Differentiation potentiality of rat visceral yolk sac in organ culture

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SUMMARY

Visceral yolk sacs removed at day 12 of pregnancy in the rat were kept in organ culture for as long as 28 days. During this *in vitro* culture, proliferation of the endoderm and the mesoderm as well as of poorly differentiated cells was observed. The latter displayed neither the characteristics of endodermal nor mesodermal cells and their presence was frequently associated with the development of giant trophoblast cells. The hypothesis is proposed that these trophoblast cells originate from these poorly differentiated cells that acquire *in vivo* and *in vitro* the potentiality to differentiate.

INTRODUCTION

The visceral yolk sac is a foetal membrane which possesses the remarkable potentiality, after displacement, to proliferate and to differentiate into either benign teratomas consisting of various adult tissues (Sobis & Vandeputte, 1974, 1977, 1979), or into malignant yolk sac carcinomas (Vandeputte & Sobis, 1978; Sobis, Van Hove & Vandeputte, 1983). This property was found in the three species of rodents (rat, hamster, mouse) examined. Since we were able to show that the benign as well as the malignant tumours also develop in the absence of germ cells (Sobis & Vandeputte, 1976, 1982, 1983), we postulated the appearance in the displaced visceral yolk sac of multipotential cells. The latter cells might give rise to the various embryonal and extraembryonal structures found in the benign teratomas and malignant yolk sac carcinomas.

We showed previously that during the early formation of yolk-sac-derived teratomas, a population of poorly differentiated cells appeared a few days after the surgical procedure (Sobis *et al.* 1982*a*). The presence of these cells in the displaced membrane was accompanied by partial degeneration of the visceral yolk sac. Thereafter, redifferentiation occurred mainly in the areas where granulation tissue developed. In order to verify whether interaction with the host tissues is necessary to induce these dedifferentiation and redifferentiation, we

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followed the morphological changes in the visceral yolk sac cultured *in vitro*. The results of the present experiments indicate that in appropriate culture conditions, these poorly differentiated cells do indeed appear and that some differentiation, mostly in trophoblast giant cells, occurs.

MATERIALS AND METHODS

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 uteri with embryos were taken out and put in petri dishes with PBS. The visceral yolk sacs were dissected free from Reichert membrane, amnion and placenta in a medium composed of PBS supplemented with 5 % heat-inactivated horse serum. After washing two times in the same medium the whole visceral yolk sacs or separate fragments cut from it either proximal to, or distal from, the placental disc region were put in 50 ml plastic tubes (Falcon 2070) containing 20 ml of NCTC-135 medium (Gibco) supplemented with 20 % horse serum, 1 % sodium pyruvate, 0·1 % glucose and antibiotics. The tubes containing the membranes were incubated for 2 h at 37 °C in an atmosphere of 5 % CO_2, at high humidity. Afterwards the tubes were closed and further incubated on a roller system at 37 °C. Every 24 h the medium was changed and the tubes flushed with 5 % CO_2. In a preliminary experiment the yolk sacs were fixed after 24, 48 or 72 h of culture in these roller tubes, in order to determine by morphological criteria, the optimal incubation period previous to the organ cultures.

Since we found that 48 h culturing in roller tubes gave the best results, all organ cultures were established starting from yolk sacs pre-cultured for this period of time. The yolk sacs were then put on lens paper on stainless steel grids. The grids were placed in organ tissue culture dishes (Falcon 3037) containing 1 ml NCTC-135 medium supplemented as described and incubated at 37 °C with 5 % CO_2. The medium was changed twice a week. After 7, 10, 13, 18, 22 and 28 days of culture, the pieces of yolk sac were fixed in formol, embedded in paraffin and cut serially. The sections were stained with erythrosin–haematoxylin or PAS.

The presence of Δ^5-3β-hydroxysteroid dehydrogenase was verified on cryostat sections with pregnolone as substrate and nicotine amide dinucleotide (NAD) as cofactor by the method previously described (Sobis *et al.* 1982*b*). Control sections were made by incubating tissues in media lacking the substrate (Botte,


Fig. 1. Visceral yolk sac after 48 h culture in roller tubes. Some proliferation of endo- and mesoderm is seen. H and E. ×140.

