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Development of separated germ layers of rodent embryos on ectopic sites: a reappraisal

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ABSTRACT **The method of separation of germ layers of rodent embryos by treating the embryonic shields with proteolytic enzymes and by microsurgery with the subsequent transplantation to ectopic sites has helped to gain a more detailed insight into what is going on during gastrulation in mammals. The space under the kidney capsule of adult animals seems to be the most appropriate ectopic site for transplantation of early postimplantation rat embryos or separated germ layers. After transplantation the grafts develop into teratomas whose complex histological structure reflects the initial developmental capacities of the graft. At the pre-primitive streak and the early primitive streak stages the primitive ectoderm differentiates into tissue derivatives of all three definitive germ layers, often in complex organotypic combinations. This is indirect evidence that all cells of the embryonic body originate from the primitive embryonic ectoderm. Halves of the primitive ectoderm obtained by a longitudinal or transverse cut through the egg cylinder give the same result. At the head fold stage the capacity for differentiation of the ectoderm is restricted to ectodermal and mesodermal derivatives. One day before gastrulation the isolated primitive ectoderm is not able to differentiate as renal isograft. The mesoderm isolated at the head fold stage and at later stages when its segmentation occurs, differentiates almost exclusively into the brown adipose tissue. The embryonic endoderm differentiates only in combination with the mesoderm. After transplantation the embryonic ectoderm loses its epithelial organization and breaks up into a mass of mesenchyme-like cells in which epithelial structures subsequently appear and differentiate in a way reminiscent of the reaggregation of cells in mixed cell suspension *in vitro*.**

KEY WORDS: *germ layers, transplantation, differentiation, rodent embryo*

Introduction

The main obstacle in manipulating embryos of rats and mice at early postimplantation stages is their cup-shaped embryonic shield («egg cylinder») with inverted germ layers: the ectoderm inside and the endoderm outside the wall of the cylinder (Fig. 1 a,b,c,d). This unusual feature makes the separation of germ layers very difficult. Grobstein (1952) mechanically isolated the embryonic ectoderm of the primitive streak stage mouse embryo and cultivated it *in vitro* and subsequently *in vivo*, in the anterior chamber of the eye. The main conclusion from his pioneer experimental approach – that the primitive or primary embryonic ectoderm (for terminology see Svajger *et al.*, 1986) contains prospective cells of the definitive embryonic endoderm – was confirmed later on by more elaborate methods.

About two decades ago we improved the method of separating germ layers of the rodent embryo by introducing the treatment of embryonic shields with proteolytic enzymes before microsurgery (Levak-Svajger *et al.*, 1969; Svajger and Levak-Svajger, 1975). The results obtained by using this procedure followed by grafting of isolated germ layers to ectopic sites were reviewed elsewhere (Skreb and Svajger, 1975; Beddington, 1983, 1986; Svajger *et al.*, 1986). Here we will only give a brief account of the principles of the experimental approach and a survey of results, with special emphasis on the reliability of the method and difficulties and dilemmas in the interpretation of results.

Separation of germ layers

The whole procedure has been described in all detail elsewhere (Svajger and Levak-Svajger, 1975 and several manuals on methods in developmental biology of mammals). This is a modification of the common method of separation of embryonic epithelia from the underlying mesenchyme. It consists of the incubation of embryonic shields in a mixture of proteolytic enzymes (0.5% trypsin + 2.5% pancreatin dissolved in calcium- and magnesium- free Tyrode's

