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The human gestational sac as a choriovitelline placenta during early pregnancy; the secondary yolk sac and organoid models

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ABSTRACT

The yolk sac is phylogenetically the oldest of the extra-embryonic membranes and plays important roles in nutrient transfer during early pregnancy in many species. In the human this function is considered largely vestigial, in part because the secondary yolk sac never makes contact with the inner surface of the chorionic sac. Instead, it is separated from the chorion by the fluid-filled extra-embryonic coelom and attached to the developing embryo by a relatively long vitelline duct. The coelomic fluid is, however, rich in nutrients and key co-factors, including folic acid and anti-oxidants, derived from maternal plasma and the endometrial glands. Bulk sequencing has recently revealed the presence of transcripts encoding numerous transporter proteins for these ligands. Mounting evidence suggests the human secondary yolk sac plays a pivotal role in the transfer of histotrophic nutrition during the critical phase of organogenesis but also of chemicals such as medical drugs and cotinine. We therefore propose that the early placental villi, coelomic cavity and yolk sac combine to function physiologically as a choriovitelline placenta during the first weeks of pregnancy. We have derived organoids from the mouse yolk sac as proof-of-principle of a model system that could be used to answer many questions concerning the functional capacity of the human yolk sac as a maternal-fetal exchange interface during the first trimester of pregnancy.

1. Introduction

The human yolk sac remains an enigma. For many years it has been considered vestigial and of little importance, despite the fact that the yolk sac is known to play an essential role in maternal-fetal nutrient transport in many animal species. Interest was rekindled with the advent of ultrasonography, for the yolk sac is the first structure to be observed within the gestational sac and indicates successful early pregnancy development. Many uncertainties remain, however, concerning its formation, function, and contribution to embryonic development. This is largely due to the difficulty of obtaining specimens and the major species differences that limit extrapolation of data from animal models. New techniques, including the generation of human blastoids, spatial transcriptomics and organoid cultures open unique possibilities to address these questions. We anticipate that in future the human yolk sac will no longer be considered a largely vestigial structure but be recognised as an essential organ of maternal-fetal exchange for nutrients and co-factors during the earliest stages of pregnancy. Indeed, we propose that the yolk sac combines with other components of the gestational sac to function physiologically as a choriovitelline placenta essential to the histotrophic transfer pathway before the maternal intervillous circulation is established towards the end of the first trimester.

1.1. Phylogeny

The yolk sac is the oldest of the extraembryonic membranes phylogenetically. In therian mammals the sac does not contain significant yolk yet it still serves a vital role in the transfer of nutrients during the period of embryogenesis in the majority of species. This function is enabled by the yolk sac being in contact with the inner aspect of the chorion, the outer wall of the blastocyst, during the earliest stages of development. Blood vessels differentiating in the extraembryonic mesoderm of the yolk sac vascularise the chorion, forming a choriovitelline placenta (Mossman, 1987). This is often a transient structure, for as the yolk sac regresses and the allantois enlarges contact between the yolk sac and chorion is lost. By then, the chorion is vascularised through the allantoic

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mesoderm, creating the definitive chorioallantoic placenta characteristic of eutherian mammals. In marsupials, the choriovitelline placenta remains the dominant form of placentation (Freyer and Renfree, 2009).

It is widely asserted that the human does not pass through this temporary phase of choriovitelline placentation, for the vascularised yolk sac never makes contact with the inner surface of the chorion (Mossman, 1987). Whilst the morphology is incontrovertible, we nonetheless argue that in the human the trophoblast, coelomic cavity and secondary yolk sac function together as choriovitelline placenta during the first weeks post-implantation. Before doing so, we consider the development of the human yolk sac.

1.2. Development

Although early human embryos are hard to obtain, the generally accepted view is that there are two phases to the development of the yolk sac, the primary yolk sac and the secondary yolk sac. The primary yolk sac is formed around days 9–10 post-conception (p.c.) when squamous cells from the hypoblast layer grow out beyond the margins of the bilaminar germ disc and over the inner surface of the trophectoderm. Almost immediately, a loose meshwork of spindle-shaped cells becomes interposed between these two cell layers (Fig. 1). The nature and origin of these cells remain controversial. They have long been considered to be extraembryonic mesoderm, but in part the confusion arose because they predate the appearance of the primitive streak. Early investigators suggested that they delaminate from the inner surface of the trophectoderm (see Luckett, 1978; Enders and King, 1993 for review), but this seems unlikely since ultrastructural studies revealed that the two tissues are separated by a basement membrane (Knoth and Larsen, 1972). Alternatively, it has been proposed that they arise from the endoderm of the primary yolk sac and differentiate into mesoderm in situ (Enders and King, 1993).

A third intriguing possibility, promoted on the basis of comparative studies, is that the meshwork represents a yolk sac that has been constrained by the invasive, interstitial mode of implantation displayed by the human (Luckett, 1978). Thus, the meshwork fills most of the blastocyst cavity in the human and chimpanzee but is absent in the rhesus macaque which has superficial implantation and hence a larger blastocyst. Equally, in closely related species of the galago family of strepsirrhine primates (*Galagoides demidoff* and *Galago senegalensis*) that exhibit contrasting patterns of implantation the meshwork is only present in those where the blastocyst becomes embedded in the uterine wall

