Cellular events during early formation of yolk-sac-derived teratomas

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SUMMARY
A sequential morphological study of the initial cellular events in teratoma induction by displaced visceral yolk sac after foetectomy in rats was undertaken. This study led to the observation that apart from proliferation of cells displaying definite endodermal or mesodermal characteristics, a population of poorly differentiated cells appeared some days after the surgical procedure. It is very likely that these poorly differentiated cells are stem cells from which differentiated structures originate afterwards by a process of redifferentiation. The development of granulation tissue rich in capillaries seems to enhance this process. Similarities and differences with blastema formation are discussed.

INTRODUCTION
We have previously described the induction of teratomas by displaced visceral yolk sac after foetectomy in rats, mice and hamsters (Sobis & Vandeputte, 1974, 1977, 1979). These benign tumors were shown to contain a great variety of well-differentiated tissues when foetectomy was performed not later than day 12 of pregnancy in the rat, day 11 in the mouse and day 9 in the hamster. Since these teratomas are not of germ cell origin (Sobis & Vandeputte, 1976), we concluded that at this stage of development the endoderm and mesoderm of the foetal membrane are less committed than those of the embryo proper. Hence the possibility of a process of 'transdifferentiation – a switch of previously differentiated cells into other types of cells' (Eguchi, 1976) has to be considered. In order to reach such a conclusion, one has, however, to verify the morphological changes that appear at the cellular level in the earliest stages of teratoma formation as well as their relationship with the environmental tissues. Since in our previous study we investigated the development of different types of endodermal, mesodermal and ectodermal structures at 2–3 days interval, the earliest stages were not investigated in much detail. Moreover, in the initial study the uterine wall was sutured after foetectomy. This procedure rendered the making of serial sections rather difficult in the early stages and also induced

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a non-specific foreign body reaction against the threads which hampered the morphological interpretation.

In the present study suturing was omitted since we have found it not necessary. Moreover, the earliest stages in teratoma development were studied at much shorter intervals (6 h). We also verified the relationship between the differentiation stages of the endoderm and the expression of an antigenic marker specific for extraembryonal endoderm (Van Hove et al. 1978). We could show that after an initial stage of partial degeneration and necrosis in the displaced yolk sac, poorly differentiated cells appear in this extraembryonic membrane. These poorly differentiated cells then give rise to various tissues of mesodermal, endodermal and ectodermal origin. This differentiation process seems to be influenced by the development of granulation tissue.

MATERIALS AND METHODS

Sixty-eight rats of the inbred strain R (Wistar albino) were used. Twelve days after mating (the day when copulation plug was found, was counted as day 0) the foetuses were removed together with the amnion. After foetectomy the visceral yolk sac was pulled through the incision and left outside the uterine wall (Sobis & Vandeputte, 1974). The uterus was not sutured.

The animals were killed at 6 h intervals from the second to the ninth day after foetectomy. For histological examination the whole uterus was fixed in formol. For electron microscopical study one uterine horn was immersed in glutaraldehyde and postfixed in osmium, the contralateral horn was fixed in ethanol: glacial acetic acid (99:1, v/v) at 4 °C (Sainte-Marie, 1962) for immunohistochemical studies.

One-hundred and ninety paraffin blocks of uterine horns with an adhering nodule were cut serially. The sections were stained with erythrosin–haematoxylin or PAS.

Serial 1 μm-thick sections prepared from 38 nodules embedded in Araldite were stained with methyl blue and safranin. Thin sections cut on an OMU-Z Reichert ultramicrotome were stained with lead hydroxide and examined with a Zeiss EM 10 electron microscope.