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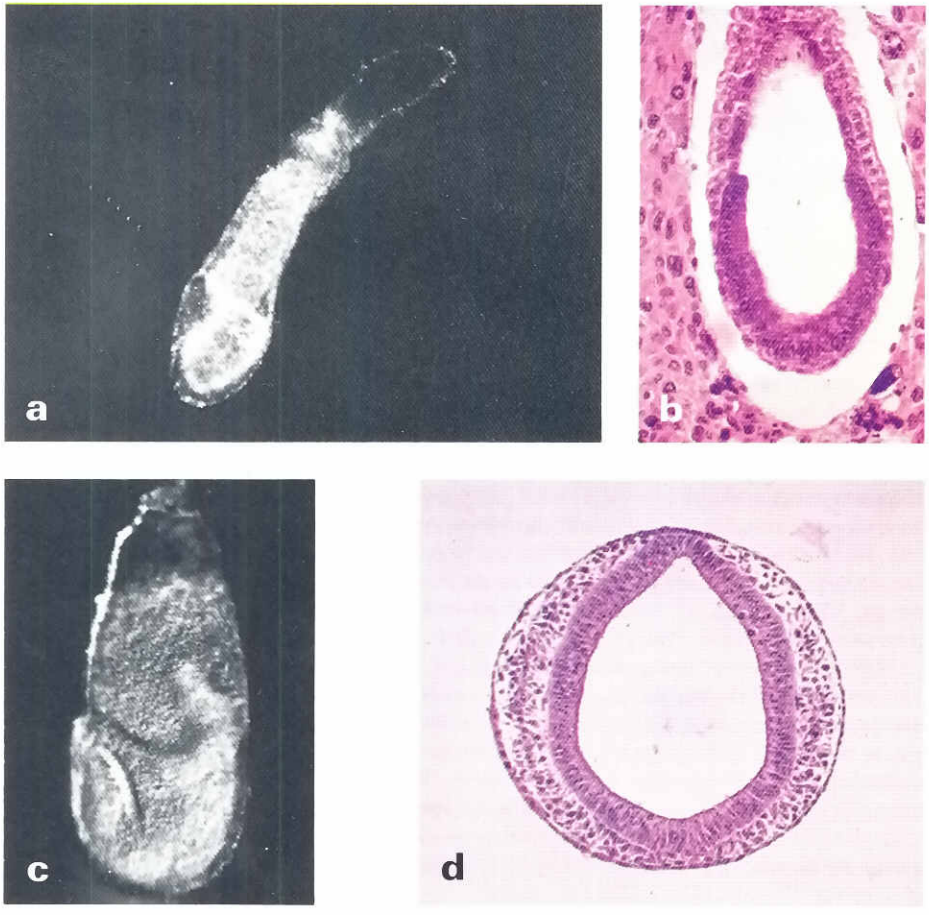

Fig. 1. Rat egg cylinders.(a) *The pre-primitive streak stage (8 days). The embryonic part (bottom) is sharply demarcated from the extraembryonic part (middle) with the ectoplancental cone (top). x50.* **(b)** *Longitudinal section through the 8-day egg cylinder. The thick inner cell layer of the embryonic part (primitive embryonic ectoderm) is lined on the outside by the thin primitive embryonic endoderm. The rest of the cylinder (upper half) is the extraembryonic part. x75.* **(c)** *The head fold stage (9 days). The embryonic part (lower half) with the head fold (left) is demarcated by amnion from the extraembryonic cavity (upper half) into which the allantois protrudes (right). x75.* **(d)** *Cross section through the 9-day embryo. All three germ layers are visible. The amniotic cavity is lined by the ectoderm. The primitive streak (bottom) is situated opposite to the neural groove (top). x40. Figs. 1a and 1c have been reproduced in a modified form from Svajger and Levak-Svajger, 1975, with permission of Springer-Verlag, Berlin.*

saline - CMF) at +4°C for 15-30 minutes. Subsequently the germ layers are separated mechanically with electrolytically sharpened and polished tungsten (wolfram) needles and grafted to an appropriate ectopic site (Fig. 2 a,b,c; Fig. 3 a,b,c,d,e,f).

Trypsin digests the glycoproteins laminin and fibronectin (Zimmermann *et al.*, 1982), the constituents of basement lamina which exist between germ layers at very early stages (Pierce, 1966; Adamson and Ayers, 1979; Mitrani, 1982). Pancreatin (probably DNA-ase which it contains) removes the viscous material which appears as a consequence of disruption of some cells during mechanical and enzymatic treatment (Moscona *et al.*, 1965). The incubation in enzymes at a low temperature ensures the separation of cell layers without their dissociation into individual cells (Szabó, 1955).