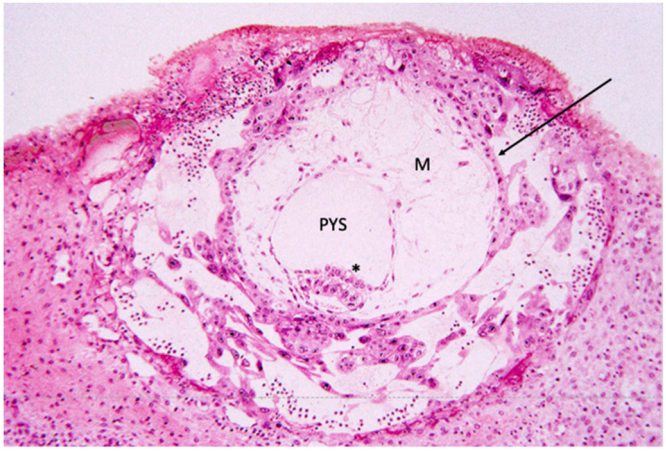

Fig. 1. Photomicrograph of an implanted conceptus at the advanced lacunar stage of development (Carnegie stage 6). The primary yolk sac (PYS) can be seen extending from the hypoblast layer (asterisk) of the bilaminar germ disc. A meshwork of cells and alveolar-like spaces (M) fills the space between the yolk sac and the trophectoderm of the original wall of the blastocyst (arrowed). Specimen from the Carnegie Collection and image by courtesy of the late Allen Enders.

(Luckett, 1978). Luckett argued that interstitial implantation restricts the size of the blastocyst, and that evolution of the yolk sac has not yet caught up with this fact and so the sac becomes crumpled within the blastocoele. Supporting his argument is the observation that the main cavity of the primary yolk sac communicates at points around its perimeter with the alveolar-like cavities present in the meshwork, and all are filled with the same flocculent material (Luckett, 1978). Only when the blastocyst enlarges as gestation advances can the yolk sac unravel into a sphere.

This is an interesting phylogenetic and developmental conundrum, and one that use of contemporary sequencing techniques should be able to solve. Interestingly, recent studies of the equivalent cells in the cynomolgus macaque (*Macaca fascicularis*) show the presence of the endoderm markers *GATA4* and *GATA6*, and absence of the mesoderm marker *T* (*brachyury*) (Nakamura et al., 2016). Consequently, Ross and Boroviak (2020) concluded that the meshwork is of endodermal origin. Similarly, fate-mapping of the posterior embryonic-extraembryonic interface in the mouse showed that a segment of the hypoblast located at the base of the yolk sac and overlying the allantois contributes significantly to the extraembryonic mesoderm in rodents (Downs, 2022; Rodriguez and Downs, 2017). Further studies are required to confirm that the same holds true for the human, while the question of folding could be addressed by culturing human blastocysts or blastoids and constraining their expansion to different degrees by 'implanting' into endometrial assembloids or hydrogel matrices of varying consistencies (Oldak et al., 2023; Rawlings et al., 2021).

Between days 12–15 p.c. the primary yolk sac appears to break down into a number of smaller vesicles, some of which cluster at the abembryonic pole of the blastocyst cavity while the portion in contact with the germ disc forms a smaller sphere, the secondary yolk sac. What factors initiate this transition or how it is achieved mechanistically are not understood, but it is tempting to speculate that it may represent separation of the original primitive endoderm cells derived from the hypoblast from the definitive endoderm formed during gastrulation. At the same time, the meshwork of cells filling the blastocyst cavity collapses and two layers of extraembryonic mesoderm become defined, one lining the inner surface of the trophectoderm forming the chorion and the other covering the outer surface of the secondary yolk sac. Between the two is the fluid-filled extra-embryonic coelom or coelomic cavity.

Development of the secondary yolk sac can be followed using ultrasonography (Fig. 2A). The diameter of the sac increases from around 3 mm at 5 weeks gestational age to a peak of nearly 6 mm at 10 weeks. After that it begins to degenerate, reducing to a diameter of just over 4 mm by 12 weeks (Jauniaux et al., 1991c). In cases of early embryonic demise or poor embryonic development, the yolk sac may continue to function for several days and becomes abnormally larger (>6 mm) on ultrasound examination (Fig. 2B) (Detti et al., 2020; Hoyes, 1969; Jauniaux et al., 1991c). This is probably due to the yolk sac continuing to transport proteins and water which accumulate in its lumen instead of being transferred to the embryo via the vitelline circulation. A similar phenomenon is observed in the mesenchymal villous tissue (oedema) in case of embryonic demise or in complete hydatidiform moles (cavitation) where a villous circulation never forms (Jauniaux et al., 1998b). These findings provide indirect evidence of an active role of the yolk sac in transport from the extraembryonic coelom into the embryonic circulation.

1.3. Structure

The structure of the secondary yolk sac has been well described at the histological and ultrastructural levels in a number of publications and so will only be briefly considered here (Hesseldahl and Falck-Larsen, 1969; Hoyes, 1969; Jauniaux and Moscoso, 1995; Jones and Jauniaux, 1995). The sac is a three-layered structure, with an inner endodermal epithelium, a layer of mesenchymal cells and an outer covering of mesothelial cells. The endodermal layer is composed of columnar cells joined by

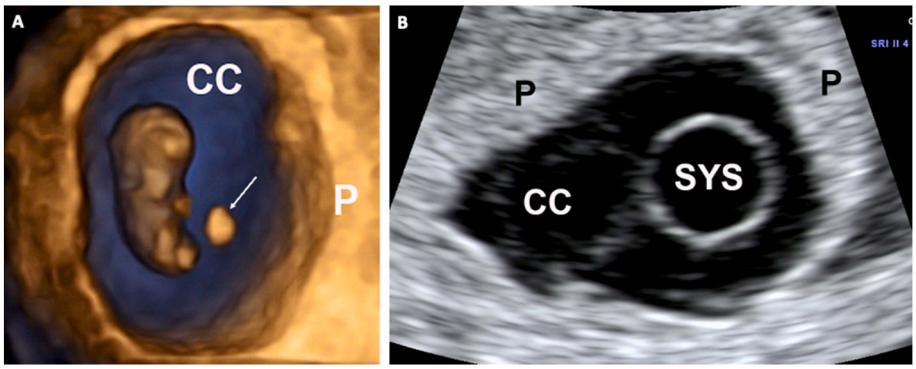

Fig. 2. Ultrasonographic images of the secondary yolk sac in normal and pathological pregnancies. A) Normal pregnancy at 9 weeks gestational age. The secondary yolk sac (arrowed) is 4 mm in diameter and clearly visible within the coelomic cavity (CC). P, placenta. B) A case of missed miscarriage at 9 weeks gestational age showing dilation (9 mm diameter) of the secondary yolk sac (SYS) within the coelomic cavity (CC). P, placenta.

