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The secondary human yolk sac has an immunophenotype indicative of both hepatic and intestinal differentiation

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ABSTRACT Although the microscopy of the secondary human yolk sac (SHYS) is well known, few studies have addressed its immunohistochemical profile. The SHYS is involved in the synthesis, absorption and transfer of various proteins and behaves as a temporary liver and intestine. The objective of this study was to evaluate the presence of immunohistochemical markers of hepatic and intestinal function in the SHYS. We performed a retrospective histological and immunohistochemical study of 26 SHYS from spontaneous abortions and tubal pregnancies, 15 of which were from the 7th to 8th week. The antibodies used were against α-foetoprotein (AFP), glypican 3 (GLP3), hepatocyte-paraffin-1 (HepPar-1), villin, CDX2, SALL4 and podoplanin (D2-40). Early SHYS from the 5th to the 8th week revealed a network of intracellular vesicles communicating with the lumen of endodermal tubules that were highlighted by intense membrane AFP expression. Endodermal cells consistently expressed AFP, GLP3, SALL4, hep-par-1, villin and CDX2, while mesothelial cells only expressed D2-40. The endodermal layer of the SHYS from the 5th to the 8th week revealed a transient canalicular network which was highlighted by strong membranous AFP expression; this may represent the substrate of a SHYS transport system during its period of maximal activity. The synthetic and transfer functions of the yolk sac endoderm were reflected in a hybrid immunophenotype in which proteins characteristic of hepatic function such as AFP, GLP3, SALL4 and hep-par-1 were coexpressed simultaneously with others such as villin and CDX2, indicative of an intestinal role.

KEY WORDS: *secondary human yolk sac, hepatic function, intestinal differentiation marker, AFP, HepPar-1*

The secondary human yolk sac (SHYS) is an organ which plays a crucial role in early development. Although it has not attracted the attention it deserves in the literature, its optic microscopy and ultrastructure are well known (Hesseldahl *et al.,* 1969; Jones *et al.,* 1995a; Nogales-Fernandez *et al.,* 1977; Pereda *et al.,* 1999; Takashina *et al.,* 1993). Its clinical relevance in relationship with early pregnancy loss has been studied both ultrasonographically (Ferrazzi *et al.,* 1988; Hustin *et al.,* 1987; Jauniaux *et al.,* 2005; Jauniaux *et al.,* 1991; Kucuk *et al.,* 1999) and histologically (Nogales *et al.,* 1992; Nogales *et al.,* 1993; Nogales *et al.,* 1995).

This essential structure is vital for protein synthesis, as can be seen from its production of a wide range of substances (Gitlin *et al.,* 1969; Gitlin *et al.,* 1970; Gitlin *et al.,* 1972; Jones *et al.,* 1995b; Shi *et al.,* 1985), especially those contributing to blood formation (Gitlin *et al.,* 1969; Gulbis *et al.,* 1994). Furthermore, the types of proteins it synthesizes are evidence that, for a short period of time, the SHYS is involved in absorptive and transfer roles, thus behaving as a temporary liver and intestine (Gulbis *et al.,* 1998; Gulbis *et al.,* 1994; Gulbis *et al.,* 1992; Shi *et al.,* 1985). Indeed, it is the principal route of entry of many proteins and iron to the embryo. However, in the last decade, little information about the immunohistochemistry of the SHYS has been provided, with only few studies dealing with the shared immunohistochemical expression of some proteins (Preda *et al.,* 2011) by the SHYS and human yolk sac tumours in order to prove the vitelline identity of these neoplasms (Nogales *et al.,* 2012; Preda *et al.,* 2011).

The present work reports, for the first time, the demonstration and location in the SHYS of highly characteristic immunohistochemical markers that are associated with hepatic (glypican 3 and hepatocyte-paraffin-1) and intestinal (villin and CDX2) functions, thus providing a morphological basis for its temporary physiological role as an active interface between the exocoelomic cavity and the developing embryo. Additionally, SALL4, a pluripotency marker and podoplanin, a mesothelial marker, were also analyzed.

This hypothesis is supported further by the expression, distribution and location of transport proteins such as α-foetoprotein

Abbreviations used in this paper: AFP, alpha-foetoprotein; GLP, glypican; H&E, Hematoxylin and eosin stain; HepPar, hepatocyte-paraffin; SHYS, secondary human yolk sac.

