A Morphological Analysis of the Transition Between the Embryonic Primitive Intestine and Yolk Sac in Bovine Embryos and Fetuses

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KEY WORDS yolk sac; primitive intestine; morphological analysis; embryos; fetuses

ABSTRACT The yolk sac (YS) is the main source of embryonic nutrition during the period when the placenta has not yet formed. It is also responsible for hematopoiesis because the blood cells develop from it as part of the primitive embryonic circulation. The objective of this study was to characterize the transitional area between the YS and primitive gut using the techniques of light microscopy, *transmission electron microscopy*, and immunohistochemistry to detect populations of pluripotent cells by labeling with Oct4 antibody. In all investigated embryos, serial sections were made to permit the identification of this small, restricted area. We identified the YS connection with the primitive intestine and found that it is composed of many blood islands, which correspond to the vessels covered by vitelline and mesenchymal cells. We identified large numbers of hemangioblasts inside the vessels. The mesenchymal layer was thin and composed of elongated cells, and the vitelline endodermal membrane was composed of large, mono- or binucleated cells. The epithelium of the primitive intestine comprised stratified columnar cells and undifferentiated mesenchymal cells. The transitional area between the YS and the primitive intestine was very thin and composed of cells with irregular shapes, which formed a delicate lumen containing hemangioblasts. In the mesenchyme of the transitional area, there were a considerable number of small vessels containing hemangioblasts. Using Oct4 as a primary antibody, we identified positive cells in the metanephros, primordial gonad, and hepatic parenchyma as well as in YS cells, suggesting that these regions contain populations of pluripotent cells. *Microsc. Res. Tech. 76:756–766, 2013.*

INTRODUCTION

The yolk sac (YS) is an extra-embryonic membrane that plays an important role in the early development of many mammalian species (Pereda et al., 2010; Wolf et al., 2003). It is the main source of nutrition for the embryo during the period when the placenta is not fully formed. Additionally, it is important in hematopoiesis because it is associated with the development of blood cells and is part of the circulatory system in the early embryo (Wolf et al., 2003; Santos et al., 2012). The YS circulation of sheep and cattle is responsible for the transfer of metabolites from the mother to the embryo before the development of the main placenta (Matsumoto et al., 2012, Rüsse et al., 1992). Thus, this structure is important for proper embryonic development because it is required for nutrition, protection, and the development of organs that are crucial for embryo survival. For example, the primitive intestine develops from the YS (Rüsse et al., 1992).

The bovine YS completes its development on approximately the 20th day of gestation and rapidly regresses (Godkin et al., 1988; Mossman, 1987; Noden and Lahunta, 1990; Rüsse et al., 1992). According to Wrobel and Suess (1998), the YS develops from 18 to 23 days of gestation. In ruminants, the YS regresses and degenerates over a short period of time (Mossman, 1987; Noden and Lahunta, 1990). It is reduced to an insignificant structure by day 30 of gestation concomitant with the development of the allantoic sac (Wooding and Flint, 1994). It cannot be detected through the rest of gestation (Latshaw, 1987; Noden and Lahunta, 1990; Wooding and Flint, 1994).

Assis Neto et al. (2012) concluded from their studies that the bovine YS decreases irreversibly in total length and that the YS disappears completely from days 50 to 70 post-insemination in most cases.

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TABLE 1. Group/age/greatest lengths (Crown-rump) of bovine embryos at 24–38 days of development that were used in histology, immunohistochemistry, and transmission electron microscopy

Group	Gestation period (day)	Cr-length (mm)	Histology	Immunohistochemistry	Transmission electron microscopy
I	24–26	3–5	3	2	1
II	27–29	6–10	6	1	1
III	30–32	11–15	5	1	2
IV	33–35	16–20	6	2	2
V	36–38	21–25	5	0	2

However, vestiges of the central part of this sac can be observed during this time. Assis Neto et al. (2009a,b, 2011) have described other features of membrane development in bovines.

Stem cells from the YS are primitive and exhibit a homogeneous morphology. YS stem cells temporarily migrate to the fetal liver and finally localize in the bone marrow, which is the permanent site of blood cell formation.

The protein Oct4 (octamer-binding transcription factor 4) is a transcription factor encoded by the Pou5f1 gene and is widely used as a marker of cellular pluripotency (Vejsted et al., 2006). Transcription factors are proteins that bind to DNA to activate or repress the expression of their target genes. The Oct4 expression is influenced by genes expressed during early embryonic development. Therefore, it may be an important factor in the processes of development and cellular differentiation (Pesce and Scholer, 2001).

This study aimed to characterize the morphology of the transitional area between the intestine and the primitive YS and to use Oct4 immunohistochemistry to identify areas with possible populations of pluripotent cells.

MATERIALS AND METHODS

Conceptus Collection

Uteri were obtained from pregnant cows of various breeds at a local slaughterhouse. The fetal crown-rump length (CR) was measured to estimate fetal age. Tissues were collected from pregnant cows (estimated fetal age ranging from 24 to 50 days; $37 \pm$ SD 7.8 days). The craniocaudal cervix of the pregnant horn was dissected, and a total of 39 concepti were collected (Table 1). The embryos or fetuses were disconnected from the extraembryonic membranes, and the crown-rump length, defined as the distance from the major point of the head to the end of the last sacral vertebra on the opposite end, was measured according to the methodology used by Winters et al. (1942), Melton et al. (1951), and Evans and Sack (1973). The embryos and fetuses were divided into groups (Groups I, II, III, IV, and V) by stage of pregnancy (days of pregnancy) according to the CR and morphological characteristics.

Light Microscopy

For histological analysis, 25 samples of embryo and fetal membranes were fixed either in PBS (phosphate buffered saline) containing 4% (w/v) paraformaldehyde or Bouin's solution for 24 h. After fixation, the material was dehydrated in increasing concentrations of ethanol (70–100%), cleared in xylene, and paraffin embedded using Histosec (Behmer, 1976; Tolosa et al., 2003). Serial 5 μm sections were stained using Picrosirius (Junqueira et al., 1979) and standard hematoxylin and eosin protocols. After staining, the slides were mounted with coverslips using Entelan as the mounting medium.