tight junctions at their apical margins. Their apical surfaces are covered by sparse short microvilli and their cytoplasm contains numerous vacuoles of various sizes and profuse rough endoplasmic reticulum. The epithelium is often invaginated to form a series of channels or tubules that communicate with the central cavity. Surrounding the invaginations are the stellate mesenchymal cells and these are covered on the outer surface by a single layer of flattened mesothelial cells. The mesothelial cells display features of an absorptive epithelium, being linked by tight junctions and bearing long microvilli on their apical surface with evidence of coated vesicles and micropinocytosis. It is notable that a network of capillaries lies in the mesenchymal layer and is closely approximated to the base of the outer mesothelial layer. This network coalesces to form the vitelline vein that runs in the yolk sac stalk directly into the differentiating liver within the septum transversum (Fig. 3) (Hamilton and Mossman, 1972).

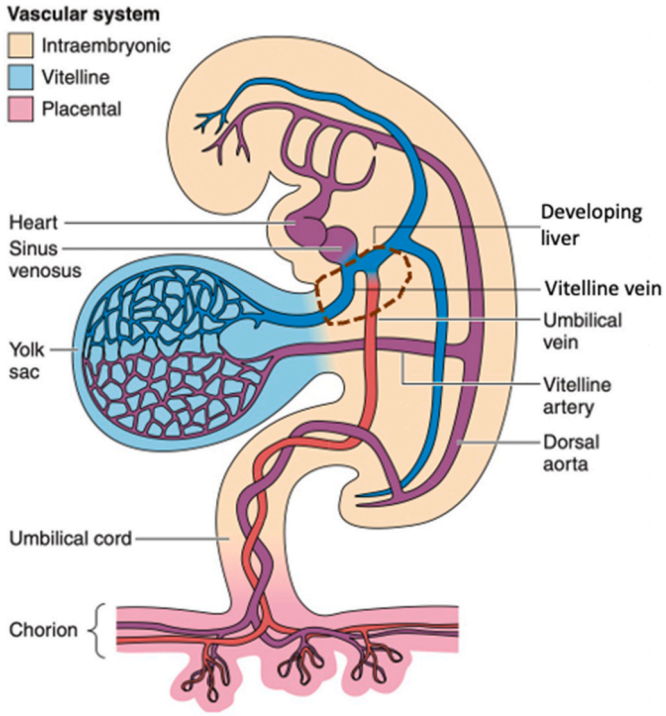

Fig. 3. Diagrammatic representation of the vitelline circulation serving the secondary yolk sac (depicted in blue). The vitelline vein delivers nutrients to the developing liver, denoted by the brown dashed line, in the septum transversum before entering the sinus venosus of the heart. Modified from Burton et al. (2016) with permission.

1.4. Transport functions

The morphological features of the yolk sac, in particular those of the mesothelial layer at the ultrastructural level, suggested to early researchers that it has an absorptive function and may play a role in the transport of maternal proteins to the embryo (Hoyes, 1969;Exalto, 1995; Gonzalez-Crussi and Roth, 1976). Others rejected this notion, citing the fact that the yolk sac never comes into contact with the decidua and that the chorion and coelomic cavity are always interposed between the two (Hesseldahl and Falck-Larsen, 1969).

These objections were nullified, however, once the composition of the coelomic fluid was analysed and compared to that of maternal serum and amniotic fluid. Molecules that transfer through the villous trophoblast in the first two months of pregnancy are likely to end-up in the coelomic cavity as there is no barrier between the villous mesenchyme and the coelomic cavity. During that period of development, there are very few villous capillaries in direct contact with the trophoblastic layer (Jauniaux et al., 1991a), and the embryonic-villous circulation is restricted by a high resistance to flow due to the high proportion of nucleated red cells present in the embryonic circulation (Jauniaux et al., 1991b). The coelomic fluid contains significantly higher concentrations of oestradiol, progesterone, human chorionic gonadotrophin (hCG), interleukin-6 and ferritin than both maternal serum and amniotic fluid whereas α -fetoprotein (AFP), which is synthesised by the yolk sac and embryonic liver, is significantly higher in both the coelomic and amniotic fluids compared to maternal serum (Table 1). Importantly, it was found that although the concentration of total protein was lower in the coelomic fluid than in maternal serum, the concentrations of 19 of the 24 individual amino acids measured were higher (Jauniaux et al., 1998a). Positive correlations were found between maternal serum and coelomic fluid concentrations for α-aminobutyric acid, tyrosine and histidine, suggesting a preferential trophoblastic transfer from the maternal circulation into the coelom. In addition, coelomic fluid contained particularly high concentrations of maternal proteins derived from the decidua, including glycodelin (PP14) and IGFBP-1 (Docherty et al., 1996; Jauniaux and Gulbis, 2000a; Jauniaux et al., 1998a; Santolaya-Forgas et al., 2006). Coelomic fluid also contains concentrations of free thyroxine, which like other thyroid hormones is not synthesised by fetal tissues until the end of the first trimester, that are a least a third of those found in the maternal serum of a euthyroid mother (Calvo et al., 2002). By contrast to the physiological decrease in maternal serum protein concentration associated with blood dilution, the coelomic concentration of total protein and pre-albumin increases as pregnancy advances during the 1st trimester (Jauniaux and Gulbis, 2000a). The presence of these maternal proteins is particularly relevant as it is now appreciated that

Table 1
Comparison of the concentrations of different proteins among the maternal serum, coelomic fluid and amniotic fluid.

