate panthothenate, thiamine	Vitamins A, E, B12, folate, cobalamin, retinol binding protein 4, vitamin D binding protein (21, 27, 29, 33-35)
Neuro-transmitters	SLC44A2*, SLC44A4*, SLC6A6*#, SLC44A1*#, SLC36A1*#, SLC6A9*#, SLC1A3*, SLC6A13*, SLC44A3#, SLC17A2, SLC25A22*#, SLC25A18, SLC25A12*#, SLC17A4, SLC6A12	noradrenaline, serotonin, dopamine, glutamate, glycine, aspartate, choline	Glutamine, glutamic acid (21, 25)
Inorganic ions	SLC25A3*#, SLC9A3R1*, SLC4A1*#, SLC20A1*, SLC12A7*#, SLC4A2*#, SLC9A3R2*, SLC34A2*#, SLC12A4*#, SLC26A6*, SLC9A1*, SLC20A2*, SLC9A6*#, SLC4A1AP*, SLC9A9#, SLC26A11*#, SLC9B2, SLC26A2*, SLC24A3, SLC9A8*, SLC12A8, SLC8B1*, SLC12A2*#, SLC24A1, SLC4A7*, SLC12A6*, SLC4A3, SLC26A10, SLC26A1*, SLC5A5, SLC9A7P1, SLC9A5*, SLC8A1, SLC4A4*	Na^+, K^+, Cl^-, HCO_3^-, Ca^{2+}, phosphate	Na^+, K^+, Cl^-, HCO_3^-, Ca^{2+}, phosphate (28-29)
Thyroid	SLC16A2#, SLC7A5*#, SLC16A10*#	iodide, iodothyronines	Tyrosine, thyroxine (26)
Organic ions	SLCO2B1#, SLC22A7, SLC10A1, SLC10A3*, SLC22A23*#, SLC51A*, SLC22A9, SLC22A17*, SLCO4C1*, SLCO2A1*, SLC22A31, SLC22A18*#, SLC22A4*, SLC22A5*#, SLC10A7*#, SLC51B, SLCO1B3, SLC22A15, SLC22A18AS, SLCO1B1, SLCO3A1*#, SLC17A3, SLC22A3*	oestradiol, glucuronide, bilirubin	bilirubin
Miscellaneous	SLC25A5*, SLC5A12, SLC25A39*#, SLC25A1*#, SLC16A3*, SLC35B2*, SLC25A20*, SLC17A5*#, SLC16A1*#, SLC16A4#, SLC13A5, SLC35B3*, SLC6A8*, SLC27A2*#, SLC25A24*#, SLC25A23*, SLC25A11*, SLC25A44*, SLC25A25*, SLC45A3*, SLC15A1*, SLC25A46*, SLC25A27, SLC25A42*, SLC27A3*, SLC25A17*#, SLC25A43*#, SLC35A1*#, SLC12A9*#, SLC16A5, SLC25A29#, SLC18B1*, SLC16A13*#, SLC25A30*#, SLC27A4*#, SLC16A9*#, SLC48A1*#, SLC33A1*#, SLC25A4*, SLC25A14*#, SLC25A33*, SLC25A16*#, SLC25A40*, SLC25A34*, SLC27A1*#, SLC25A26*, SLC47A1*#, SLC15A3, SLC16A12*#, SLC25A45*, SLC5A11*, SLC25A51*, SLC13A3*#, SLC16A14, SLC25A21-AS1, SLC16A7#, SLC16A6*, SLC15A2, SLC27A5*, 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