Scanning Electron Microscopy

For transmission electron microscopy, eight samples of embryo and fetal membranes were fixed in 2.5% glutaraldehyde diluted in PBS (pH 7.4–0.1 M) for 24 h. After fixation, the fragments (embryos and attachments) were washed in PBS and post-fixed in 1% osmium tetroxide (osmium tetroxide 4% w/w solution in water, Polysciences) for 1 h and washed in PBS. The fragments were then dehydrated in increasing concentrations of ethanol (70–100%) and in propylene oxide (EM Grade, Polysciences) as the final dehydration step. The samples were incubated for 12–16 h in a 1:1 mixture of propylene oxide and Spurr's resin (Spurr's kit-Electron Microscopy Sciences) followed by incubation in pure Spurr's resin for an additional 4–5 h under constant agitation. The blocks were cut with an ultramicrotome (Leica® model Ultracut UCT). Semithin sections of 1 μm were obtained and stained with a hot aqueous solution of 1% sodium borate in distilled water containing 0.25% toluidine blue.

Immunohistochemistry

For an immunohistochemical analysis of Oct4 expression, a total of six embryos were fixed in PBS containing 10% formaldehyde at 24 to 26 days ($N = 2$; CR = 3–5 mm), 27 to 29 days ($N = 1$; CR = 6–10 mm), 30 to 32 days ($N = 1$; CR = 11–15 mm), and 33 to 35 days ($N = 2$; CR = 16–20 mm) of pregnancy. Embryos were routinely processed by paraffin embedding (Behmer, 1976; Tolosa et al., 2003), and 5 μm tissue sections were placed on silane-treated slides. Immunohistochemistry to identify putative pluripotent cells was performed using a polyclonal, primary antibody, goat anti-human Oct4 (Santa Cruz Cat. No. NB 11085544) diluted at 1:500 in PBS containing 1.5% BSA (w/v). Briefly, sections were sequentially incubated with peroxidase-blocking buffer [histochemistry buffer with 3% H_2O_2 in methanol (v/v)] for 20 min and washed three times in PBS. The slides were then placed in 0.1 M citrate buffer at pH 6.0 and subjected to microwave irradiation for 15 min for antigen retrieval. Nonspecific binding sites were blocked using Dako Protein Block (Cat. No. X090930) for 20 min. The sections were then incubated with primary antibody overnight at 4°C in a humidified chamber. The sections were then washed three times for 5 min using PBS and incubated with a biotinylated secondary antibody for 45 min and streptavidin-HRP for 45 min. The binding was visualized using aminoethyl carbazole for 3–5 min (Cat. No. SK 4200) as the chromogen and counterstained with hematoxylin. The slides were washed