Protein	Maternal serum	Coelomic fluid	Amniotic fluid
Total proteins (median g/L)(Gulbis et al., 1992)	70.00	3.09	0.13
Albumin (mean g/L)(Jauniaux et al., 1994)	45.5	1.7	ND
Pre-Albumin (mean g/L)(Jauniaux et al., 1994)	1.14	0.04	ND
Oestradiol (mean pg/mL)(Jauniaux et al., 1993)	916	8470	986
Progesterone (mean pg/mL)(Jauniaux et al., 1993)	17	340	7
Intact hCG (mean IU/L)(Jauniaux et al., 1993)	67553	189866	1000
AFP (mean kIU/L)(Jauniaux et al., 1993)	5.9	18696	22890
Relaxin (median ng/L)(Johnson et al., 1994)	10000	122	9
Transferrin (median g/L)(Gulbis et al., 1994)	2.50	0.22	ND
Ferritin (median μg/L)(Gulbis et al., 1994)	49	287	2
IL-6 (median pg/mL)(Jauniaux et al., 1996)	ND	87.5	16.5

hCG = human chorionic gonadotropin; AFP= Alpha-fetoprotein; IL-6 = Interleukin-6; ND= Not detectable.

the human placenta is supported principally by histotrophic nutrition from the endometrial glands and decidua during the first trimester (Burton and Jauniaux, 2023; Burton et al., 2002). The coelom may therefore be viewed as a liquid extension of the villous mesenchyme and a reservoir of nutrients in which the secondary yolk sac floats (Fig. 2A).

To investigate the possible absorptive role of the yolk sac the composition of the fluid within the yolk sac was compared to that in the coelom (Gulbis et al., 1998). The similarity between the two fluids indicated that free transfer occurs between the two compartments, and this was confirmed by the fact that the placental hormone human chorionic gonadotropin was present in yolk sac fluid whilst the encoding mRNA is not expressed by the yolk sac tissues (Gulbis et al., 1998). Thus, the evidence suggests both the outer mesothelial and inner endodermal layers are exposed to maternal nutrients and may potentially be involved in their uptake. The higher levels of most proteins and amino-acids, but also of drugs, cotinine and inulin, between the coelomic and amniotic fluids (Table 1) suggest that transfer through the amniotic membrane separating the two cavities is limited (Jauniaux and Gulbis, 2000b; Jauniaux et al., 1997, 1999), further supporting the concept that the yolk sac is the main route between the coelomic cavity and the fetal tissues.

In order to gain firmer evidence of uptake we performed immunohistochemical staining for transporter proteins. Unfortunately, the repertoire of commercially available antibodies raised against these proteins is severely limited. Nonetheless, we tested for the multifunctional endocytic receptors megalin and cubilin that have been demonstrated to be important for uptake in the endodermal layer of the inverted yolk sac of rodents (Fisher and Howie, 2006). Staining for cubilin was strong within the endoderm cells whereas that for megalin was weak and patchy (Burke et al., 2013). These findings are consistent with the concept that decidual glycoproteins can pass into the yolk sac cavity where they may be taken up the endoderm cells and broken down within their lysosomal complexes (Jones and Jauniaux, 1995). Contrary results were obtained for specific transport receptors, and tocopherol transfer protein, the folate receptor alpha and glucose transporter 1 all localised predominantly to the outer mesothelial epithelium (Burton and Jauniaux, 2022; Jauniaux et al., 2004, 2007).

A more comprehensive study involving bulk mRNA sequencing compared the transcript profile of the human yolk sac with that of the mouse and published databases for the chicken (Cindrova-Davies et al., 2017). Transcripts encoding a multitude of transporters were identified, including a number of apolipoproteins and lipoprotein receptors, members of the ABC transporter family, and 259 members of the solute carrier (SLC) family. A high degree of conservation of these transcripts was found across with the mouse and chicken, supporting our concept as there is irrefutable evidence that the yolk sac plays an essential role in nutrient transfer in these species (Ornoy and Miller, 2023). In addition, ligands for many of the receptors were found to be present in the coelomic fluid on proteomic analysis (Cindrova-Davies et al., 2017). These data do not localise the transporters to a specific epithelium, and further immunohistochemical or higher-throughput spatial transcriptomic studies are required to do so.

Nonetheless, the weight of morphological, transcriptomic, proteomic, and immunohistochemical evidence to date make it extremely likely that the human yolk sac is involved in nutrient uptake during the period of embryogenesis.

1.5. *The first-trimester gestational sac as a choriovitelline placenta*

In view of these findings, we have proposed that the human placenta functions as a choriovitelline placenta during the first trimester of pregnancy (Cindrova-Davies et al., 2017) (Fig. 4). Although contact is not established between the vascularised mesoderm of the yolk sac and the chorion, we speculate that nutrients are nonetheless taken up and transported to the embryo through the vitelline circulation during the period of embryogenesis. There are a number of steps in this potential pathway for which evidence exists. Firstly, we have shown that maternal proteins secreted by the decidua and endometrial glands are endocytosed by the syncytiotrophoblast covering of the placental villi and that vesicles containing these proteins enter the digestive lysosomal pathway (Burton et al., 2002; Hempstock et al., 2004). Free amino acids released by the digestion may then pass out of the basal surface of the syncytiotrophoblast which is rich in transporter proteins (Cleal et al., 2018). There are few villous capillaries at this stage of development to take up the nutrients and the fetal placental circulation is not yet established. Instead, we propose the nutrients are carried in the longitudinal fluid-filled channels within the stromal core of the villi (Castellucci and Kaufmann, 1982), and it is notable that Hofbauer cells (macrophages) present within the channels are immunopositive for maternal decidual proteins (Burton et al., 2002). These channels connect with the coelom at the proximal ends of the villi where they arise from the developing chorionic plate (Burton et al., 2001). The nutrients accumulate in the coelomic fluid at high concentrations (Jauniaux and Gulbis, 2000a), from where amino acids, lipids, vitamins and other factors may be taken up by a variety of transporters on the mesothelial layer of the yolk sac and passed to the underlying capillaries. This pathway would be analogous to that seen in the yolk sac of the rat where it has been estimated that ~95% of the amino acids supplied to the embryo during organogenesis are derived from uptake and breakdown of maternal proteins rather than transfer of individual amino acids (Brent and Fawcett, 1998).