For the immunohistochemical study the peroxidase–antiperoxidase method was used as previously described (Van Hove et al. 1978). Serial paraffin sections from 28 blocks fixed in alcohol–acetic acid were incubated first with anti-endodermal (Van Hove et al. 1978) or normal rabbit serum, then with goat anti-

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Fig. 1–3. The visceral yolk sac left outside the uterus for 2 days after foetectomy.
Fig. 1. Proliferation of endoderm with mitosis (arrow). H. and E. × 1600.
Fig. 2. Presence of ‘empty’ cells in the endoderm. H. and E. × 660.
Fig. 3. Lipid degeneration of endodermal cells.
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### Table 1. Appearance of different structures in displaced visceral yolk sac

<table>
<thead>
<tr>
<th>Tissue observed</th>
<th>Days after foetectomy</th>
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<td></td>
<td>2</td>
</tr>
<tr>
<td>No. of examined nodules</td>
<td>31</td>
</tr>
<tr>
<td>Proliferation of mesoderm</td>
<td>8</td>
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<tr>
<td>Proliferation of endoderm</td>
<td>10</td>
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<tr>
<td>Poorly differentiated cells</td>
<td>10</td>
</tr>
<tr>
<td>Cartilage</td>
<td>4</td>
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<tr>
<td>Muscle</td>
<td>1</td>
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<tr>
<td>Endodermal structures</td>
<td>1</td>
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<tr>
<td>Epithelial nests</td>
<td>1</td>
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<tr>
<td>Neural structures</td>
<td>2</td>
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<td>Gut</td>
<td></td>
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<td>Pancreas</td>
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<tr>
<td>Proliferation of parietal yolk sac</td>
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rabbit IgG and finally with a peroxidase-antiperoxidase complex prepared in rabbit (Nordic, Tilburg, Netherlands). The sections were counterstained with methylene green.

### RESULTS

**Macroscopy**

No nodules were observed with the naked eye in animals killed between the second and the fourth day after foetectomy. Using a stereomicroscope, however, in nearly all places where the visceral yolk sac was left outside the uterus, some attached membrane was found. In the rats killed later, small nodules from 1 to 5 mm in diameter were easily seen. In early stages haemorrhages sometimes accompanied the nodules. In the uterus, placentas were present in nearly all places where the foetus had been removed. From day 7 on after foetectomy the placentas became smaller and at day 9 they were no longer observed.

**Histology and ultrastructure**

In order to avoid lengthy morphological descriptions, we characterize in detail the structures seen in the early stage of the experiments. For the later stages, only new findings are reported. Summarized results are shown in Table 1.
Two days after foetectomy in almost all places operated on, the visceral yolk sac was found outside the uterus in continuity with the membrane which remained attached to the placenta. The exteriorized part of the yolk sac was partially necrotizing and frequently infiltrated by poly- and mono-nuclear cells. In some cases pieces of parietal yolk sac with trophoblastic giant cells were also present. The latter cells were always accompanied by haemorrhages. In most sections proliferation of endoderm with mitoses was observed (Fig. 1). Apart from these proliferative endodermal cells, cells with small, frequently asymmetrically placed nucleus and empty-looking cytoplasm were found (Fig. 2). The mesenchyme also seemed to proliferate but no mitoses were seen. The basement membranes separating the epithelial from the mesenchymal cells, and the mesenchymal cells from the mesothelium were clearly visible. Nucleated and adult erythrocytes were present in the blood vessels. The morphology of the placenta and the intrauterine part of the yolk sac was similar to the one observed in normal pregnancy at day 14. The foetal blood vessels of the placenta contained, however, less blood cells.

On the ultrastructural level the majority of endodermal cells presented the usual picture of 12- to 14-day-old visceral yolk sac in the rat. The cells, interconnected with desmosomes, possessed regular, long microvilli, nuclei situated basally, well-developed rough endoplasmic reticulum, Golgi apparatus and mitochondria. Some cells, however, seemed to be less differentiated and contained abundant free ribosomes. The cells which looked empty in light microscope, were found to be degenerating cells filled with lipid droplets (Fig. 3). The mesenchymal cells possessed free ribosomes and a well-developed rough endoplasmic reticulum. In the vessels, typical nucleated erythroblasts and erythrocytes were observed. The serosal basement membrane containing collagen fibres, separated the vessels from mesothelial cells. The latter cells were characterized by elaborated ergastoplasm (R.E.R.) and numerous free ribosomes or polyribosomes.