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(AFP) in a network of intracellular channels connected with the endodermal tubules that may represent the substrate of a transfer system between embryo and the exocoelomic cavity.

Results

Hematoxylin and eosin stain (H&E)

The SHYS histology was assessed according to its developmental stage: the only available *early sac from the 5-6th week* showed a trilaminar wall structure with an inner linear endoderm and an external mesothelial layer with haemopoietic islands present in its intervening mesenchymal layer. The endodermal cells had rounded large nuclei with macronucleoli and an eosinophilic, granular cytoplasm populated by numerous intracytoplasmic vesicles. Frequent mitoses were present.

In sacs from the 7-8th week, the thickness of the endodermal layer was substantially increased and proliferated to form downwards columns of cells that eventually became hollow, empty tubules (Fig. 1A). Their cytoplasm exhibited abundant intracellular lumina (Fig. 1B) often in close apposition with the endodermal tubules and the haemopoietic islands present in the mesenchyme. Some anisokaryosis was often present (Fig. 1B). The mesothelium was unremarkable.

Involuting sacs from the 9-12th week revealed a flattened or coarsely vacuolated endoderm with collapse and flattening of the endodermal tubules and disappearance of the intracellular vesicles. Blood islands progressively disappeared while the mesothelial layer became prominent and vacuolated. At the end of this period, endodermal cells had become atrophic and been desquamated into the endodermal cavity as amorphous eosinophilic granular debris. Parietal fibrosis and calcification were present.

TABLE 1

ANTIBODIES USED IN THIS STUDY

Antibody	Clone	Dilution	Vendor
Glypican 3 (GLP3)	SP86	Prediluted	MasterDiagnostica, Spain
α-foetoprotein (AFP)	Polyclonal	Prediluted	DAKO, Denmark
Hepatocyte paraffin 1 (HepPar-1)	OCH1E5	Prediluted	DAKO, Denmark
CDX2	DAK-CDX2	Prediluted	DAKO, Denmark
Villin	1D2 C3	Prediluted	DAKO, Denmark
Podoplanin (D2-40)	D2-40	Prediluted	DAKO, Denmark
Calretinin	DAK-Calret 1	Prediluted	DAKO, Denmark
Anti-Mesothelioma antibody	HBME-1	Prediluted	DAKO, Denmark

Immunohistochemistry

α-*foetoprotein* (AFP) secretion was seen as early as the 5th week and remained positive throughout the evolution of the SHYS, even in the atrophic or shed endodermal cells of the involuting sacs of 10-11 weeks. Strong cytoplasmic stain was exclusively found in the endodermal layer. It is worth noting that in SHYS from 5-8 weeks, the numerous intracellular vesicles were highlighted by a strong membrane AFP expression (Figs. 1 C,D). Vesicles were seen to communicate with the endodermal tubules, which also expressed a strong luminal stain (Fig. 1D). Some hematopoietic cells were also AFP positive (Fig. 1C). Mesothelial layer cells were negative, although some diffusion and background stain occurred. In the accompanying embryonal tissues, a strong expression was identified in both the liver trabeculae and some blood cells.

Glypican 3 (GLP3) staining pattern was similar in strength and location to that of AFP, albeit with a minimal background stain, remaining positive throughout the evolution of endoderm. Although the endodermal cytoplasm was well stained, it had a stronger membranous intensity than AFP (Fig. 2A). The endodermal intracellular vesicles and tubules also showed a prominent apical and luminal stain. In the available embryonal tissues, liver cells showed a constantly strong positivity. This antibody was also expressed in developing mesenchymal cells and the neuroepithelial structures of embryos.

Hepatocyte Paraffin-1 (HepPar-1) expression was reduced to cells of the endodermal layer and was also constantly present throughout the evolution of the SHYS. It had a characteristically strong, coarsely granular cytoplasmic (mitochondrial-type) staining pattern (Fig. 2B). Even atrophied or shed endodermal cells of the involutive period also showed similar reactivity. Among the embryonal tissues, only liver was specifically stained with a similar expression.