Choice of the host tissue

There are several ectopic (extrauterine) sites to which embryonic tissues may be transplanted in order to test their developmental potential. Some of them, such as the subcutaneous tissue (Usadel *et al.*,1970) and the cheek pouch of the hamster (Damjanov, 1978) were only occasionally used. Several decades ago the chorioallantoic membrane (CAM) of the chick embryo was used as the host tissue for early rat embryos (Nicholas and Rudnick, 1933). In our experience, in addition to a restricted time for growth, grafts of rat egg cylinders on the CAM display a low rate of incorporation, a poor tissue survival and growth and an incomplete differentiation (Skreb and Svajger, 1975).

The anterior chamber of the eye was also considered as a convenient site for transplantation of early postimplantation mouse and rat embryos (Grobstein, 1951, 1952). There are, however, some disadvantages to this method. The rate of successfully incorporated and differentiated grafts is relatively low and mesodermal tissues differentiate poorly when the egg cylinders are transplanted before the onset of gastrulation. The possible explanation is that the grafts float in the aqueous humour before attachment to the iris, and that the incorporation into the iris is not always complete. This assumption was reinforced by the experience of poor results obtained by introducing rat egg cylinders into the large anterior chamber of the rabbit eye (Skreb and Levak, 1960).

Intratesticular grafting has been commonly practised in the experimental embryology of mammals. It has the obvious advantages of easy accessibility of the host organ and the immediate implantation of the graft into an abundantly vascularized environment. If one neglects the somewhat lower temperature within the scrotum in comparison with that in the rest of the body, the disadvantage is that the graft is obscured by the thick capsule of the testis and its development is difficult to follow continuously.

Already our first experiments with transplantation of whole rat egg cylinders under the kidney capsule showed the superiority of this site over the anterior chamber of the eye. The rate of successful implantation was high and tissue derivatives of all three germ layers