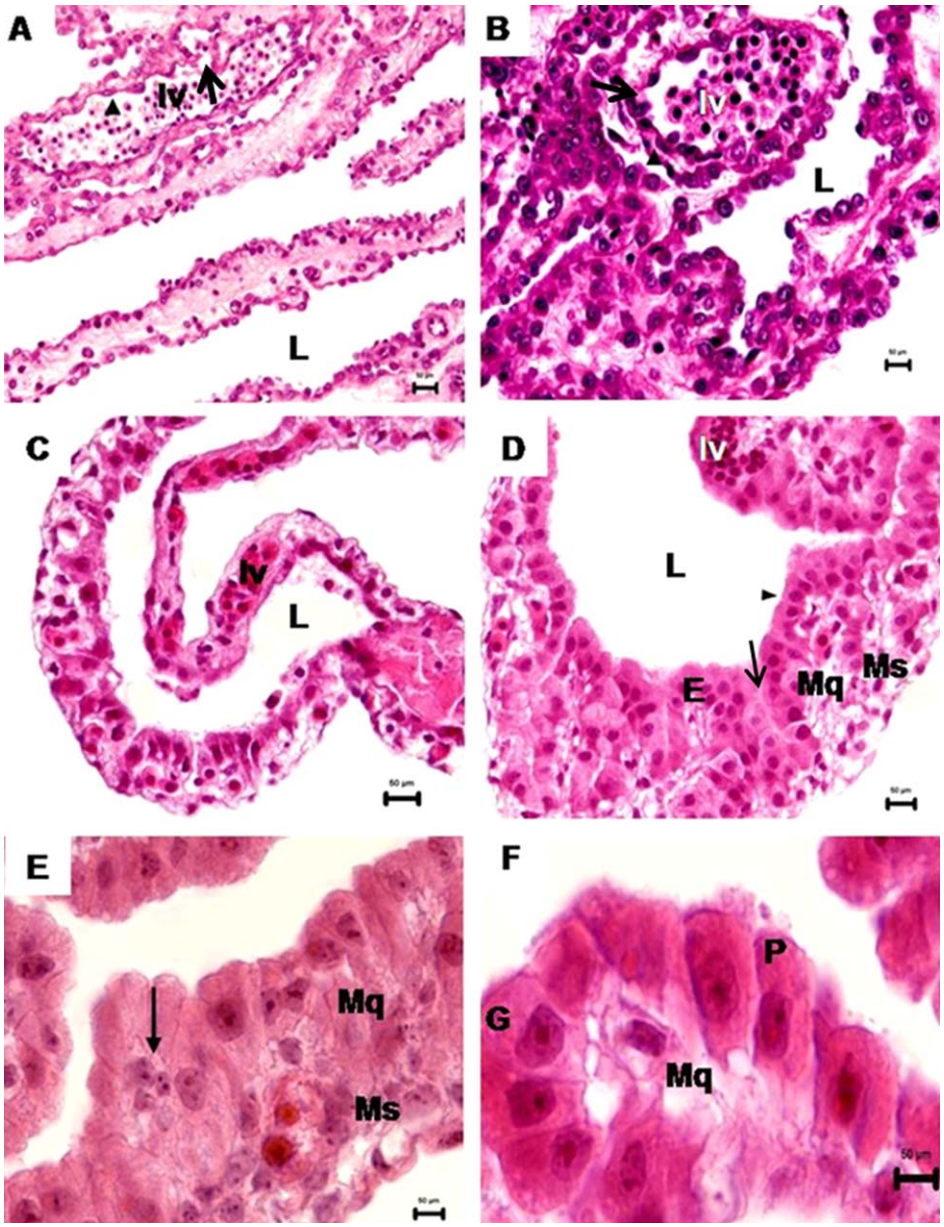

Fig. 1. Photomicrograph of bovine embryonic YS of groups: **A**: group I (CR 4 mm); **B**: group II (8 mm); **C**: group III (CR 12 mm); **D**: group IV (18 mm), and **E**: group V (CR 21 mm). A, B, and C, island vessels (iv) coated with endothelial cells (arrowhead) and the primitive blood cells, primary erythroblasts (arrows), and lumen (**L**). In 1D, note that the YS consists of three layers: endoderm (**E**), mesoderm (Ms), and mesenchyme (Mq). Yolk epithelium with tissue tucked into the lining of the YS forming small tubules (arrow). **E**: the endodermal cells may have two nucleoli (arrow). **F**: detail showing the cells of the endoderm as globoid (G) and prismatic (P) in shape. Staining: hematoxylin and eosin. Bar = 50 micrometers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

under tap water, the cover slips were mounted, and the slides were examined for staining by light microscopy.

The region corresponding to the anatomical connection of the YS and the primitive intestine was photographed with a digital camera (Nikon Eclipse E-400). The nomenclature used to describe the structures was established by the International Committee on Veterinary Gross Anatomical Nomenclature (2005), the International Committee on Veterinary Gross Histological Nomenclature(2005), and the International Committee on Veterinary Embryological Nomenclature (1994).

RESULTS

Microscopically, the YS was a trilaminar structure. A single layer of endodermal cells lined the YS cavity; a simple mesothelial layer faced the exocoelom, and there was a vascular mesenchymal middle layer. The endoderm was composed of endodermal cells, which were supported by the embryonic mesenchyme and mesothelium (Figs. 1A–1F). Primitive blood cells were

most widely distributed in the embryonic blood vessels.

In the embryonic samples from group I (24–26 days of gestation), hemangioblast cells were identified inside the vascular islands, which were located in the embryonic mesenchyme of the YS. The YS epithelium presented globular to columnar cells arranged in a single layer and supported by the embryonic mesenchyme, which formed small folds (Fig. 1A).

The YS of embryos from group II (27–29 days of gestation) and group III (30–32 days of gestation) showed similar characteristics. In both groups, vascular islands were preeminent and contained red blood cells (hemangioblasts). Moreover, these islands corresponded to blood vessels formed by YS endoderm and mesoderm (Figs. 1B–1D).

The YS cells were arranged in a single layer and displayed globular to columnar shapes. The vitelline epithelium in Group I showed fewer and more defined folds than that in the other two groups. The mesenchyme formed a thin layer of elongated cells. The endoderm was composed of large, mononucleated or binucleated cells, often showing two nucleoli. The mesenchymal region was full of blood cells (Fig. 1F).

The YS of embryos from Group IV (33–35 days of gestation) and Group V (36–38 days of gestation) comprised columnar and cubic cells that showed faint staining and were binucleate in some cases. The YS epithelium and connective tissue folded deeply into the YS, forming small tubules with a thick layer of epithelium (Fig. 1E).

The YS epithelium and connective tissue formed folds, which resulted in canalicular structures. These cells had round, often oval nuclei, with a prominent nucleolus (Figs. 1E and 1F).

At the ultrastructural level, we observed abundant euchromatin (euchromatic nuclei), indicating that these cells had a high transcriptional activity (Fig. 2A). The mitochondria were primarily situated between the nucleus and the luminal surface of the cells. Large amounts of rough endoplasmic reticulum were distributed sparingly, and small vesicles were observed throughout the cytoplasmic region (Figs. 2B–2D). Occasionally, intercellular spaces were observed between the endodermal cells and the large amount of rough endoplasmic reticulum and small vesicles in the cytoplasm (Fig. 2E). This observation may indicate fenestration of the endothelial lining. Primary erythroblasts were observed inside the vessels (Fig. 2F).

The initial differentiation of the primitive intestine comprised stratified columnar cells with a brush border (endoderm), followed by mesenchymal stem cells derived from the mesoderm (Figs. 3A, 3B, and 3D). The details of the lumen of the primitive intestine, with secretions in its interior and prismatic stratified epithelium, were observed (Fig. 3C). In embryonic samples, active cells were observed in the YS and in the area where it connects to the primitive intestine (proximal YS portion).

The primitive intestine was divided into three regions: the cranial intestine, caudal intestine, and midgut. The midgut (primitive) had a close relationship with the YS through the vitelline duct (or YS pedicle). The vitelline duct remained temporarily attached to the YS. In the ventral region of the embryo, the apex of the intestinal loop was inside the extra-embryonic cavity of the umbilical cord attached directly to the vitelline duct (Fig. 4A).

The transitional area between the primitive YS and the primitive intestine was narrow in relation to both structures and contained cells of irregular shape, such as flat or globose. We observed hemangioblasts and structures indicative of the passage of these cells to the mesenchymal layer of the lumen. In the mesenchyme of this transitional area, several small capillaries were observed within the hemangioblast (Fig. 4B).

Sections were made of the entire transitional area between the YS and the primitive intestine. We could divide all the sections into five groups: (a) the central region of the YS, (b) one end of the YS region, (c) the center of the YS duct (transitional area), (d) the beginning of the primitive intestine, and (e) the region of the intestinal loop (Figs. 5A and 5B). Around the central region of the YS, we observed that the arteries and veins and allantoic duct that form the umbilical cord were all enveloped by the embryonic mesenchyme (Figs. 4A, 5A, and 5B).

According to the plane of the section shown in Fig. 5, the endodermal epithelium in the region closest to the yolk duct showed three different shapes of epithelial cells: globular, cuboidal, and columnar with large and elongated nuclei supported by the mesenchyme layer (Fig. 5C). In this region, we observed the mesoductus lateral to the central portion of the YS surrounded by vessels containing cells with erythroblast characteristics (Fig. 5D). In the plane of section E–F, the lumen of the YS duct was almost nonexistent, and blood vessels were observed in the mesenchymal region (Fig. 5E). In the plane of section G–H, the structures characteristic of both the YS and primitive intestine were difficult to identify. A central cluster of undifferentiated cells was observed in this transition area. Furthermore, we observed blood vessels around the duct (Fig. 5F). In the plane of section I–J, we observed the development of one protrusion of the intestinal loop in the direction of the vitelline duct inside the umbilical cord and extra-embryonic coelom. This protrusion from the embryonic cavity (intraembryonic coelom) divides the primitive intestine into two parts: the cranial and caudal regions. In the center of the duct, we observed an arrangement of cells at an early stage of differentiation resembling the intestinal epithelium bordered by a vascularized mesenchyme (Fig. 5G).