Villin showed a strong cytoplasmic and membranous expression in the endoderm throughout the evolution of the

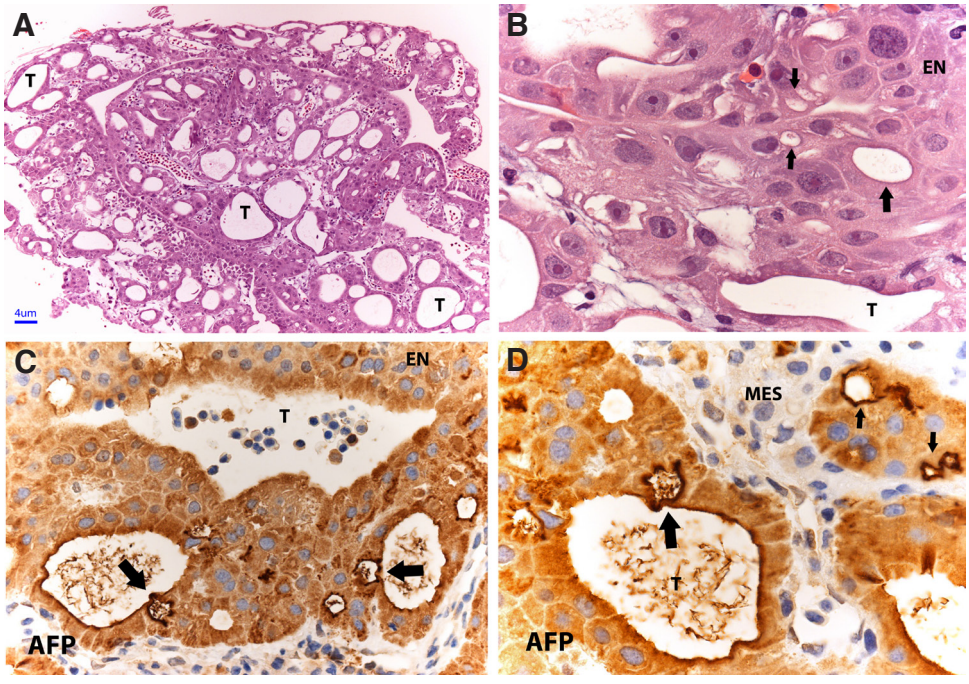

Fig. 1. A partly collapsed secondary human yolk sac from the 8th week. (A) *Note the numerous endodermal tubules(T).* **(B)** *Higher magnification of the endodermal epithelium (EN). Cells have large, irregular nuclei with prominent nucleoli.* **(C,D)** *Their ample cytoplasm reveals lumina (arrows) whose membranes are intensely AFP immunoreactive (arrows).* **(D)** *AFP immunoreactivity is absent in the mesenchyme (MES).* **(C,D)** *Intracellular lumina communicate with the large endodermal tubules; see arrows in (C,D).*

TABLE 2

SHYS IMMUNOPHENOTYPE

		ANTIBODIES						
Week	**#**	**AFP**	**GLP3**	**HepPar-1**	**Villin**	**CDX2**	**SALL4**	**D2-40**
5-6	1	1/1	-	-	-	-	-	-
7-8	15	15/15	15/15	12/14*	11/12*	10/14*	10/13*	15/15
9-11	10	10/10	10/10	9/10	5/9*	9/10	3/8*	8/9*

All antibodies, except for podoplanin D2-40, were expressed in the endodermal layer. Only podoplanin was positive in the mesothelium.

(*) In some cases, step sections failed to produce a sufficient number of slides to complete the study of some antibodies

TABLE 3

IMMUNOHISTOCHEMICAL EXPRESSION OF CONCOMITANT EMBRYONAL TISSUES

		ANTIBODIES					
Weeks	**#**	**AFP**	**GLP3**	**HepPar-1**	**Villin**	**CDX2**	**SALL4**
7-8	6	L 6/6	L 6/6 Mes 6/6 Neu 6/6	L 6/6	L 6/6 * Gut 3/3	* Gut 2/6	* L 1/4 Neu 6/6
9-11	4	L 4/4	L 4/4 Mes 4/4 Neu 4/4	L 4/4	L 4/4	-	Neu 4/4

(*) Not all embryonal organs were analyzed, due either to poor section orientation or absence of material in successive slides. KEY: L, liver; Mes, mesenchyme; Neu, neuroepithelium.

endodermal layer (Fig. 2C). In the embryonal tissues it was markedly positive in the cytoplasm of liver cells and in the apical membranes and cytoplasm of the intestinal lining.