Figs 2 & 3. Visceral yolk sac after 7 days in organ culture.
  Fig. 2. Proliferation of endoderm. H and E. ×660.
  Fig. 3. Proliferation of mesoderm. Mitotic figure (arrow) is seen. H and E. ×660.

Fig. 4. Visceral yolk sac after 13 days in organ culture. Large areas of poorly differentiated cells. H and E. ×660.
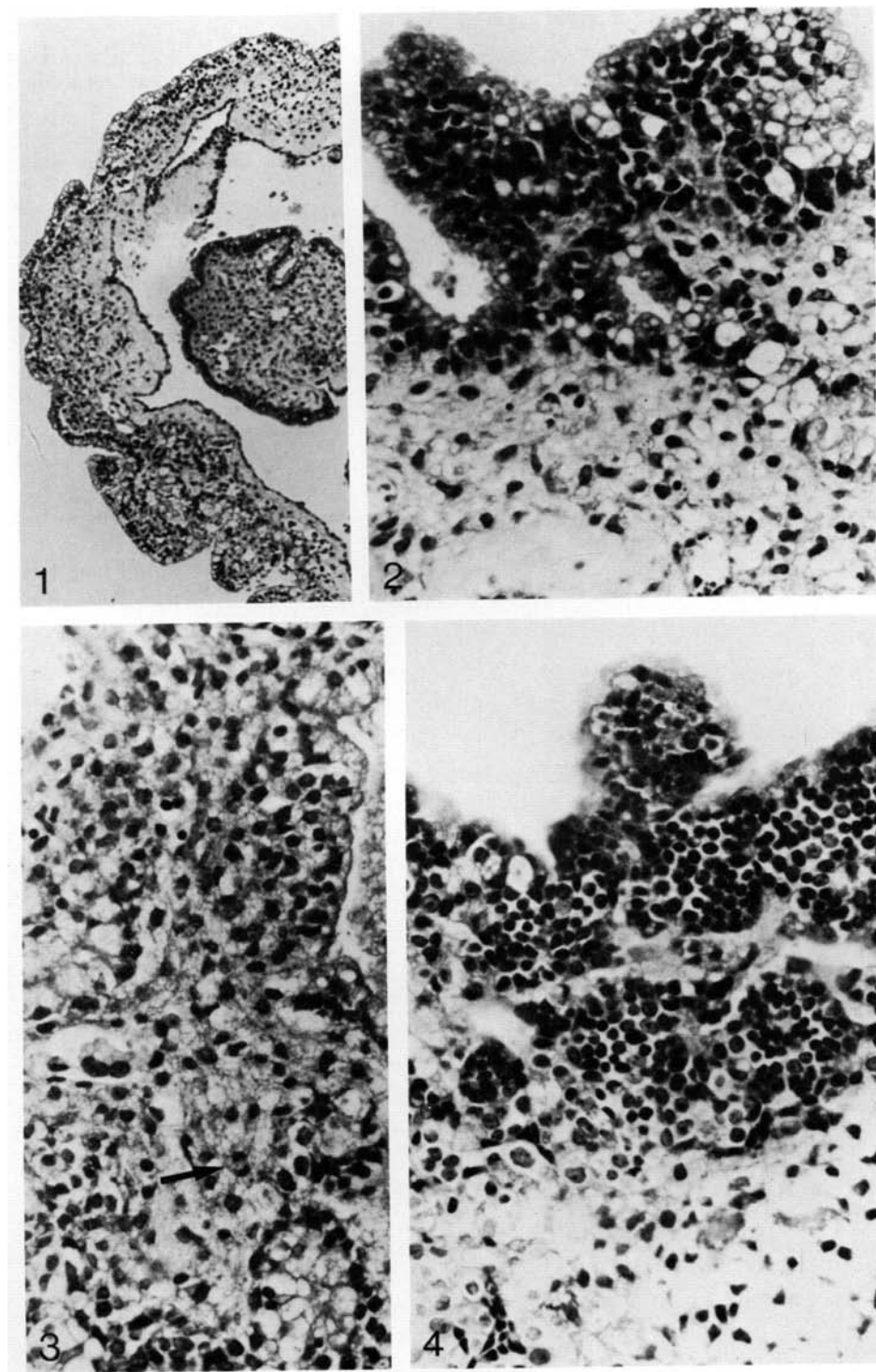
Figs. 1–4

Table 1. *Morphological characteristics of visceral yolk sacs cultured* in vitro

Days in organ culture*	7	10	13	18	22	28	Total
Number of yolk sacs	9	10	9	9	4	4	45
Proliferation of mesoderm	3	5	5	5	1	1	20
Proliferation of endoderm	4	4	4	2	1	1	16
Poorly differentiated cells	3	5	6	5	2		21
Trophoblast giant cells	4	5	2	4	2		17
Haemopoiesis	3	2	1				6
Squamous epithelium			5	7	3	2	17

* The period of organ culture was in all cases preceded by 48 h of culture in roller tubes.