Analysis of the coelomic fluid reveals that a proportion of the maternal proteins pass through the trophoblast and enter the coelom intact. This raises the alternative possibility that the proteins may pass into the yolk sac cavity and be taken up and broken down by the endoderm cells of the yolk sac or developing gut. These two pathways are not mutually exclusive and may operate in tandem to transport specific nutrients, or at different stages of gestation.

In our proposal maternal nutrients are still taken up by the vitelline capillaries in the extraembryonic mesoderm of the yolk sac wall, as in a

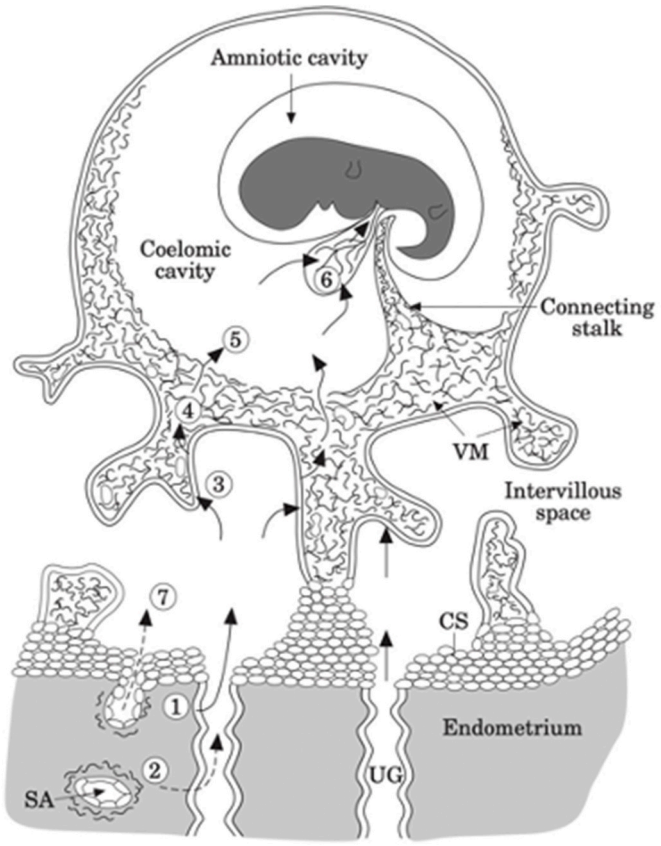

Fig. 4. Schematic representation of the histotrophic nutrition pathway during early pregnancy. (1) Secretions from the uterine (endometrial) glands (UG) are delivered through openings in the cytotrophoblastic shell (CS) into the intervillous space of the placenta. (2) They are supplemented by maternal plasma percolating through the aggregates of endovascular trophoblast occluding the spiral arteries (SA) and by decidual proteins. (3) Nutrients are taken up by the syncytiotrophoblast covering the placental villi and pass intact or as digests along stromal channels (4) in the villus mesenchymal core (VM) into the coelomic cavity (5). (6) From there they are taken up by the yolk sac and pass to the embryo via the vitelline circulation. (7) Towards the end of the first trimester maternal blood enters the intervillous space freely, enabling the onset of haemotrophic nutrition. Reproduced from Burton et al. (2001) with permission.

physical choriovitelline placenta, and delivered to the embryo by the vitelline vein. The only difference is that the coelomic cavity is interposed between the trophoblast and the yolk sac. As the coelomic cavity does not contain oxygen carriers, oxygen can only reach fetal tissues, including the yolk sac, by simple diffusion. Thus, in addition to molecules with an antioxidant capacity present in coelomic fluid (Jauniaux et al., 2004), the coelom may provide an additional barrier to excessive oxygen exposure for the embryonic tissues (Jauniaux et al., 2003).

1.6. Future directions

Although a functional role for the human secondary yolk sac in early maternal-fetal exchange is an attractive concept, there are currently no physiological or experimental data confirming it operates in this way in vivo. Ideally, the pathway would be traced from uptake from the coelomic fluid to transfer to the embryonic circulation. The relative contributions of the mesothelial and endodermal layers would be determined, and the importance of simple diffusion, facilitated diffusion and active transport tested for different ligands using competitive inhibitors. The specificity and transport kinetics of the different pathways would in turn be calculated. Such studies require access to fresh material, and obtaining fresh human yolk sacs is challenging both ethically and practically. Their extremely delicate nature means that they usually collapse during the evacuation procedure, making identification difficult. Equally, few species have the same morphological arrangements as the human, with the yolk sac floating in the coelomic cavity. One is the Little Brown Bat, *Myotis lucifgus*, and when horseradish peroxidase was injected into the coelom it was endocytosed by the mesothelial cells covering the yolk sac (Enders et al., 1976). Whilst this experiment provides evidence of uptake of proteins from the coelomic fluid, the authors were unable to confirm transfer into the vitelline capillaries.