CDX2 was strong and diffusely expressed in the nuclei of early and sacs from the 7th to 8th week, becoming weaker and more focal in distribution during the involution period (Fig. 2D). In embryos showing gut structures, lining cells had a strong nuclear positivity which was, however, absent in liver and other tissues.

SALL4 broadly expressed a similar nuclear staining pattern to CDX2 in both chronology and intensity (Fig. 2E). In the embryonal tissues it was strongly expressed in neuroepithelium.

Podoplanin (D2-40) expression was reduced to the mesothelial layer (Fig. 2F), which failed to stain for other markers. In the 3 instances where sections were still available, mesothelium was negative for other characteristic mesothelial antibodies such as calretinin and HBME-1.

The immunohistochemical findings of both SHYS and embryos are shown in Tables 2 and 3. As a summary, Fig. 3 A-E shows the comparative expression of the antibodies in a 7th week SHYS.

Discussion

Clinical interest in SHYS morphology has been focused mainly on its ultrasound appearance during the first trimester. Although it has been proposed that SHYS changes may act as markers for some chromosomal abnormalities (Schmidt *et al.,* 2011), it seems that the predictive value of SHYS measurements in determining the outcome of an early pregnancy is limited, as the alterations in SHYS size are the consequence of poor embryonic development or embryonic death rather than being the primary cause of early pregnancy failure (Jauniaux *et al.,* 2005). This clinical perception agrees with morphological studies of the SHYS in spontaneous pregnancy loss, which revealed only non-specific, degenerative features related to embryonal death and retention (Nogales *et al.,* 1992; Nogales *et al.,* 1993; Nogales *et al.,* 1995).

In the present paper, we analyze SHYS material originating from spontaneous pregnancy loss. Sacs from all evolutive periods were included and there were only minimal degenerative changes. Due to the limits imposed by such scanty material, we focused on the demonstration of a short series of readily available antibodies characteristic of the presumed secretory, synthetic and absorptive