Tramontana & Chieffi, 1968). As positive control the frozen sections of 12-day-old rat placenta were used.

RESULTS

In order to verify the technique of dissection, several visceral yolk sacs were fixed and examined histologically. They showed the typical structure of this membrane; that is, an endodermal layer, mesenchymal tissue with blood vessels containing nucleated blood cells and mesothelium. No contamination by other structures, such as parietal yolk sac or placental tissue, was detected. After 48 h of culture in roller tubes, the visceral yolk sac was rounded up, the endoderm forming the outside layer. Some proliferation of the endo- and mesoderm could be observed (Fig. 1). The results of the histological examination of the visceral yolk sacs maintained in organ culture for different periods of time are sum-marized in Table 1. Since the histological data are similar for the entire visceral yolk sac and for its proximal or distal fragments they are put together.

Seven days after organ culture we observed proliferation of endo- (Fig. 2) and mesodermal cells (Fig. 3) as well as the appearance of poorly differentiated cells. Mitotic figures were occasionally seen. The poorly differentiated cells did not possess the cytological characteristics necessary to establish their origin as being endodermal or mesodermal (Figs 4 & 6) and were very similar to those described in the displaced visceral yolk sac *in vivo* (Sobis *et al.* 1982a). The formation of

Fig. 5. Visceral yolk sac after 13 days in organ culture. Large area of poorly differen-tiated cells is seen in the upper right part. Area of trophoblast giant cells in the centre. H and E. ×140.

Fig. 6. Area of poorly differentiated cells from Fig. 5 at higher magnification. ×660.

Fig. 7. Area of trophoblast giant cells from Fig. 5 at higher magnification. ×660.