The successful in vitro culture of spare human blastocysts up to day 14 opens new possibilities, but still raises ethical concerns. The generation of human blastoids from stem cells is perhaps more widely applicable, but the technique is still in its infancy and many technical challenges remain. Although cells of the different germ layers are present, so far no blastoids have shown proper structural organization. If this limitation can be overcome the models may permit similar tracer studies as in the bat after the injection of labelled ligands into the coelomic cavity. A more practical and technically easier option might be the organoid approach.

Organoids are three-dimensional, self-organising culture systems that faithfully reflect the structure and function of the original tissue. These genetically stable, long-term cultures enable investigation of normal development and disease modelling, and have been derived from many organs and tissues (Schutgens and Clevers, 2020). Their physiological responsiveness enables them to be used to predict drug effects, and they open new avenues for personalised regenerative medicine (Wu et al., 2023). Importantly in this context, they have been used successfully in transport studies (Zietek et al., 2020). A recent study reports generating organoids from canine, bovine and porcine yolk sacs (Pereira et al., 2023). These 3D cultures (using Matrigel or hanging drops) form fluid-filled spheres of flattened cells bearing microvilli that resemble morphologically the mesothelial cells of the YS. They express zinc and cholesterol transport markers and haemoglobin subunit zeta-like (HBZ), but low expression levels of alpha-fetoprotein (AFP) again suggest they are more mesothelial than endodermal in nature.

As proof-of-principle, we generated yolk sac organoids from the mouse yolk sac. These organoids are cystic, inverted structures, consisting of a single layer of largely cuboidal epithelial cells (Fig. 5A). The organoids express transport proteins, including transthyretin (Ttr; transports thyroxine and retinol), retinol binding protein 4 (Rbp4), apolipoprotein a1 (Apoa1), as well as markers of endoderm, Afp, Sox7 and H19 (Fig. 5B and C and 6D). Derivation of the YS organoids required the stimulation of the MAPK signalling (EGF, FGF10, HGF), WNT signalling (R-spondin), and blocking of BMP (Noggin). Interestingly, inhibition of TGF-β signalling promoted organoid expansion (Fig. 6A–C), increased Apoa1 protein level (Fig. 6E) and Apoa1 and Afp mRNA expression (Fig. 6D), and reduced the mRNA expression of the markers of primitive endoderm, Sox7 and H19. We also tested the effect of LIF, which was inconsistent. The high expression of AFP, Sox7 and H19 suggests that these murine yolk sac organoids are likely to be of endodermal origin.

In future, such organoids could be utilised in uptake studies, either directly as spheres or used to seed transport membranes incorporated into microfluidic devices. A potential limitation is, however, access to fresh YS tissue from which to generate the organoids. The ability to genetically derive a human embryoid model from induced pluripotent stem cells may circumvent this problem (Hislop et al., 2024). The cells in this model self-organise and form an amniotic cavity, bilaminar disc, and an anterior hypoblast pole and posterior domain, closely mimicking human embryogenesis but lacking trophoblast. This highly reproducible model captures the reciprocal co-development of embryonic tissue and the extra-embryonic endoderm and mesoderm niche with early haematopoiesis. The multilineage yolk sac tissue undergoes distinct waves of haematopoiesis and as it expresses endoderm and mesoderm fates it