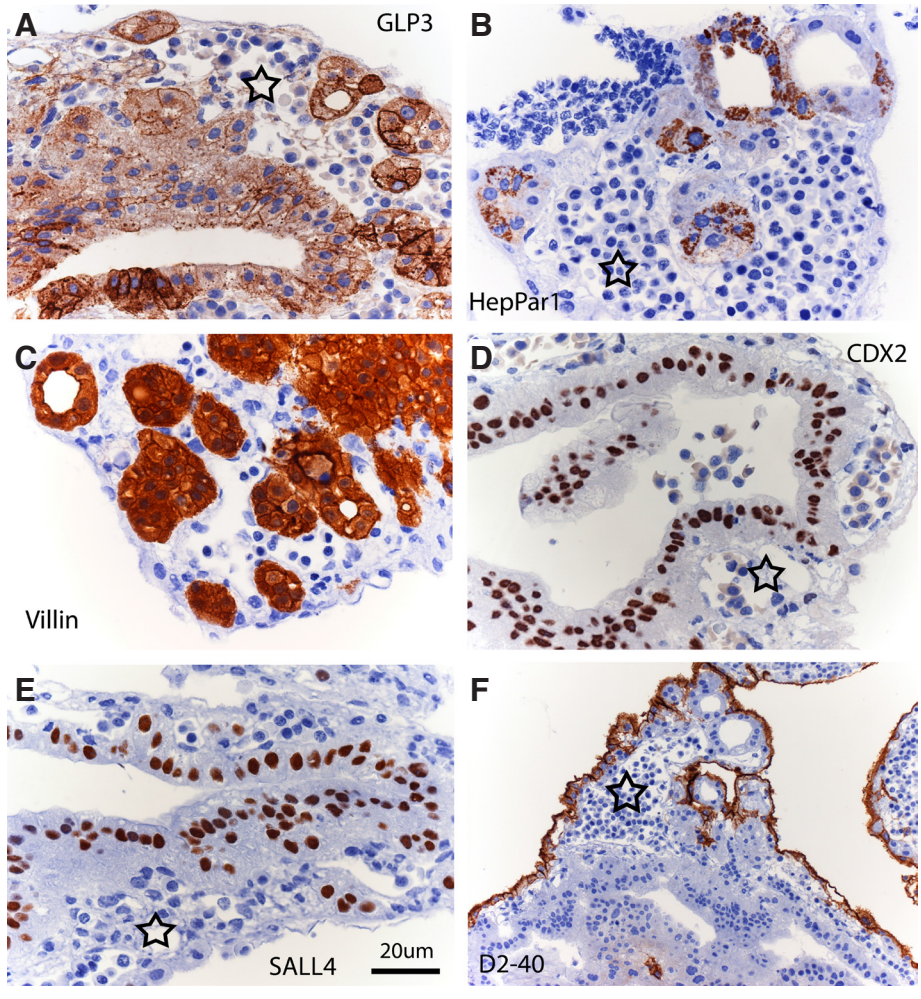

Fig. 2. Immunophenotype of another secondary human yolk sac from the 8th week, during which haematopoiesis (*) is prominent. (A) *GLP3 delineates endodermal membranes.* **(B)** *Granular cytoplasmic (mitochondrial) positivity for HepPar1 is prominent.* **(C)** *Villin shows strong membrane and cytoplasm expression, while CDX2* **(D)** *and SALL4* **(E)** *reveal nuclear positivity.* **(F)** *D240 podoplanin is only expressed in mesothelial cells.*

functions of the SHYS as a temporary liver and intestine and also used in the diagnosis of yolk sac tumours (Nogales *et al.,* 2012).

SHYS has been considered a transfer organ between the embryo and the exocoelomic cavity (Gulbis *et al.,* 1998), implying a role of active synthesis, absorption and transference of proteins during a short but crucial period of ontogenesis.

Optic microscopy shows the endoderm lining the yolk sac cavity to have a progressively complex structure, developing short columns of cells that contain an abundant network of intracellular vesicles. These structures are present in the earliest SHYS of this study at the 5th week, but they eventually collapse and disappear in involutive sacs. Indeed, they seem to be present only during its period of maximum activity.

Ultrastructurally, SHYS endodermal cells share many common features with hepatic ones, having a glycogen-rich cytoplasm, well-developed Golgi complex, and abundant rough endoplasmic reticulum profiles (Jones *et al.,* 1995a; Pereda *et al.,* 1999; Takashina *et al.,* 1993). Well-developed apical microvilli are present on the tubular surface and also line the numerous intracellular vesicles (Takashina *et al.,* 1993) that will eventually coalesce to form a complex system of endodermal tubules. They are likely to have a role in transport of various substances due to their close relationship with blood islands, mesenchymal capillaries and mesothelium.

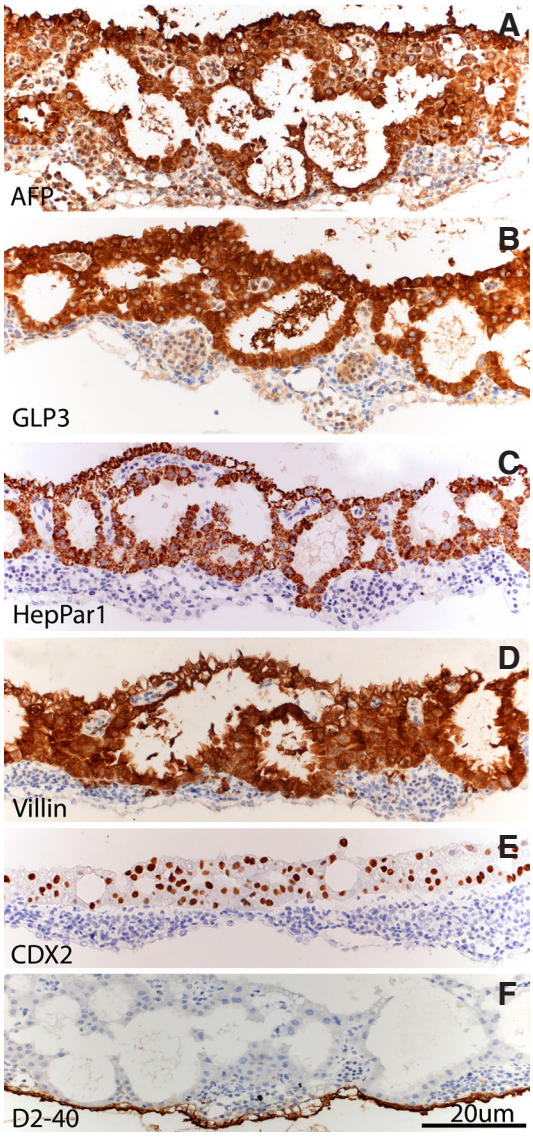

Fig. 3. Immunophenotype of a secondary human yolk sac from the 7th week. *The endoderm is strongly positive in both cytoplasm and membrane for* **(A)** *AFP and* **(B)** *GLP3.* **(C)** *HepPar1 exhibited a coarse granular expression.* **(D)** *Villin immunoreactivity is intense in the cytoplasm and membrane.* **(E)** *CDX2 labeling is intense in the nucleus.* **(F)** *Only the mesothelial layer presented podoplanin immunoreactivity.*