Two days and 6 h after foetectomy the histological picture of the visceral yolk sac outside the uterus was nearly the same as that found 6 h earlier. There were, however, more proliferating cells in all layers: endoderm, mesenchyme, mesothelium and also endothelial cells. This proliferation seemed to be more pronounced in the parts of yolk sac adhering to necrotized membrane. Ultrastructurally, lipid degeneration in endodermal and mesodermal cells as well as phagocytosis of erythrocytes and necrotized cells by endoderm was observed.

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Fig. 7. The yolk sac 4 days after operation. Poorly differentiated cell between necrotized cells.

Fig. 8. The yolk sac 4 days and 18 hr after foetectomy. Proliferation of poorly differentiated cells. H. and E. x 660.

Fig. 9. High magnification of the same case. Mitotic figure is seen (arrow). H. and E. x 1600.
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In the animals killed 2 days and 12 h after operation, the infiltration of necrotizing visceral yolk sac by mononuclear and polynuclear cells was more pronounced. The proliferating endodermal cells seemed to be less differentiated. This was confirmed on ultrastructural levels; the cytoplasm of the cells was rich in ribosomes but nearly devoid of other cellular organelles. The cells, however, were still interconnected with desmosomes (Fig. 4).

In the yolk sac left outside the uterus for 2 days and 18 h the proliferation of the endodermal and mesodermal cells increased. In the cases where the membrane was not in continuity with the placenta this proliferation was not observed.

At three days after foetectomy we started to observe the appearance of poorly differentiated cells. These cells were seen between the proliferating mesodermal and endodermal cells. They did not, however, possess the cytological characteristics necessary to establish their origin as being endodermal or mesodermal (Fig. 5). Their number markedly increased in the next 24 h as followed at 6 h interval. During this interval (from 3 to 4 days after foetectomy) we also observed a gradual thickening of the basement membranes, the infiltration of the visceral yolk sac by lymphocytes and finally the appearance of granulation tissue (Fig. 6). This granulation tissue rich in capillaries was located outside the uterine wall and adhered to it.

Four days after foetectomy, areas of necrosis next to areas of proliferating endodermal and mesodermal cells were still present. Poorly differentiated cells were found in increasing numbers in the basal layer of endoderm and in the mesoderm near the necrotized tissue (Figs. 7, 8, 9). At the same time the first signs of differentiation appeared. These consisted mainly in the development of foci of cartilage adhering to granulation tissue. In one case we also found a structure very similar to primitive neural tube and comprising elongated cells surrounding a central lumen. In nodules examined 12 h later we observed similar structures. At that time, however, mitotic figures were clearly seen at the inner border of the tube, a characteristic feature for neural tube. In the nodules examined at 4 days and 18 h, we found, apart from the co-existence of necrotizing and proliferative areas in the yolk sac, the presence of numerous poorly differentiated cells and the presence of the previously described differentiated structures (cartilage and neural tissue), the development of young

Fig. 10. Five days and 6 h after foetectomy. Presence of young neural tissue. H. and E. × 660.

Fig. 11. Six days after foetectomy. New endodermal cells with poorly developed microvilli and some elaborated cytoplasmic organelles.

Fig. 12. Six days and 12 h after operation. Parietal yolk sac proliferation with secretion of hyalin outside the uterus. H. and E. × 660.

Fig. 13. Seven days after foetectomy. A part of necrotized yolk sac in the uterus (arrow) and granulation tissue outside, with cartilage, epithelial nests and pancreatic tissue. PAS. × 65.
Fig. 14. Eight days and 18 h after operation. The neural tissue. H. and E. ×1600.
Fig. 15. Nine days after foetectomy. Neural cells along external uterine wall. H and E. ×180.
Fig. 16. The yolk sac outside the uterus 3 days after operation. The presence of endodermal antigen(s) at the apical pole of non-proliferating endodermal cells. PAP reaction. ×660.
Fig. 17. Five days after operation. The endodermal cells of new structure presence of antigen on entire cell surface. PAP reaction. ×660.
Fig. 18. Five days after foetectomy. Poorly differentiated cells do not express endodermal antigen(s). PAP reaction. ×1600.
Fig. 19. Six days after operation. Presence of endodermal antigen(s) on cells of primitive endodermal structure. PAP reaction. ×660.
endodermal structures. Moreover, in two nodules small foci of parietal yolk-sac cells embedded in hyalin (PAS-positive, diastaze-resistant) were observed.