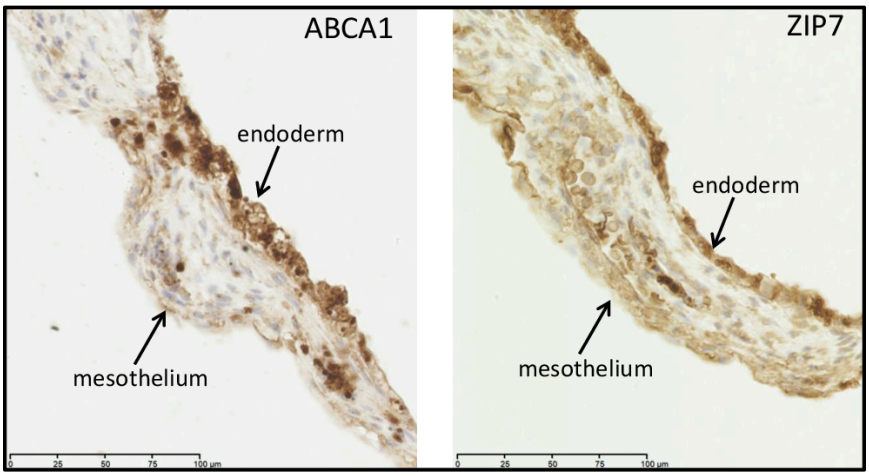

Fig. 3. Immunolocalization of ABCA1 and SLC39A7/ZIP7 transporter proteins in the human yolk sac at 11 weeks gestational age. Sections were immunostained with anti-ABCA1 or anti-ZIP7 antibodies. In both cases, staining was present in the inner endodermal and outer mesothelial layers, although it was stronger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

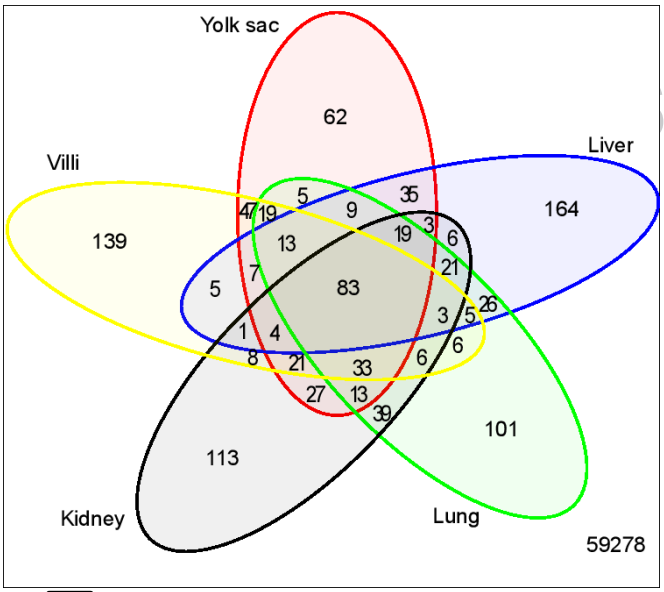

Fig. 4. Venn diagram comparing the most abundant 400 transcripts in the human yolk sac, with first trimester placental villi and adult liver, lung and kidney. Transcripts shared by all five tissues (83) encoded principally house-keeping proteins, whereas those shared uniquely with liver (35) encoded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

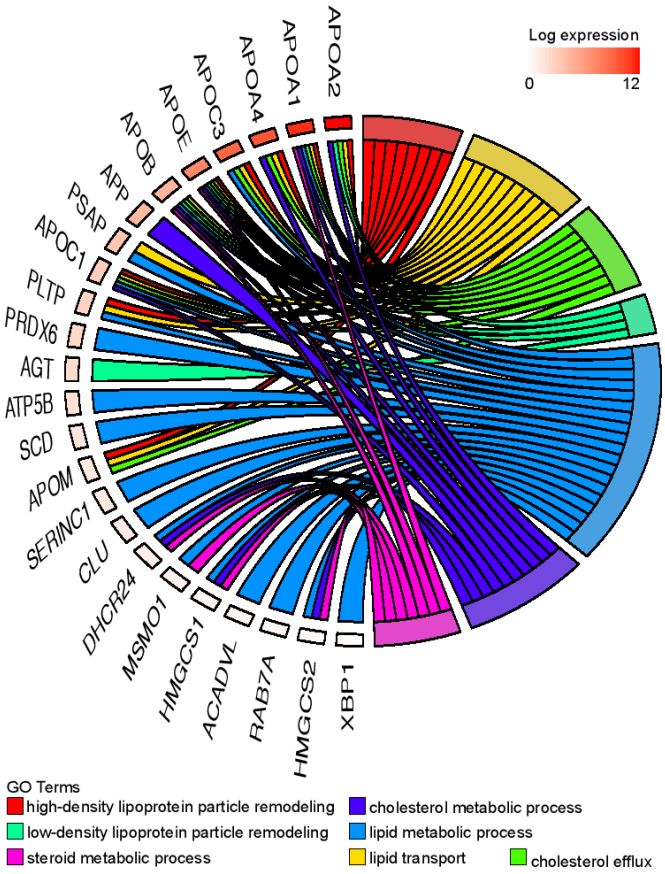

Fig. 5. Chord plot connecting GO Biological Process terms associated with "lipid metabolism" and genes encoding transcripts that are shared by the human yolk sac and adult liver. The overlap suggests the yolk sac may perform hepatic functions while the fetal liver differentiates.