In this paper we demonstrate that this transient canaliculotubular complex displays a strong apical membrane staining for both AFP and GLP3, possibly indicating an active transport of these important proteins.

The functional similarities of the SHYS endodermal cells with hepatic and intestinal ones lie in their shared synthesis of proteins such as AFP, prealbumin, albumin, caeruloplasmin, fibrinogen, plasminogen, lipoproteins, α1-protease inhibitor, transferrin, GLP3, etc. (Gitlin *et al.,* 1969; Gitlin *et al.,* 1970; Gitlin *et al.,* 1972; Gulbis *et al.,* 1998; Gulbis *et al.,* 1992; Preda *et al.,* 2011; Shi *et al.,* 1985). Among these, AFP and GLP3 are only expressed by developing hepatocytes (Kandil *et al.,* 2007; Mizejewski *et al.,* 2001) and have a similar distribution in both SHYS and liver. AFP function resides in the binding and transport of various ligands and recently an additional role as a growth regulator has been recognized (Mizejewski *et al.,* 2001). Glypican 3 also acts as a modulator, activating intracellular signaling proteins and growth factors (Filmus *et al.,* 2008). Their similar location and staining patterns in SHYS would reflect a synergic relationship, possibly related to cell growth control.

HepPar-1 is an empirically obtained monoclonal antibody (Wennerberg *et al.,* 1993) raised against formalin-fixed, paraffin embedded hepatic tissue that is highly specific of both adult and embryonal liver cells, both normal and neoplastic. Outside the liver, it is only focally present in the glands of the small intestine and shows a weak expression in gastric glands (Lugli *et al.,* 2004). Recently, the antigen for this antibody has been shown to be carbamoyl phosphate synthetase 1, an enzyme in the urea cycle located in mitochondria (Butler *et al.,* 2008). This hepatic antigen has not been previously studied in the SHYS. Here, it was consistently present throughout the development of the SHYS from the 7th week onwards and was also exclusively expressed by the liver cells of the accompanying embryos.

Villin is a Ca^{2+} regulated actin-binding protein that is expressed early during embryogenesis, being present in the mouse yolk sac in early stage visceral endodermal cells (Maunoury *et al.,* 1988). In the human embryo, it is found at the 8th week in the early intestinal tube. It is considered an early marker of endodermal cell lineage and it is identified in gastrointestinal, renal and urogenital epithelial cells (Robine *et al.,* 1985). Villin is regarded as an early marker of committed intestinal absorptive cells (Khurana *et al.,* 2008), being expressed also by liver ducts. The expression of villin in the SHYS and embryonal liver cells is consistent with both an intestinal and hepatic phenotype for the SHYS, where it is present in both the free membrane surfaces delineating endodermal tubules and the intracellular vesicles. A diffuse cytoplasmic stain was also present in both the SHYS and embryonal liver cells.

CDX2, a caudal-like homeodomain-containing transcription factor, is expressed in intestinal endoderm posterior to the stomach throughout gestation and adult tissues, where its strong expres-

sion is mostly found in the nuclei of small and large intestine and pancreatic ducts (Moskaluk *et al.,* 2003). Metaplastic conditions that reproduce intestine are also CDX2 positive (Nicolae *et al.,* 2011) as well as some unusual metaplasias such as morules in various organs (Houghton *et al.,* 2008). Its early functions include promotion of trophoblast differentiation (Stringer *et al.,* 2012) and determination of blastocyst polarity (Jedrusik *et al.,* 2008). Later on, it is involved in the differentiation and development of the intestine, but is not expressed by liver, even at an early stage of differentiation, as confirmed in this study by its absence in the hepatic tissue of all our accompanying embryos. It is worth noting that its expression was strong and diffuse in sacs from the 7-8th week but diminished to a weak and focal stain in the older involuting ones.