Fig. 8. Visceral yolk sac after 18 days in organ culture. Area of trophoblast giant cells with focus of poorly differentiated cells (arrow). H and E. ×660.
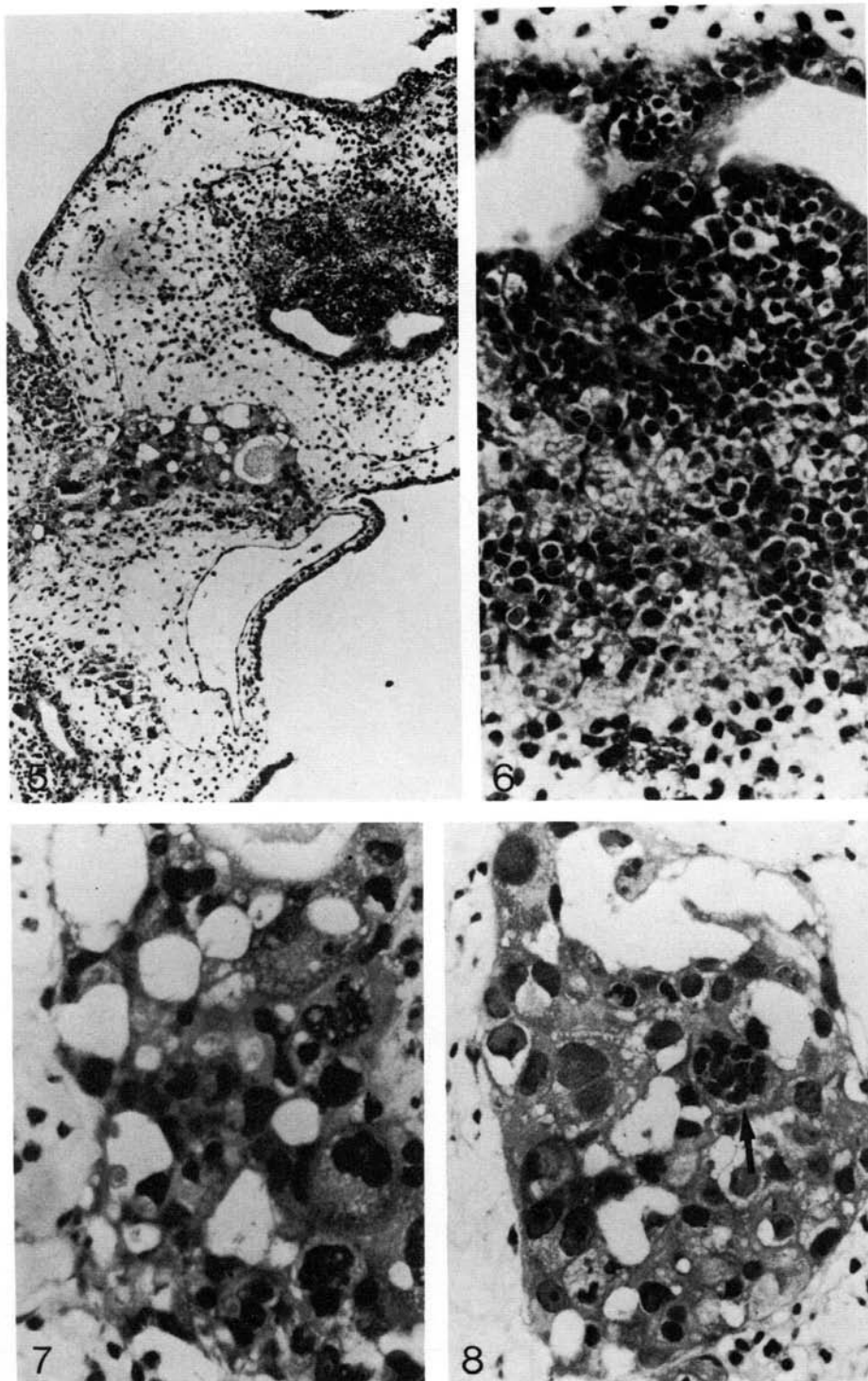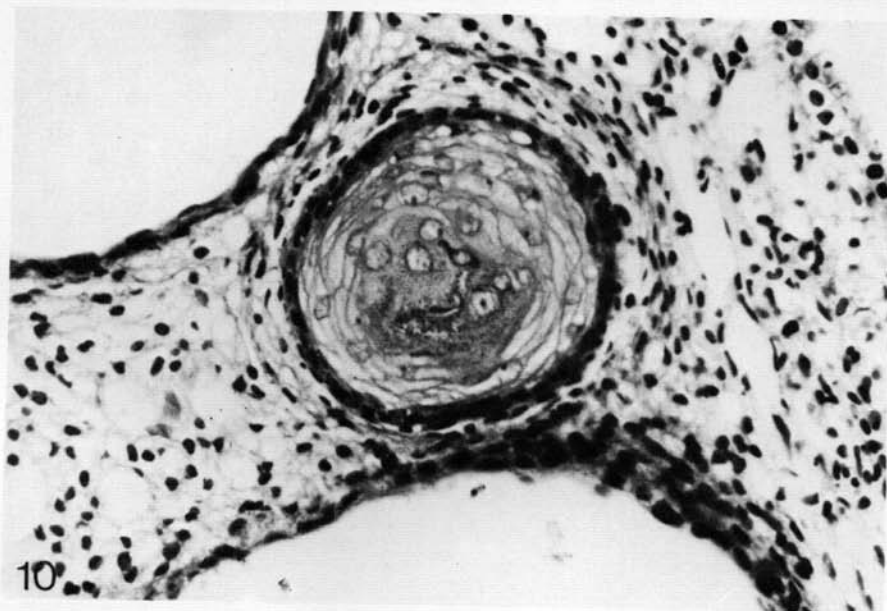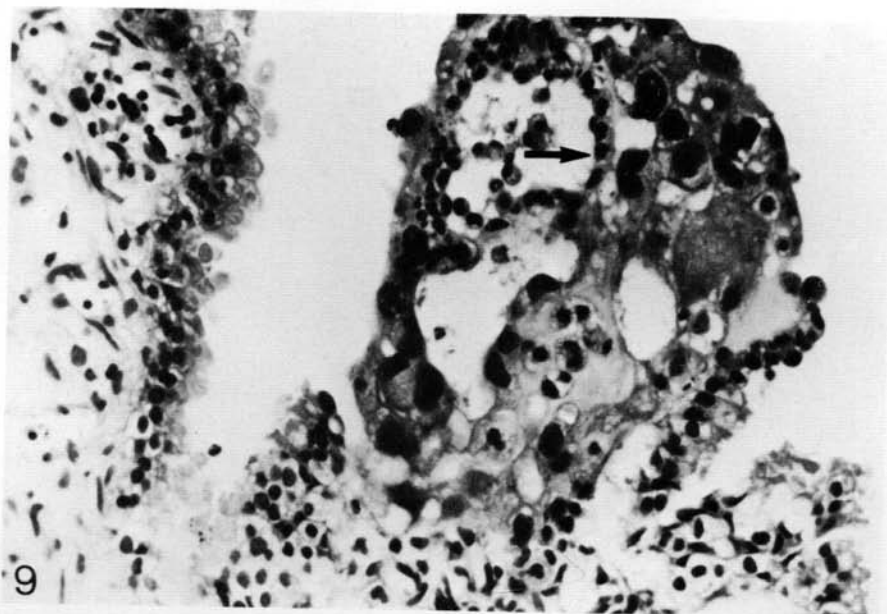
Figs. 5–8