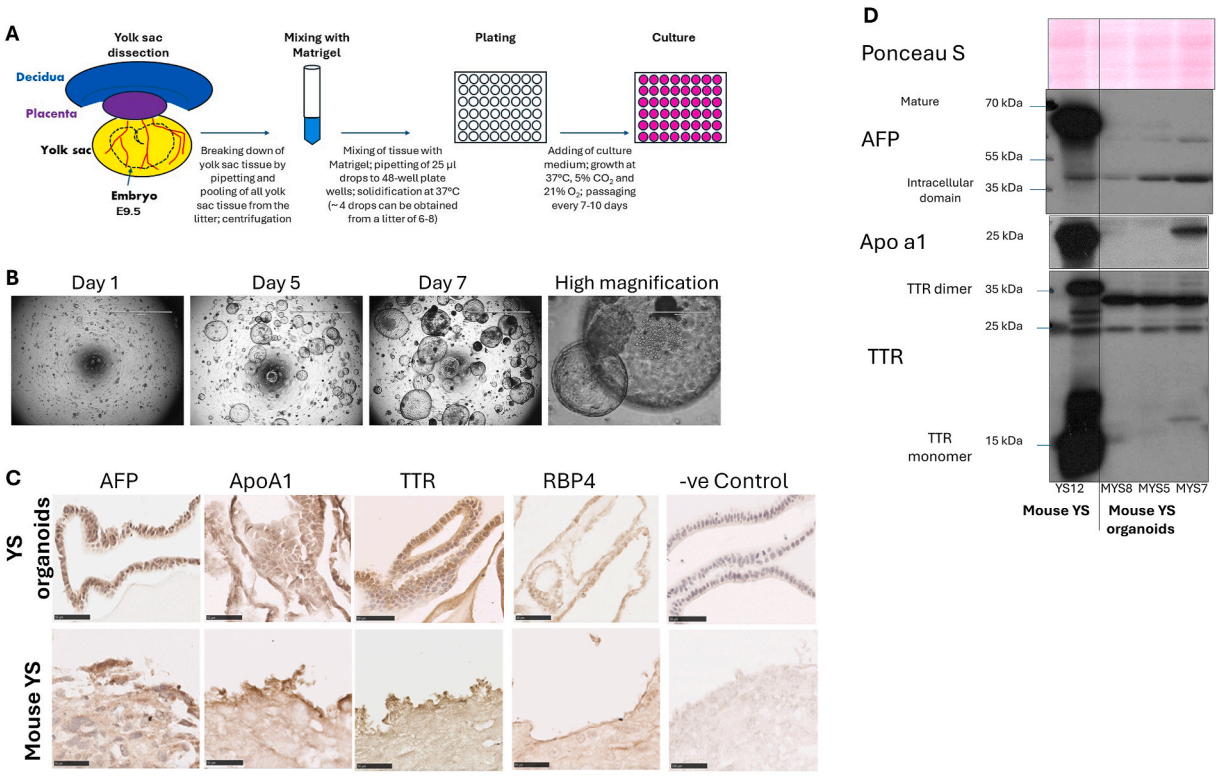

Fig. 5. Derivation and characterisation of mouse yolk sac organoids. A) Mouse yolk sac organoids were derived from dissected yolk sac tissue of pregnant mice at E9.5. Each gestational sac was separated and yolk sac tissue dissected out, broken down by pipetting, centrifuged, resuspended in Matrigel and plated out in a 48-well plate. 250 µl of culture medium was added to each well. B) The organoids were grown at 37 °C, 5% CO2 and 21% O2. The culture medium was replaced every 2 to 3 d. The organoids were passaged approximately every 7 to 10 d. The culture medium contained factors which simulate MAPK signalling (EGF, FGF10, HGF), WNT signalling (R-spondin), and block BMP (Noggin). Scale bars = 2000 µm (first three images) and 200 µm (high magnification image). C) Organoids were grown for 7 d, processed and expression of various markers, including α-fetoprotein (AFP), apolipoprotein A1 (ApoA1), transthyretin (TTR), retinol binding protein 4 (RBP4), was tested by immunohistochemistry. Scale bars = 50 µm. D) Western blotting for the markers. Note that mature murine AFP is 69–73 kDa; Murine AFP also contains an alternate start site at Met281 that may generate an intracellular 37 kDa form.

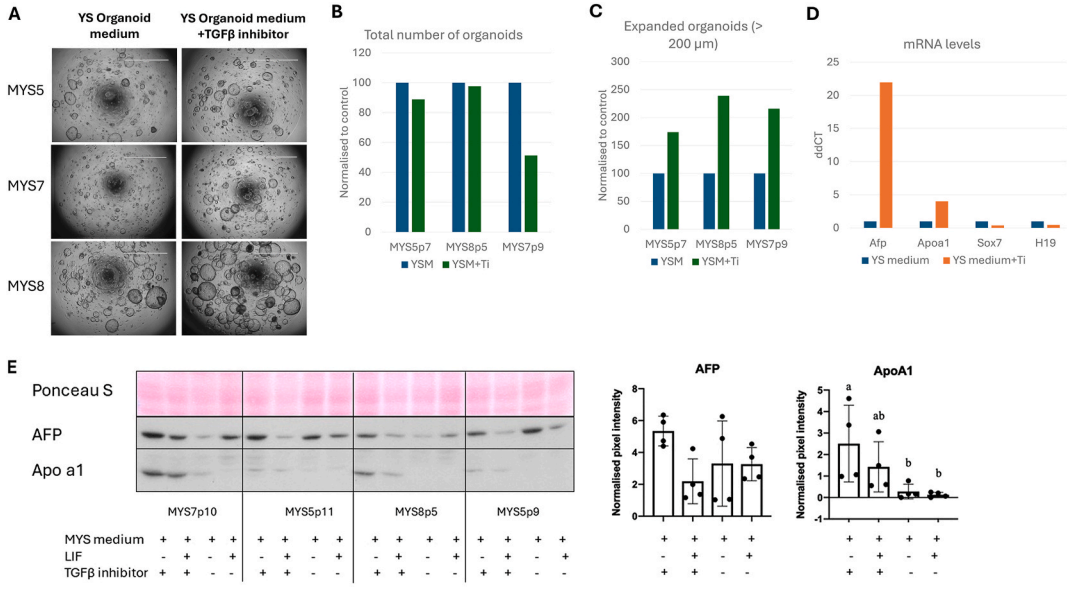

Fig. 6. Organoid efficiency assays from single cells to assess the effects of TGF-β inhibition on YS organoid expansion and marker expression. 5000 organoid single cells were plated in a Matrigel drop per culture condition in triplicate and the number of organoids formed after 7 d was scored and assessed for markers. Inhibition of TGF-β signalling promoted organoid expansion (A–C), increased mRNA levels of Apoa1 and Afp, and reduced mRNA levels of Sox7 and H19 (D). TGF-β inhibition also resulted in significantly increased protein expression of Apoa1 (E). Addition of leukemia inhibitory factor (LIF) had no significant or consistent effect (E). Scale bars = 2000 µm.

may provide starting material for the derivation of organoid cultures.

2. Conclusions

Mounting evidence over the last decade supports an active role of the human secondary yolk sac in early pregnancy as part of the histotrophic nutritional pathway essential for normal embryonic and fetal development. Despite there being no direct contact between the vascularised mesoderm of the yolk sac and the chorion, we propose that the early placental villi, the coelomic cavity, and the secondary yolk sac combine to function as a physiological equivalent of a choriovitelline placenta. The secondary yolk sac is thus likely to be essential for the transfer of maternal molecules such as thyroid hormones, folic acid, oligo-elements and essential amino-acids before the fetal tissues are able to synthesise them.

Morphological abnormalities of the secondary yolk sac have been reported in 70% of first trimester human miscarriages (Nogales et al., 1993), but separating cause from effect is impossible. For example, it has been proposed that yolk sac dysfunction secondary to hyperglycaemic vasculopathy contributes to birth defects in cases of maternal diabetes (Dong et al., 2016). New and emerging models of the yolk sac will enable many of the questions pertaining to the role of the yolk sac in early post-implantation development, disease pathologies and protection against xenobiotics to be addressed.