The epithelium of the YS endoderm contained cuboidal cells, and it formed folds with the mesoderm and mesenchyme. The mesenchymal layer showed blood islands containing erythrocytes. Some cells stained more clearly than others using hematoxylin and eosin, and certain cells contained two nucleoli (Fig. 5C).

The embryo as a whole and its attachments were subjected to an immunohistochemical analysis. We observed several regions containing cells that were strongly immunopositive for Oct4. In the primitive kidney formed by both ridges of the meso- and metanephros, we observed Oct4-positive cells in the renal tubule and in its mesenchyme. The cells of the hepatic cords, the YS mesenchyme, and the gonadal region also showed a positive reaction to the Oct4 antibody, showing locations with possible populations of pluripotent cells (Figs. 6B, 6D, 6F, and 6H).

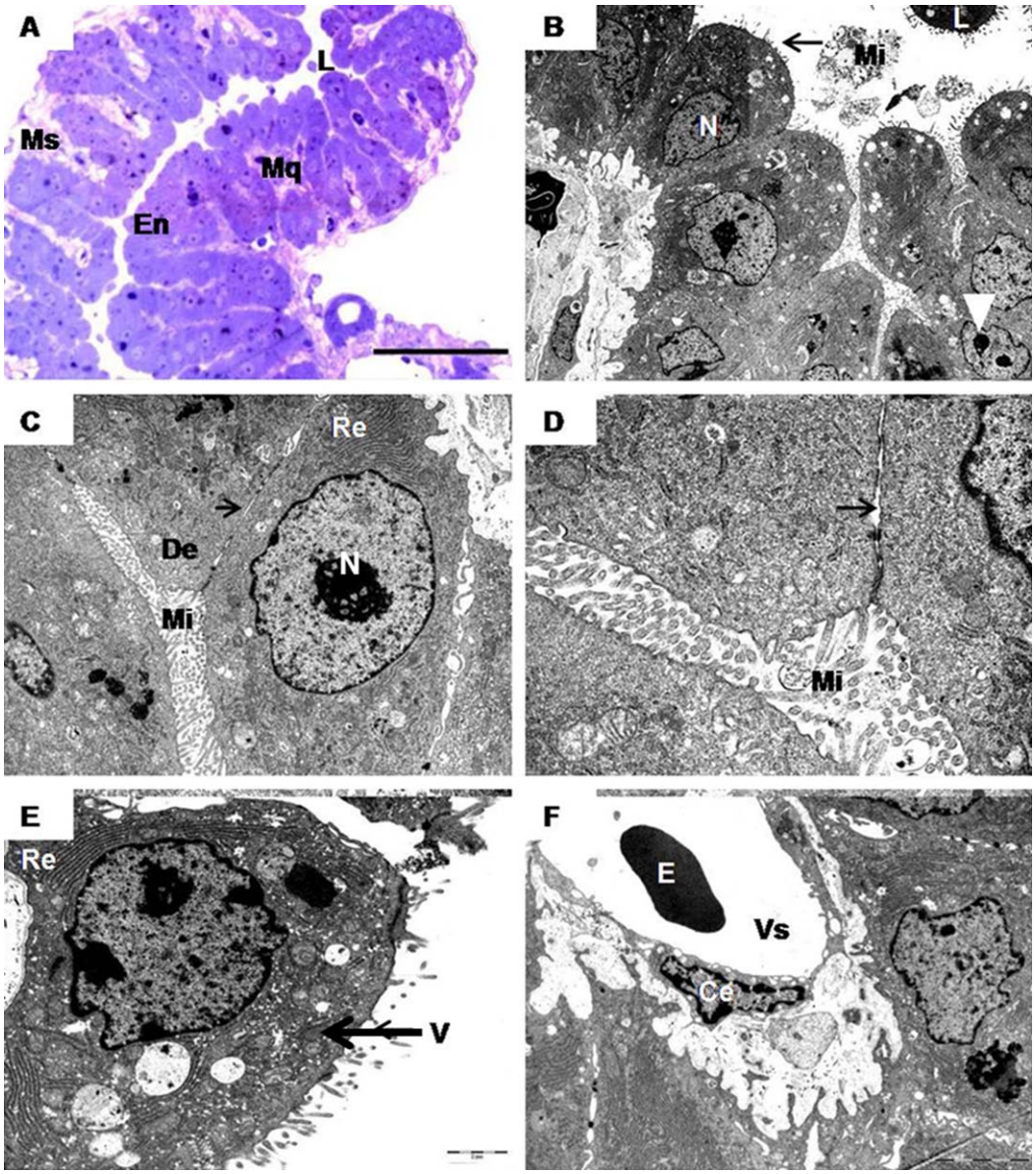

Fig. 2. Photomicrograph and electron photomicrograph of the YS of a bovine embryo with a CR of 23 mm at an estimated gestational age between 36 and 38 days. **A**: semifine sections of the bovine embryo YS, showing its trilaminar structure composed of layers of endoderm (En), mesoderm (Ms), and mesenchyme (Mq) in the intermediate zone. Lumen (L). Staining: Toluidine Blue, 40X. **B**: Endodermal cells containing nuclei (N) of irregular shape, euchromatic, mitochondria, presence of microvilli on the apical membrane (Mi), 3500x. **C**: the zona occludens (arrows) in the endodermal cells of the YS. Abundant rough endoplasmic reticulum is present in the cytoplasm (Re). Desmosomes (De) and a nucleus (N), 4400x. **D**: the zona occludens (arrow) and the microvilli in the lumen (E), 1100x. **E**: large amount of rough endoplasmic reticulum (Re) and small vesicles (V) in the cytoplasm, 5600x. **F**: erythroblasts (E) located inside the blood vessel (Vs) and endothelial cells (Ce), 5600x. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

The YS of the bovine embryo develops at ~18 to 23 days of gestation. It starts as a trilaminar disc formed by the ectoderm, mesoderm, and endoderm and develops into a cylindrical body (Wrobel and Suess, 1998). The initial age of the embryos studied herein was 24 days of gestation; for this reason, we could not observe the early development of the YS. According to Rüsse et al. (1992), this phase starts at 22 days of gestation.