Fig. 9. Visceral yolk sac after 22 days in organ culture. Trophoblast giant cells with parietal endodermal cells (arrow) and hyalin. H and E. ×660.

Fig. 10. Visceral yolk sac after 18 days in organ culture. Nest of squamous epithelium surrounded by connective tissue. H and E. ×660.

blood cells was also observed. In most sections partial degeneration of the foetal membrane involving both the endo- and mesoderm was seen. In half of the visceral yolk sacs, trophoblast giant cells characterized by their typical nuclear

and cytoplasmic structure, were also detected (Figs 5 & 7). These trophoblast giant cells were mainly observed in close association with an area of proliferating poorly differentiated cells (Figs 5 & 8). They were also detected in visceral yolk sacs kept in organ culture for 10, 13, 18 and 22 days. The trophoblastic nature of these giant cells was confirmed by the detection of Δ^5-3β-hydroxysteroid dehydrogenase, a histochemical marker for trophoblast (Botte *et al.* 1968; Chew & Sherman, 1975). Using pregnolone as substrate the giant cells displayed the presence of many blue-staining granules spread over the cytoplasm. The reaction was similar to that observed in the giant cells of rat placenta used as a positive control. In control sections incubated in media without pregnolone no reaction was observed. Whereas giant trophoblast cells are easy to recognize on a morphological and histochemical basis, this is not the case for cytotrophoblast. Although we suspected their presence in close association with the giant trophoblast cells we have no definite proof for their development since they do not display specific cytological markers. In one case we also observed proliferation of a parietal yolk sac structure associated with an area of giant trophoblast cells (Fig. 9). In the visceral yolk sac kept for 10 days in organ culture the histological findings were similar to those described at day 7. Starting at day 13 of organ culture, nests of squamous epithelium with keratin were seen (Fig. 10). These structures were even found as late as 28 days of organ culture. At that time the visceral yolk sac seemed still well preserved, although the cellular proliferation was less pronounced. Whereas the visceral yolk sac was kept well preserved for 4 weeks in organ culture, pieces of parietal yolk sac and of placental tissue cultured in the same conditions, were completely necrotic.

DISCUSSION

The results of this experiment demonstrate that the visceral yolk sac can be well preserved for at least 28 days in organ culture. This period is far beyond the normal survival time of the visceral yolk sac since this membrane was taken out at day 12 of pregnancy, cultured for 2 days in roller tubes and then put in organ culture for as long as 28 days. Like in the *in vivo* displaced visceral yolk sac (Sobis *et al.* 1982*a*) one observes the proliferation of the endoderm and mesoderm as well as the appearance of poorly differentiated cells. The presence of haemopoiesis until 13 days after organ culture may reflect the prolongation of normal yolk sac function occurring *in vivo*. After 13 days stratified squamous epithelium and cysts filled with keratin appeared. Haemopoiesis and squamous epithelium have also been described in long-term cultures of visceral yolk sac by Sorokin & Padykula (1964). Although, as suggested by the authors, the squamous epithelium is likely to derive from the endoderm by a process of metaplasia, we cannot dismiss the possibility that this epithelium may also originate from the poorly differentiated cells. Indeed, in many cases there was no spatial association with the endoderm of the visceral yolk sac and only