In all stages described until now, the visceral yolk sac left inside the uterus displayed areas which were structurally intact next to areas with definite signs of necrosis. Infiltration by polymuclear and mononuclear cells and development of granulation tissue were, however, not recorded inside the uterus. The structure of the placentas was similar to that in normal pregnancy, but starting 4 days after foetectomy the Duval sinuses became more pronounced mainly by proliferation of the mesenchymal tissue.

In the rats killed at 6 h intervals on the fifth day after foetectomy, some parts of the yolk sac were still well preserved containing proliferative endodermal and mesodermal cells next to poorly differentiated cells and some degenerative endodermal cells filled with lipid droplets. Degenerated and necrotized parts of the yolk sac were embedded in granulation tissue. Inside the latter tissue further development of differentiated structures (cartilage, small glandular structures and young neural tissue, Fig. 10) took place. The glandular structures were progressively surrounded by a basement membrane. Muscle and nests of epithelial cells were observed for the first time. One day later, cysts lined by either endodermal or by squamous keratinizing epithelium appeared in the granulation tissue. At the ultrastructural level the cells lining the endodermal cysts were less differentiated than the endoderm of the visceral yolk sac: the microvilli were not so regular, desmosomes were not always present and the cytoplasmic organelles not well elaborated. The cysts were surrounded by a thin basement membrane (Fig. 11). Proliferation of parietal yolk-sac cells embedded in hyalin (Fig. 12) was found in seven cases. At this stage the placentas became progressively smaller, partially degenerated and invaded by connective tissue. In the cases in which it was still possible to observe continuity between the visceral yolk sac inside and outside the uterus a marked difference between these two parts was recorded. Whereas the intraterine part was completely necrotized, the exteriorized part, embedded in granulation tissue, proliferated and differentiated (Fig. 13).

In the nodules examined 7 days after foetectomy elaborated structures like glands and gut with the mucosa surrounded by a muscular layer were observed. As mentioned previously, all these differentiated tissues were found in association with granulation tissue surrounding a residue of necrotized yolk sac.

The sections from nodules taken at 8 and 9 days after operation showed the presence of all previously described structures at a further stage of differentiation; some cysts were lined by ciliar epithelium, other cysts possessed well-structured layers of squamous epithelium as in the skin. This higher degree of differentiation was confirmed at the ultrastructural level. At that stage the young neural tissue comprising elongated cells with fusiform nuclei (Fig. 14) no longer displayed the typical structure of a neural tube. Indeed, it consisted of closely packed cells grouped in a wavy pattern and suggesting cell migration
The granulation tissue was progressively replaced by denser connective tissue.

**Immunological studies**

As previously described the endodermal cells of the 12-day-old intrauterine visceral and parietal yolk sac strongly reacted with antiserum made against yolk-sac carcinoma (Vandeputte et al. 1979). At this stage the endodermal antigen(s) is evenly spread over the cell surface. In the rats killed 2 days after foetectomy the endodermal antigen(s) was concentrated in the intrauterine as well as in the exteriorized part of the visceral yolk sac at the apical pole of the endodermal cells. This staining pattern was observed in the non-proliferating areas of the yolk sac (Fig. 16). In the areas, however, where the endodermal cells were proliferating and still kept the morphological characteristics of endoderm the antigen(s) was shown to be spread over the whole cell surface as in the 12-day-old yolk sac (Fig. 17). Cells displaying this staining pattern were numerous in the nodules developing 2–5 days after operation. The poorly differentiated cells which showed no definite endodermal or mesodermal characteristics did not express endodermal antigen(s) (Fig. 18). As described above, these cells were first seen 3 days after foetectomy and rapidly increased in number in the following days.