The YS is located in the exocoelomic cavity in the ventral portion of the embryo near the umbilical cord and remains connected to the hindgut through its central portion (Assis-Neto et al., 2010). The YS develops as an annex of the embryonic midgut and consists of a layer of endodermal epithelium followed by a vascularized fetal mesenchyme (Leiser and Kaufmann, 1994). Rüsse et al. (1992) observed that during the initial phase of YS elongation, the endoderm differentiates into flat and prismatic-shaped cells in a region of the cytoplasmic membrane, aligned to the trophoblastic cells.

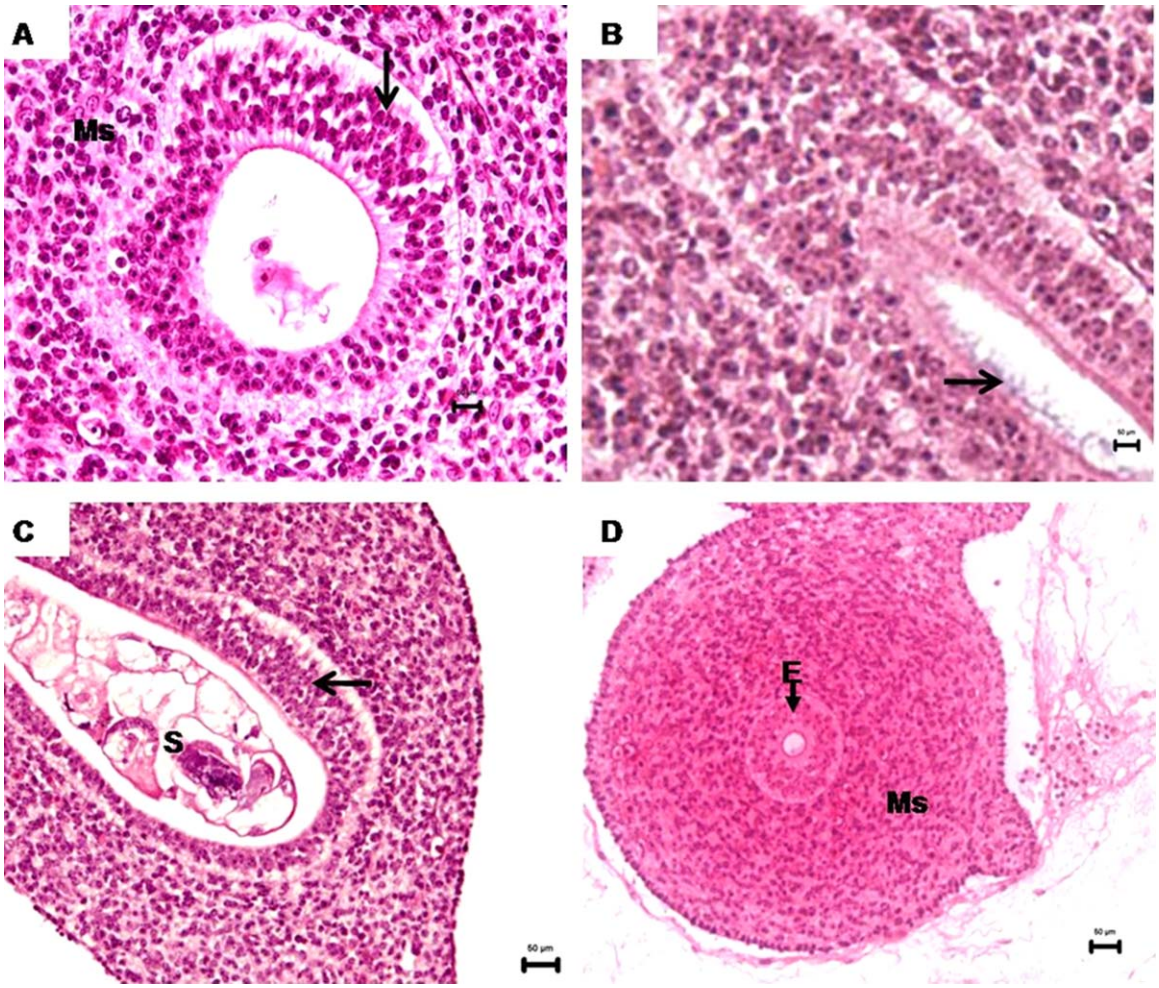

Fig. 3. Photomicrographs of the primitive intestine of bovine embryos in group II (CR 10 mm), estimated gestational age between 27 and 29 days. **A**: cross section of the epithelium showing early differentiation (arrow) and mesenchymal cells (Ms). **B**: longitudinal section of the primitive intestine, luminal area in the brush border (arrow). **C**: detail of the primitive intestine with secretion (S) in its interior and prismatic stratified epithelium (arrow). **D**: cross section of the intestine of an embryo with CR 21 mm, mesenchymal cells (Ms), and the developing epithelium (**E**). Staining: hematoxylin and eosin. Bar: 50 micrometers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In our studies, the YS epithelium consisted of a single layer of cells that were prismatic in shape and supported by the embryonic mesenchyme, forming small folds. These same characteristics were observed by Assis-Neto et al. (2010) in the bovine.

The YS regresses and degenerates very early in cattle (Mossman, 1987; Noden and Lahunta, 1990); in horses and carnivores, the YS functionally persists (Rüsse et al., 1992).

The macroscopic characteristics of YS involution in cattle have been given contradictory characterizations by several authors. In sheep, YS involution occurs during the third week according to Barone (1986) and during the 25th day of gestation according to Bryden et al. (1972). The YS is reduced to a solid group of cells, and the remnants of these cells are not found throughout the remainder of the pregnancy (Latshaw, 1987; Noden and Lahunta, 1990; Wooding and Flint, 1994). We observed the YS in all samples investigated (24 to 50 days of gestation). Assis et al. (2012) concluded that the bovine YS decreases irreversibly in total length and that the YS disappears completely from 50 to 70 days post-insemination in most cases. However, vestiges of the central part of this sac can be observed during this time. These results were also found in our studies of bovine embryos derived from natural mating.