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, 8) and 18 genes in the mouse with candidate Atf4 binding sites (Datasets S9, 10). The evidence used for the assignment of GO terms varies and for 2 genes (IGF2 and BHLHE40) depends on a Non-traceable Author Statement (NAS, Dataset S3). Furthermore, binding motifs are not available for all candidate factors even in the most recent JASPAR database.

Yolk sac and villi compared to 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 human yolk sac transcript profile with tissues where function has been better defined experimentally. The placental villi serve similar functions to 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 (Figure 4). The transcripts that are unique to each tissue and those shared among these tissues are listed in the Dataset S11. As expected, the transcripts shared by all 5 tissues encoded abundant house-keeping 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 cor-

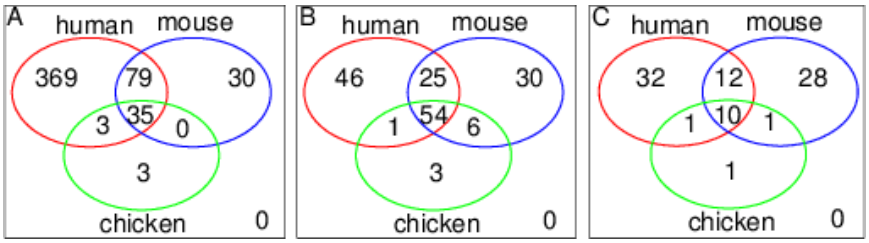

Fig. 6. Venn diagrams illustrating the overlap among overrepresented GO terms associated with the 400 most abundant transcripts in each of the human, mouse and chicken yolk sacs: A, "biological process"; B, "cellular component" and C, "molecular function". The considerable overlap among the species in all three categories suggests conservation of functions.