SALL4 is a transcriptional activator of Pou5f1 and has a critical role in the maintenance of cell pluripotency by modulating Oct4 expression (Zhang *et al.,* 2006). In the liver, SALL4 plays a decisive role in controlling the lineage commitment of hepatoblasts not only by inhibiting their differentiation into hepatocytes but also driving their differentiation toward cholangiocytes (Oikawa *et al.,* 2009). So, it would seem that SALL4 is crucial in liver cell differentiation and its strong expression in early SHYS could be related to its early hepatic function.

Bile secretion only takes place at the 12th week (Crawford *et al.,* 2002) and is not needed at this developmental stage; consequently it is neither present in the SHYS nor in the embryonal liver, only appearing in the foetal stage.

The role of SHYS mesothelium has been assessed as being active in protein transfer (Gulbis *et al.,* 1998; Jauniaux *et al.,* 2000), although others have proposed a merely protective role (Pereda *et al.,* 1999). The only marker that was positive in the mesothelium of the SHYS was D2-40, a monoclonal antibody against podoplanin (Kalof *et al.,* 2009). However, other frequently used mesothelial markers such as calretinin and HBME-1 were negative, although they were not performed in all sacs due to the depletion of tissue in the paraffin blocks.

The above results reveal that the SHYS endodermal cells have a hybrid immunophenotype of both liver and intestinal cells that parallel their synthetic and transport functions. Their predominantly hepatic features of differentiation are present at a histological level (intracytoplasmic vesicles configuring a canalicular system) and immunohistochemically (expression of specific liver cell markers such as AFP, HepPar-1 and GLP3). Moreover, it seems that expression of SALL4 is crucial in liver cell differentiation. The intestinal phenotype is represented by the expression of villin, which is present in both intestine and early liver. Furthermore, CDX2 is characteristic of intestinal cells but absent in liver cells.

Materials and Methods

26 SHYS were obtained from the routine histopathological archives of our hospital between 1986 and 2007, corresponding to 24 products of conception from spontaneous abortions and 2 tubal ectopic pregnancies. Gestational ages were assessed by taking into account both clinical data and morphological milestones. The earliest sac corresponded to a 5 week tubal pregnancy, 15 were in the 7th to 8th week range and 10 corresponded to the involutive period of 9-11 weeks. All had a good histological preservation, with minor changes of maceration in only 3. Concomitant embryos were found in 11 cases, of these, 8 were fresh and in only 3 were there minor maceration changes.

The SHYS and embryos were formalin fixed and subsequently embedded in paraffin and stained with H&E. Step sections were performed.

Immunohistochemistry was done using the antibodies listed in Table 1. Functionally, these antibodies recognize the following functional proteins: *α-foetoprotein (AFP),* a protein is expressed in the early endoderm that binds and transports various ligands, being highly characteristic of yolk sac and immature liver; *Glypican 3 (GLP3),* a cell surface heparan sulphate proteoglycan is expressed by both yolk sac and liver that acts as a modulator, activating intracellular signaling proteins and growth factors; *Hepatocyte-paraffin-1 (HepPar-1)*, a mitochondrial urea cycle enzyme highly characteristic of embryonal and adult liver cells; *Villin*, a Ca^{2+} regulated actin-binding protein present in both embryonal and adult intestinal cells; *CDX2*, a protein from a ParaHox gene which interacts in trophoblast differentiation, axial development and particularly, in gut differentiation; *SALL*, a stem cell nuclear transcriptional factor, expressed in early development as part of a transcriptional core network that maintains the pluripotent properties and self-renewal capacities of embryonal stem cells and *Podoplanin (clone D2-40)*, a membrane glycoprotein with mucin-like characteristics that is expressed in the apical membrane of the mesothelium.

Their nuclear, cytoplasmic or membranous expression was analyzed in both the endodermal and mesothelial layers and in the accompanying embryonal tissues when these were available. In some cases, due to the small amounts of tissue, serial sections failed to produce a sufficient number of viable slides for immunohistochemistry. Additionally, in some embryos, the initial haphazard paraffin wax inclusion and block orientation precluded a detailed study of every embryonal organ.

Author's roles

Francisco F Nogales designed the study and participated in the analysis, execution and manuscript drafting and critical discussion. Isabel Dulcey retrieved archive and clinical material, performed the immunohistochemical and bibliographical analysis and participated in the manuscript drafting and critical discussion.

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