occasionally did we observe nests of squamous epithelium lying between the columnar epithelium of the endodermal layer. The most interesting finding in this experiment was, however, the observation of foci of trophoblast giant cells. The trophoblastic nature of the giant cells is illustrated by the presence of Δ^5-3β-hydroxysteroid dehydrogenase using pregnolone as a substrate. This histochemical reaction is similar to that observed in a first generation of giant cells in rat placenta and is considered to be specific for trophoblastic giant cells. The presence of Δ^5-3β-hydroxysteroid dehydrogenase in the giant cells indicate their role in the production of progesterone (Sheth, Koszalka & Brent, 1978). These trophoblast cells are not a contaminant due to a technical error in the dissection of the visceral yolk sac since (i) they were observed as well in pieces of the yolk sac distal from the placental disc region as in pieces proximal to it; (ii) the trophoblast cells present in the placenta or attached to the parietal yolk sac and cultured in the same conditions, were completely necrotic in organ culture; (iii) the trophoblast giant cells were never found in the visceral yolk sac prior to the organ culture and were only detected after a period of 7 days *in vitro*. Hence, we think that the trophoblast cells differentiate from multipotential cells which appear in the cultured visceral yolk sac. As previously described *in vivo*, such multi-potential cells present in the displaced yolk sac may give rise to embryonal and extraembryonal structures. From this cell population derive benign teratomas and yolk sac carcinomas. The poorly differentiated cells present in the latter tumour can differentiate, not only in visceral and parietal yolk sac structures, but also in trophoblast as recently shown in transplantable yolk sac carcinomas (Sobis *et al.* 1982*b*) and by cloning of the tumour cells (unpublished observation). In the present study the trophoblast cells developed in normal, not transformed tissue kept in organ culture. The presence of such a variety of cell types including trophoblast and in one case of parietal yolk sac cells in the long-term *in vitro* culture of visceral yolk sac is probably due to the culture conditions used. Indeed, before applying the culture conditions described, many other methods have been tried out with less-good results. Amongst the factors that may have played a positive role we mention (i) the roller system allowing the formation of three-dimensional structures previous to organ culture. The formation of such a structure may favour differentiation (Martin, 1982). (ii) The use of NCTC medium. This very rich medium may be partly responsible for the differentiation into trophoblast giant cells since it promotes the trophoblast outgrowth from blastocysts (Blake, Schindler & Sherman, 1983). (iii) The presence of alpha-foetoprotein which was detected for at least 7 days in the culture supernatants (data not shown) and was shown by Toder, Blank, Gold-Gefter & Nebel (1983) to promote the outgrowth of trophoblast. (iv) The presence of mesenchymal tissue in the cultured visceral yolk sac could also play a role in the induction of trophoblast giant cells. Indeed, proliferation and differentiation of trophoblast is influenced by the inner cell mass during early embryogenesis (Gardner, 1972) and by the allantois in later stages (Gardner,

Papaioannou & Barton, 1973; Peel & Bulmer, 1977; Snell & Stevens, 1966). Hence the possibility that the poorly differentiated cells observed in the cultured visceral yolk sac have the potentiality to form trophoblast cells in association with the mesenchyme of this membrane acting as inducer. Since it was shown that neither uterine factors nor contact with maternal cells is required for differentiation of trophoblast (Sherman & Atienza-Samols, 1979), it is after all not so surprising that under these special conditions such differentiation can occur *in vitro*.

Moreover, it is now well documented that parietal and visceral endoderm originate from primitive endoderm (Gardner, 1982). The latter develops from inner cell mass (Papaioannou, 1982) which has the potentiality to form trophoblast-like giant cells *in vivo* (Rossant & Lis, 1979) and *in vitro* (McCue & Sherman, 1982). Hence, it seems probable as suggested by Gaillard (1972, 1981) that all extraembryonic tissues (visceral and parietal yolk sac, and trophoblast) have a common origin. It remains, however, to be established whether cells present in the displaced yolk sac can *in vitro* and *in vivo* regain after dedifferentiation such a multipotentiality. The results obtained in organ culture and those described *in vivo* on yolk-sac-derived teratomas support this possibility.

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