At later stages (5 days or more) the endodermal antigen(s) was also detected at the cell surface of groups of endodermal cells forming primitive endodermal structures (Fig. 19). Once these endodermal structures developed into more differentiated derivatives like gut or endodermal cysts the antigen(s) was no longer detected in the cells. At all stages the mesenchymal cells and tissues, the Reichert membrane and the placental tissue did not react within the anti-endodermal antiserum.

**DISCUSSION**

The present studies show that the endoderm and mesoderm of the visceral yolk sac, left outside the uterus after foetectomy, already start to proliferate 2 days after operation. This phenomenon is observed in an increasing number of cases during the following 3 days and diminished afterwards. Three days after foetectomy poorly differentiated cells appear. These cells show neither mesodermal nor endodermal characteristics. They do not express endodermal antigen(s). The poorly differentiated cells were present in the displaced yolk sac during the entire period of the experiment but were most numerous at day 4 and 5. Their appearance was accompanied by partial degeneration of the membrane left extrauterine and its infiltration by mononuclear and polynuclear cells.

Four days after foetectomy newly formed young tissues were found: cartilage, nests of endodermal cells which express endodermal antigen(s) and primary neural structures. On the following days an increased number of nodules containing these tissues were observed. Preceding the earliest stages of differ-
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entiation we observed the invasion and proliferation of capillaries into the necrotizing yolk sac and the development of granulation tissue (3 days and 12 h after foetectomy). Later on (day 7) the first organoid structures like pancreas and gut were also found to develop in the granulation tissue which in the meantime had become more fibroblastic. At this stage of organoid differentiation the endodermal cells no longer display endodermal antigen(s). This also applies to the later stages in teratoma development as well as to the normal gut of the 18-day-old rat foetus (Vandeputte et al. 1979).

The following results described here add new information to those published earlier (Sobis & Vandeputte, 1975): (1) the appearance of poorly differentiated cells possessing no definite mesodermal or endodermal characteristics; (2) the presence of these dedifferentiated cells is accompanied by degeneration of visceral yolk sac; (3) differentiation of these poorly differentiated cells only occurs in areas where granulation tissue develops; (4) young neural tissue can be detected during the first days of teratoma formation. The last finding clearly indicates that the visceral yolk sac can give rise to ectodermal tissue, although this membrane comprises only endoderm and mesoderm. Cells considered as multipotential and from which derivatives of all three germ layers can originate are germ cells or early embryonal cells (Stevens, 1967; Minz, Cronmiller & Custer, 1978). Germ-cell origin of yolk-sac-derived teratomas is not likely since the part of the membrane pulled through the uterine wall is not the one near the allantois in which the primary germ cells are found at day 9 (Chiquoine, 1954). Moreover, Ożdżeński (1969) has more recently shown that the primordial germ cells do not originate in the yolk sac, but instead in the root of the allantois and in the hind region of the embryo. Furthermore, these cells, the structure of which is very characteristic (Spiegelman & Bennett, 1973; Zamboni & Merchant 1973; Eddy, 1974), were never found in serial 1 μm sections made of 12-day-old yolk sac (Sobis & Vandeputte, 1975). We also showed previously that treatment with busulphan which is known to destroy the germ cells during their migratory phase did not influence the number or the morphology of the yolk-sac-derived teratomas in rats (Sobis & Vandeputte, 1976) and mice (Sobis & Vandeputte, 1979). Moreover, the results of experiments performed on Steel mice indicate that the lack of germ cells does not inhibit the development of yolk-sac-derived teratomas (Sabid & Vandeputte, 1982). Therefore one has to postulate the appearance of multipotential cells other than germ cells in the displaced visceral yolk sac and which give rise to the various structures found in these benign teratomas. Whether these multipotential cells were initially present in the yolk sac or arose through a process of dedifferentiation is still a matter of debate. Since we never observed these dedifferentiated cells on serial sections made through many yolk sacs of the rat examined at different stages of their development during normal pregnancy, we rather favour the hypothesis that they arise by a process of dedifferentiation. Such a process of dedifferentiation followed by redifferentiation is well documented in the case of regenerative blastema.
Indeed, the regeneration of an amputated amphibian limb is preceded by dedifferentiation to form so-called blastema. This then undergoes redifferentiation to form the regenerate in a fashion at least superficially analogous to normal limb development in ontogeny (Tassava & Mescher, 1975). It seems worth mentioning that the most highly evolved animals in which limb regeneration is seen are some species of amphibians. In phylogenesis these species may be compared to the metamorphosis embryo (Witschi, 1956) – stage 26 of Witschi – this is the 12th day of embryogenesis in rats. Moreover the regeneration of amputated limb buds in 12-day-old rat embryo was described (Deuchar, 1976).