Assis-Neto et al. (2010) previously showed that during the progression of the pregnancy, the total length of the YS decreases. The long extremities gradually become vestigial and later disappear. It was not possible to evaluate the disappearance of the YS; therefore, the main focus of our study was to describe the structure until the 50th day of gestation.

We also verified that the YS is composed of three layers: a layer covering the YS cavity, the endoderm, composed of endodermal cells supported by an embryonic mesenchyme; a simple mesothelial layer facing the exocoelomic cavity, the mesothelium; and a vascular mesenchymal intermediate layer, the mesenchyme. The endoderm of the vitelline membrane was

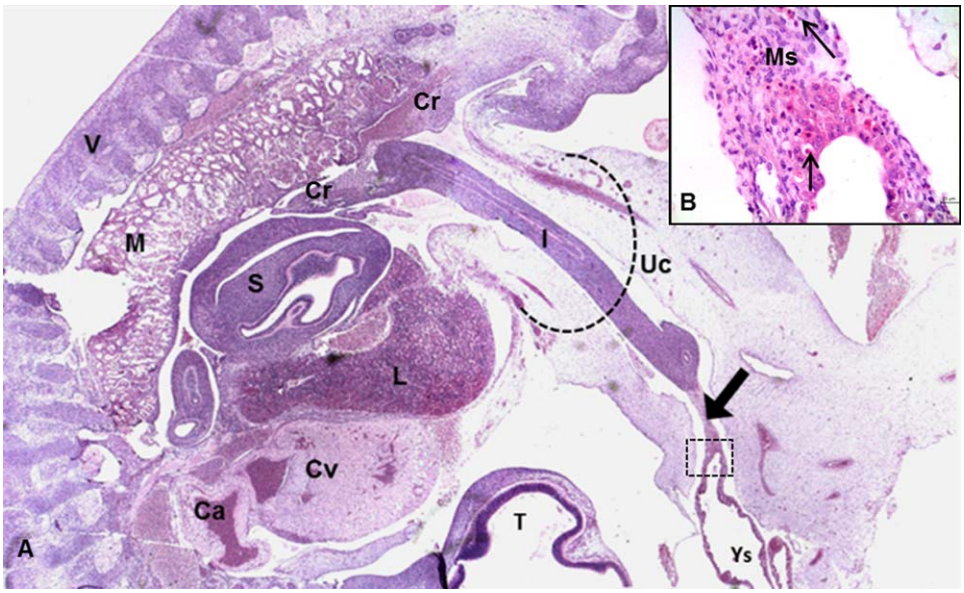

Fig. 4. Photomicrograph of a bovine embryo with a CR of 10 mm at a gestational age of ~27–29 days. **A**: vertebrae (V), mesonephros (M), stomach (S), liver (L), heart ventricle (Cv), heart atrium (Ca), telencephalon (T), umbilical cord marked by a circle (Uc), YS (Ys), primitive intestine: cranial intestine (Cr), caudal intestine (Ca), and midgut (I). Note the local connection of the YS and primitive intestine (arrow and square) 10x. **B**: mesenchymal layer (Ms) and hemangioblasts inside the capillaries (arrow). Staining: hematoxylin and eosin. 40x. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

composed of large endodermal cells, which were mononucleated or multinucleated. Primitive blood cells were widely distributed in the embryonic blood vessels. Pereda and Niimi (2008) found the same distribution in the human YS.

Zago and Covas (2006) showed that in humans, ~2–3 weeks after fertilization and implantation of the egg in the uterine endometrium, cell clusters called "blood islands" appear in the mesenchyme, lateral to the notochord. The outer cells of the blood islands differentiate into endothelial cells, whereas the internal cells differentiate into primitive blood (Choi, 2002). Our findings confirm this description. Additionally, these structures were abundant and differently shaped (CR from 4 to 10 mm). In embryos with crown-rump measurements larger than 10 mm, the blood islands were smaller than in earlier embryos. This difference may be due to a degenerative process of the YS in ruminants because the YS in ruminants is functional for only a short part of the gestational period (Noden and Lahunta, 1990). The beginning of the degenerative process of the endodermal wall of the YS is associated with the collapse of tubules present in sheep embryos with a CR of 36 mm and in bovine embryos with a CR of 42 mm. In sheep, embryos with a CR of 42 mm also showed degeneration of the YS walls (Rüsse et al., 1992).

The YS wall receives an abundant blood supply (Rüsse et al., 1992). In all embryonic samples, we observed blood vessels in the mesenchyme of the embryonic YS.

According to our results, the dorsal aorta supplies the YS, which is attached to the midgut, the allantois, and a hindgut diverticulum. The vitelline artery is followed by the paired allantoic or umbilical arteries (Noden and Lahunta, 1990). In cattle, the distal umbilical cord carries two arteries, two veins, and the allantoic duct (Miglino, 1991). In transverse sections of the YS with umbilical cord, we also found two arteries, two veins, and the allantoic duct around the central region; all vessels were surrounded by the embryonic mesenchyme.

Using transmission electron microscopy (TEM), we observed that cells of the YS showed decondensed chromatin (euchromatic nuclei), indicating that these cells have high transcriptional activity. According to Salesbury and Vandermark (1964), the embryo can survive for a short period due to the nutrients that it absorbs from its own YS and from uterine secretions. Liu et al. (1991) analyzed the proteins in the YSs of mice and found that these proteins were identical to those found in fetal bovine serum, such as transferrin, α1-fetoprotein, α1-antitrypsin, and α1-acid glycoprotein.

Using TEM, we observed microvilli on the surface of endodermal cells, as described previously by Tiedman (1979) in the YS of cats, by Assis-Neto et al. (2010) in cattle and by Pereda et al. (2010) in humans. In addition, we observed a large number of mitochondria located between the nucleus and the luminal end. A large amount of sparse rough endoplasmic reticulum was also identified. According to Rüsse et al. (1992), this structure is related to protein metabolism. The presence of rough endoplasmic reticulum and mitochondria in the vitelline epithelium is functionally associated with protein metabolism (Assis Neto et al., 2012).