3. Materials and methods

3.1. Mouse tissue collection

Yolk sacs were collected from time-mated virgin C57BL/6J mice at E9.5 (day of plug = E0.5). Experiments were carried out in accordance with the United Kingdom Animals Scientific Procedures Act 1986 which mandates ethical review. The fat was cut away from the uterus and each gestational sac was separated and transferred to PBS for greater visibility. Using a dissection microscope, the decidua of each uterine bud was cut (Fig. 5A), to allow separation of the placenta from the yolk sac and embryo at the mesometrial pole. Yolk sac tissue for RNA or protein extraction was snap-frozen and transferred to −80 °C until processing.

3.2. Derivation and characterisation of mouse yolk sac organoids

Mouse yolk sac organoids were derived from dissected yolk sac tissue of pregnant mice at E9.5. The visceral yolk sac was easily distinguishable by its vasculature and was obtained by detachment from the embryo at the yolk stalk and from the parietal yolk sac at the allantoic placenta. Yolk sac tissue was pooled together from all litter mates. After collection in medium (DMEM/F12, primocin), the yolk sacs were broken down by pipetting until no large fragments were visible, pelleted by centrifugation (600 g, 5 min) and resuspended in ice-cold Matrigel (Corning, 536231) at a ratio of 1:20 (vol:vol). Drops of 25 μl Matrigel–cell suspension were plated in the centre of 48-well plates (Costar, 3548) and allowed to set at 37 °C. About four drops can be obtained from a uterus of 6–8 gestational sacs. 250 μl culture medium was added to each well. The culture medium contained factors which simulate MAPK signalling (EGF, FGF10, HGF), WNT signalling (R-spondin), and blocking of BMP (Noggin) (Cindrova-Davies et al., 2021; Turco et al., 2017). The medium was changed every 2–3 days. Cultures were passaged by manual pipetting every 7–10 days. The organoids were grown at 37 °C, 5% CO_2 and 21% O_2.

3.3. Organoid formation efficiency assays

We followed our previously published protocol (Turco et al., 2017). Briefly, yolk sac organoids were removed from Matrigel using Cell Recovery Solution, pipetted several hundred times and trypsinised with TrypLE Express (Invitrogen, 12604-013). Single cells were derived by passing the digest washed in medium through a 40 μm cell strainer (Corning, 352340). This was followed by cell count. 5000 cells were plated per 20 μl Matrigel drop per culture condition in triplicate for the growth factor requirement experiments (Fig. 6). The number of organoids formed after 7 d was scored.

3.4. Immunohistochemistry

Immunostaining of organoids was performed as previously described (Cindrova-Davies et al., 2021). Briefly, organoids were removed from Matrigel using Cell Recovery Solution, fixed in 4% paraformaldehyde and encapsulated into 1% agarose (Melford, MB1200), dehydrated and embedded in paraffin wax. 7 μm thick sections were dewaxed, rehydrated, incubated in 3% H_2O_2 for 15 min to block endogenous peroxidase activity. If required, heat-induced antigen retrieval was performed by boiling sections in Tris-EDTA buffer (TE; 10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) in a microwavable pressure cooker for 1–2 min (microwave at 570 W). Sections were blocked for 1 h in 5% goat serum, 2% BSA solution (in TBS) to prevent non-specific antibody binding and incubated overnight at 4 °C with the appropriate primary antibodies: α-fetoprotein (AFP; Abcam, ab46799; 1:100), apolipoprotein A1 (ApoA1; Thermo Fisher Scientific, PA5-78798; TE, 1:1000), transthyretin (TTR; Thermo Fisher Scientific, PA5-80197; 1:100), retinol binding protein 4 (RBP4; Abcam, ab188230; TE, 1:200). This was followed by detection with avidin-conjugated secondary antibodies, and visualised with a Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) and SigmaFast DAB (Sigma, Poole, UK). Sections were then lightly counterstained with haematoxylin, before being dehydrated and mounted.

3.5. Western blotting

Placental lysates were processed and run on western blots, as previously described (Cindrova-Davies et al., 2007, 2021), using the following antibodies: anti-AFP (Abcam, ab46799; anti-TTR (Thermo Fisher Scientific, PA5-80197), anti-ApoA1 (Thermo Fisher Scientific, PA5-78798).

3.6. Quantitative real-time RT-PCR analysis

Total RNA was isolated from snap frozen mouse yolk sacs or mouse yolk sac organoids using RNAeasy kit (Qiagen, Crawley, UK). RNA was quantified by spectrophotometry (Nanodrop Technologies, DE, USA). In brief, 20 μg of total RNA from each sample was reverse transcribed using a master mix containing SuperScript II Reverse Transcriptase in the First Strand Buffer with 0.1M DTT (Invitrogen, Paisley, UK), 50 ng/ml random hexamers (Sigma). The DNA Engine Opticon 2 Sequence Detection System (Bio-Rad Laboratories, UK) was used to perform real-time PCR according to the manufacturer's protocols (using TaqMan–FAM1 dye). TaqMan gene expression assays (Applied Biosystems) were performed on triplicate samples as per manufacturer's instructions. Data were normalised relative to *Sdha*. All the qRT-PCR was performed using TaqMan probes.

3.7. Statistical analysis

Data are expressed as mean ± SD. Comparisons were made using ANOVA with a Tukey's multiple comparison post hoc test. Differences were considered significant at $p \leq 0.05$.

CRediT authorship contribution statement

Graham J. Burton: Writing – original draft, Conceptualization. **Eric Jauniaux:** Writing – review & editing, Resources, Conceptualization. **Tereza Cindrova-Davies:** Writing – review & editing, Investigation, Formal analysis. **Margherita Y. Turco:** Writing – review & editing,

Investigation, Formal analysis.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors used no generative AI and AI-assisted technologies in the writing process.

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Data availability

No data was used for the research described in the article.

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