The formation of blastema can be induced by numerous factors such as mechanical trauma, ultraviolet and X-irradiation (Maden, 1979), implant of normal and neoplastic tissues. All these factors, however, seem to provoke non-specific lesions of the tissues and it is likely that the post-traumatic reaction is responsible for the dedifferentiative changes (Carlson, 1974). Such post-traumatic reaction (degeneration, necrosis and infiltration by mononuclear and polynuclear cells) was also observed in the displaced visceral yolk sac in our experiment. It might be possible that this 12-day-old membrane being provided with mechanical trauma, can also undergo dedifferentiation. The dedifferentiated cells in blastema are characterized by morphological features similar to those found in undifferentiated embryonal cells (Burgess, 1974; Wallace & Maden, 1976). Cells showing such morphology are also observed in displaced visceral yolk sac at 3 days and later after operation. Their appearance is preceded by cell degeneration, a phenomenon described in blastema formation (Atkinson, Atkinson & Merrifield, 1976) and in almost all tissue of normally developing embryos (Poelmann & Vermeij-Keers, 1976). In the visceral yolk sac, as well as in the blastema, complex differentiation can be observed and in both these various differentiated tissues stem from somatic diploid cells. A similar complex differentiation can be observed in teratocarcinoma derived from 6 to 7-day-old embryo implanted into extraterine sites (Dunn & Stevens, 1970; Damjanov, Solter, Belicza & Skreb, 1971). However, the yolk sac as well as the embryo, when placed ectopically, does not differentiate in its normal way, but gives rise to benign or malignant tumours, while the blastema transplanted into amphibian with regenerative ability forms a normal limb. Whereas in the case of blastema the host is apparently able to control the redifferentiation, the adult rodent does not seem to provide such a controlling environment. In some cases the displaced yolk sac can even form a malignant tumour (yolk-sac carcinoma) (Vandeputte & Sobis, 1978; Sakashita et al. 1977). Which factors influence either the differentiation of the displaced yolk sac into various benign structures or the development of malignant tumour, is not clear yet.

Our sequential morphological study seems, however, to indicate that the development of granulation tissue favours the differentiation process as indicated by the following findings:
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(1) The first stages of differentiation in the displaced yolk sac were only observed in parts of the membrane surrounded by granulation tissue.

(2) The part of the yolk sac left inside the uterus after foetectomy never differentiated. Granulation tissue was not observed in this intrauterine milieu.

(3) In the several cases in which we found small foci morphologically similar to yolk-sac carcinoma and consisting of proliferating yolk-sac endoderm secreting hyalin, no granulation tissue was recorded. Also in these areas no differentiated organoid structures were observed.

Hence, the possibility that the blood supply may be an important environmental factor in controlling the differentiation. In a first stage this nutritional supply is probably assured by the continuity of the extrauterine part of the visceral yolk sac with the intrauterine one adhering to the placenta. During the following days, when the intrauterine membranes are destroyed, the newly formed capillaries present in the granulation tissue may take over this nutritive role. This continuous blood supply might explain the extensive differentiation observed in the yolk sac displaced after foetectomy compared to the poor or absent differentiation of yolk sac implanted in other sites. Whether a relationship exists between the absence of granulation tissue, accompanied by a deficient blood supply, and malignant transformation is still very hypothetical.

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