The cytoplasmic region showed small vesicles throughout the cytoplasm and occasionally in the region between the endodermal epithelial cells of the YS. Intercellular spaces may be one possible area of fenestration in the endothelial lining. The endothelium of blood vessels in the cat YS is fenestrated and shows a complete basement membrane; this characteristic is

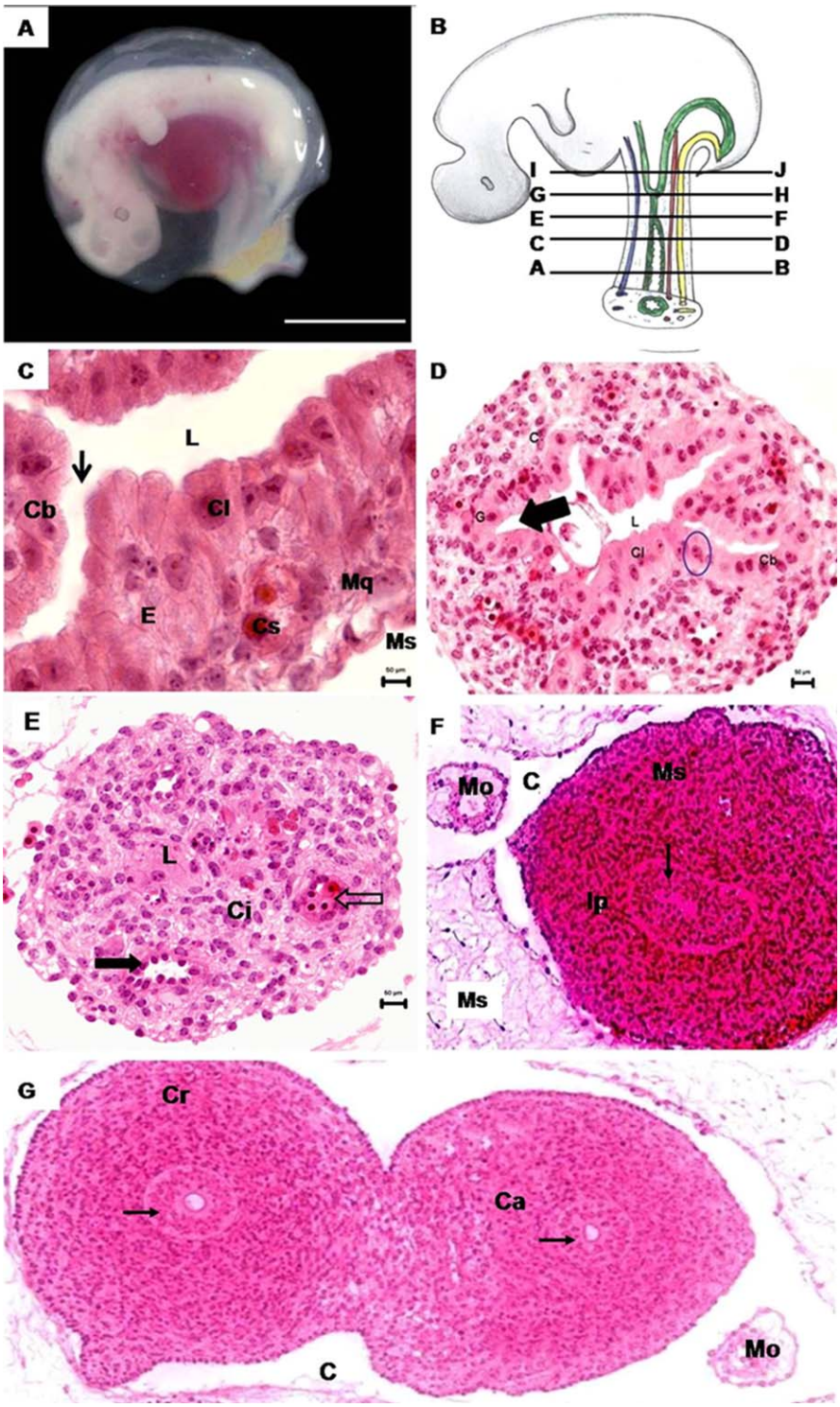

Fig. 5. Photography (**A**) and layout (**B**) of a bovine embryo in lateral view with a CR of 12 mm (group III) at an estimated gestational age between 30–32 days, representing the sequential transverse sections of the transition region between the YS and primitive intestine of A-J. B: the structures near the transition: the umbilical veins (blue), the umbilical arteries (red), allantois (yellow), YS, and primitive intestine (green). B and C, the A–B and C–D cutting planes; note the three different types of endodermal epithelial cells: globular (G), cuboidal (Cb), and columnar (Cl) with a large nucleus and the presence of two nucleoli (circle), and marked wrinkles (arrow) facing the lumen (L). **E**: the E–F cutting plane with closed vitelline duct (L), endothelial cells (arrow) in the center a cluster of undifferentiated cells (Ci), blood vessels with hemangioblast (empty arrow). **F**: the G–H cutting plane, showing the beginning of the primitive intestine (Ip) close to the vitelline duct. Note the arrangement of cells, initiating the differentiation of intestinal epithelium (arrow), mesoductus (Mo), extra-embryonic coelom (C), and mesenchyme (Ms). 5G, the I-J cutting plane; observe the primary intestinal loop beginning the differentiation of the intestinal epithelium (arrow) and the regions of primitive intestine, the cranial (Cr) and caudal intestine (Ca). Staining: hematoxylin and eosin. Bar: 50 micrometers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