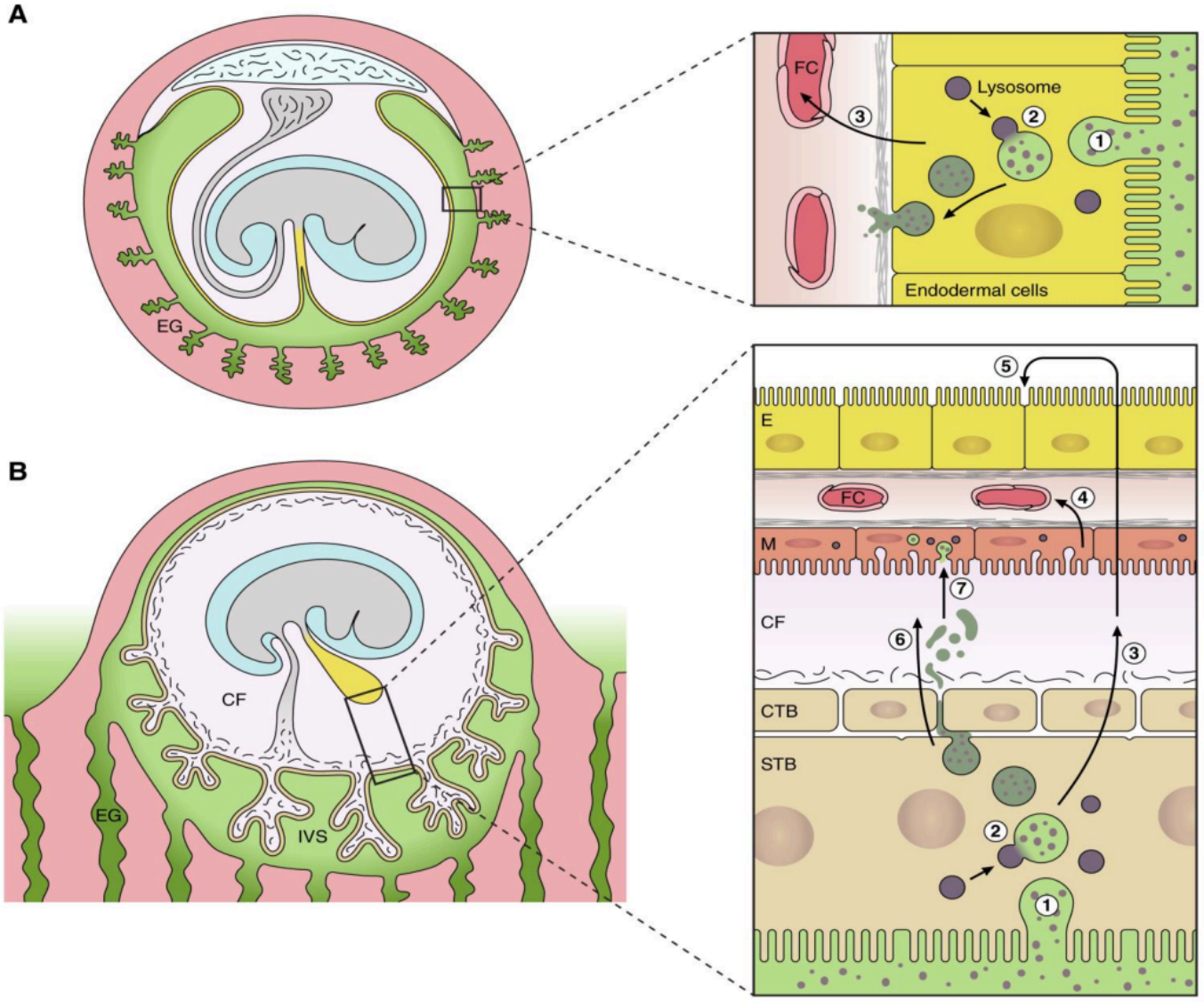

Fig. 7. Diagrammatic comparison of the nutrient pathway during early pregnancy in the mouse (A), and the speculated pathway in the human (B). In the mouse, histotrophic secretions (green) released from the endometrial glands (EG) are phagocytosed (1) by the endodermal cells (E) of the visceral layer of the inverted yolk sac (YS). Following fusion with lysosomes (2), digestion of maternal proteins leads to release of amino acids that are transported (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 (1) by the syncytiotrophoblast (STB) (ref. 42). We speculate that following digestion by lysosomal enzymes (2), free amino acids are transported (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 (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 (5). Some intact maternal proteins may also be released into the CF by exocytosis of residual bodies (6), and be engulfed by the mesothelial cells (7). CTB; cytotrophoblast cells.

rection, 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", "lipid transporter activity" (all greater than 28-fold enrichment and $P < 2x10^{-4}$ after Bonferroni correction, Dataset S13).

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 characteristic typical of an absorptive epithelium (10, 55). However, their orientation is different as the human yolk sac floats in the exocoelom with the endodermal cell layer lining the interior of the sac, whilst the rodent yolk 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 sac 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 S20. The raw and processed chicken yolk sac data were obtained directly from the authors (58). Gene ontology 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 over-represented 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 Figure 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 between species and is more limited in the chicken, which necessarily precludes detecting 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, TTR) are also present among the 400 most abundant mouse and chicken yolk sac transcripts ($P = 2.3 \times 10^{-13}$).

Conclusion

Overall, our data show that the human secondary yolk sac appears 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 of the yolk sac epithelia, and 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 co-factor in signaling pathways involved in axis determination and other fundamental developmental events, appears of particular significance. Furthermore, the high level of conservation of transcripts compared with the mouse and the chicken, where the yolk sac is known to be essential for development, suggests maintenance of function.

The secondary yolk sac is thus 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 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), but 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 both 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: 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 were presumably 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 which 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 pre-existing 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 (Figure 7).

Methods

Human tissue collection

Tissue and fluid samples were collected with informed written patient consent and approval of the Joint UCL/UCLH Committees on the Ethics of

Human Research (05/Q0505/82) from 7-12 weeks in 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°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°C until processing.

RNA extraction and RNA-Seq

RNA from human and mouse yolk sacs and human first trimester placental villi was extracted using RNeasy plus universal mini kit (Qiagen, Cat No 73404). 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 sequenced (single end 50 base reads, SE50) in several lanes of the Illumina HiSeq2500. Additional details are provided in the Supplemental Information.

Data availability

The data sets generated during the current study are available in the European Nucleotide Archive (ENA http://www.ebi.ac.uk/ena) under the accession number 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 LC-MS/MS (Dionex Ultimate 3000 RSLC nanoUPLC, Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Detailed methods are described in the Supplemental file.

Immunohistochemistry

Immunohistochemistry was performed as previously described (76) using the following primary antibodies: anti-SLC39A7 (ZIP7, Abcam, ab117560) and anti-ABCA1 (Abcam, ab7360).

Supporting Information.

Additional methods and references (57, 77-87) are in the online supplemental methods.

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