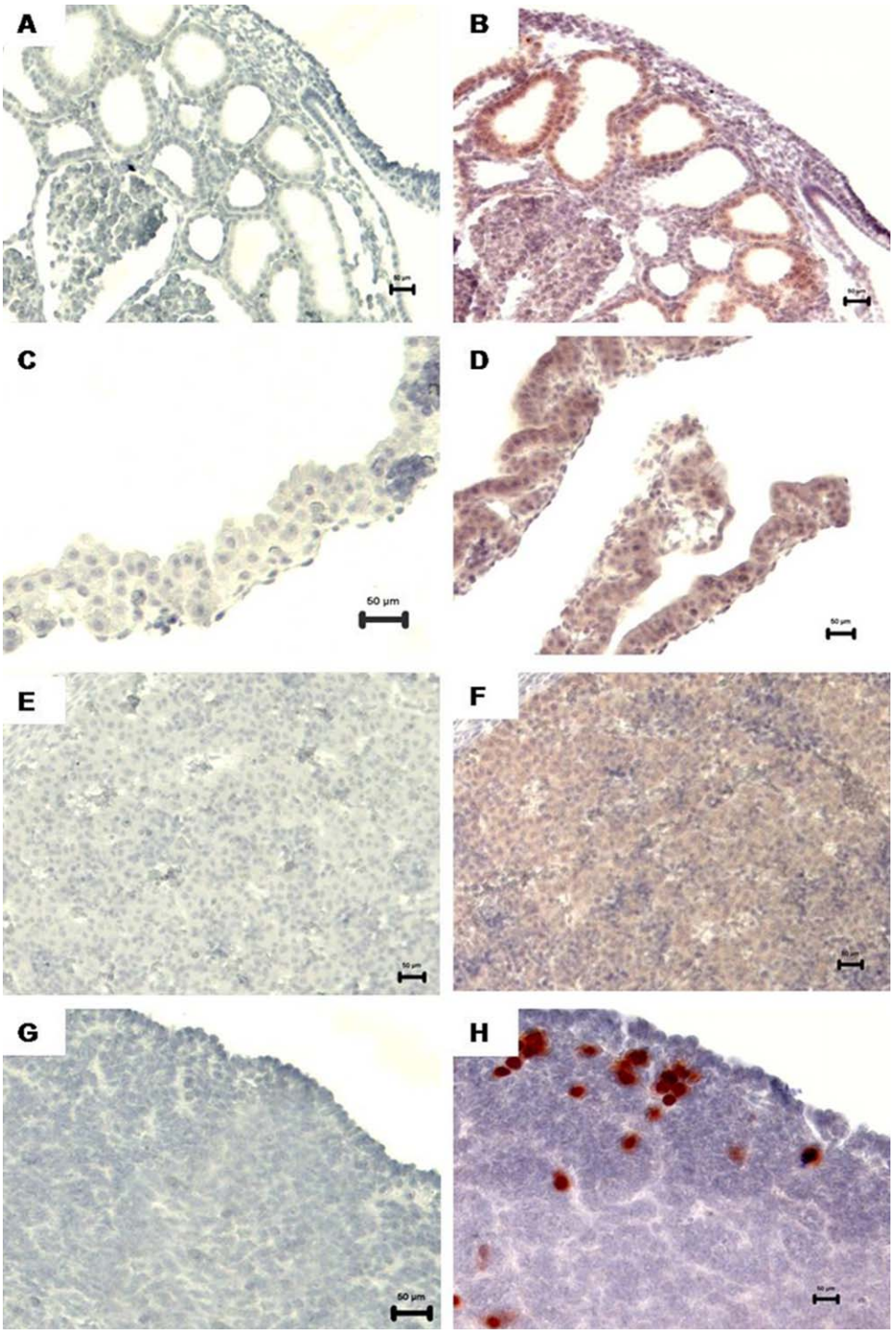

Fig. 6. Photomicrograph of bovine embryos in group IV (CR 16 mm) at an estimated gestational age of 33–35 days. Observe that **A**, **C**, **E**, and **G** are in the control group and **B** (primitive kidney), **D** (YS), **F** (liver), and **H** (gonads) identify the Oct4 positive cells. Immunohistochemistry. Bar: 50 micrometers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

favorable for the passage of proteins (Tiedemann, 1979) and blood cells (Larsen and Knothe, 1971).

According to Santa Barbara et al. (2003), the histogenic process in the intestinal epithelium may be divided into three phases: proliferation and epithelial morphogenesis, cell differentiation, and a final phase that is related to the biochemical and functional maturation of different types of epithelial cells. In our study, we observed the first and second stages of the gestational period in detail.

The primitive intestine can be divided into three regions: the cranial intestine, caudal intestine, and midgut. The midgut (primitive) is in close proximity to the YS via the vitelline duct (YS pedicle). The vitelline duct remains temporarily attached to the YS, as confirmed by Santa Barbara et al. (2003).

Oct4 is essential for identifying the pluripotential founder cell population in the mammalian embryo (Nichols et al., 2008). The transcription factor Oct4 POU (Pict-Oct-Unc) is expressed in embryonic stem cells and the germ cells of rats (Pesce and Scholes, 2001), mice and pigs (Vejsted et al., 2006), dogs (Martins et al, 2011), paca (Franciolli, 2011), and cattle (Kurosaka et al., 2004). In this study, we described and confirmed the expression of this transcription factor in bovine embryos from 24 to 50 days of gestation, suggesting possible populations of pluripotent cells.

Positive reactions to Oct4 were observed in the gonadal region and paramesonephric region, as described previously by Martins et al. (2011) in dogs. We also identified positive cells in the liver parenchyma and in the YS membrane.

Only a small number of cells express Oct4 before the inactivation of gametes (Pesce and Scholes, 2000). Our findings are in agreement with this result, as we identified a small number of positive cells during the immunohistochemical analysis.

During this study, we evaluated the morphological development of the YS and its connection to the primitive intestine. In embryos with a gestational age of 25 days, the YS exhibited a yellow color in situ and was relatively large, indicating a high functionality in embryo nutrition because proteins produced by this structure are important during organogenesis. However, a transitional point that connects the YS to the primitive intestine is necessary for these proteins to be transferred from the YS to the embryo.

The mesenchymal layer is one of the most important populations because its inductive function facilitates the transmission of metabolic information to the YS for the primitive intestine (Matsumoto et al., 2012). At approximately 35 days of gestation, the transition between the YS and the primitive intestine showed a small lumen, signifying the end of vitelline dependence and early communication with the placenta. A failure during this initial process would affect all of embryonic development and the maintenance of the maternal environment. It is possible that this mechanism represents one of the major causes of implantation failure and pregnancy loss.

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