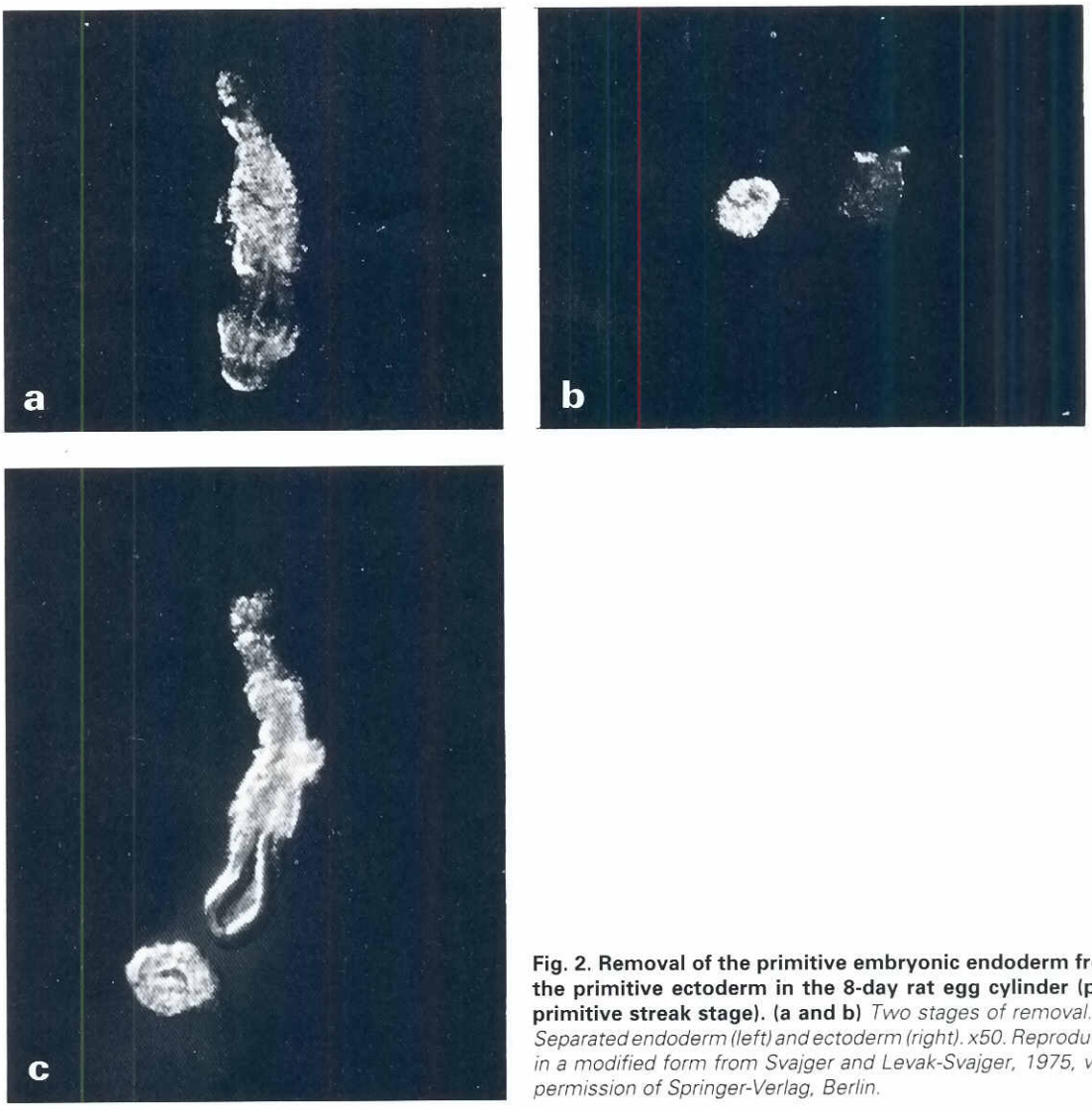

Fig. 2. Removal of the primitive embryonic endoderm from the primitive ectoderm in the 8-day rat egg cylinder (pre-primitive streak stage). (a and b) *Two stages of removal.* **(c)** *Separated endoderm (left) and ectoderm (right). x50. Reproduced in a modified form from Svajger and Levak-Svajger, 1975, with permission of Springer-Verlag, Berlin.*

differentiated regularly regardless of the initial developmental stage of grafted egg cylinders (Skreb *et al.*, 1971). The renal capsule consists of an outer connective tissue layer, a layer of atypical smooth muscle cells with adrenergic innervation and vascularized loose connective tissue adjacent to the renal parenchyma (Bulger, 1973; Kobayashi, 1978). Therefore, by the act of transplantation the graft is immediately placed between two well-vascularized tissues: the kidney capsule and the kidney parenchyme. Blood capillaries bud into it from both sides. Moreover, the graft acts as a vascular attractive (angiogenetic) factor: at the time of recovery one can always see a small artery emerging in the renal hilus and running towards the graft which is penetrated by its terminal branches. Even the smallest graft is clearly visible under the thin and transparent capsule and it can be recovered for histology, so that a «time-lapse» analysis of its differentiation is possible. The connection between the graft and the host tissues remains only vascular. As a rule, the graft does not penetrate into the renal parenchyme and at recovery it can be «cleanly» detached from the surface of the kidney (Fig. 4). For all these reasons this favorable ectopic site has been exclusively used in our investigations. The inbred Fischer strain of rats was used. The host animals were 3 month old males (in order to avoid hormonal changes during the estrus cycle).

Experimental embryonic teratomas

After transplantation of egg cylinders or parts of them an intensive cell proliferation and differentiation of tissues takes place. As a result tumors of various sizes and shapes appear as elevations on the surface of the kidney, covered with the extended capsule. Their size depends on the nature and size of the grafted material, of the time spent in the host and on various other, partially unknown factors (delayed vascularisation, partial necrosis etc.). Some tissue constituents of tumors (brain, cartilage, centers of ossification, cystic structures etc.) can be clearly distinguished through the transparent renal capsule (Fig. 5a).

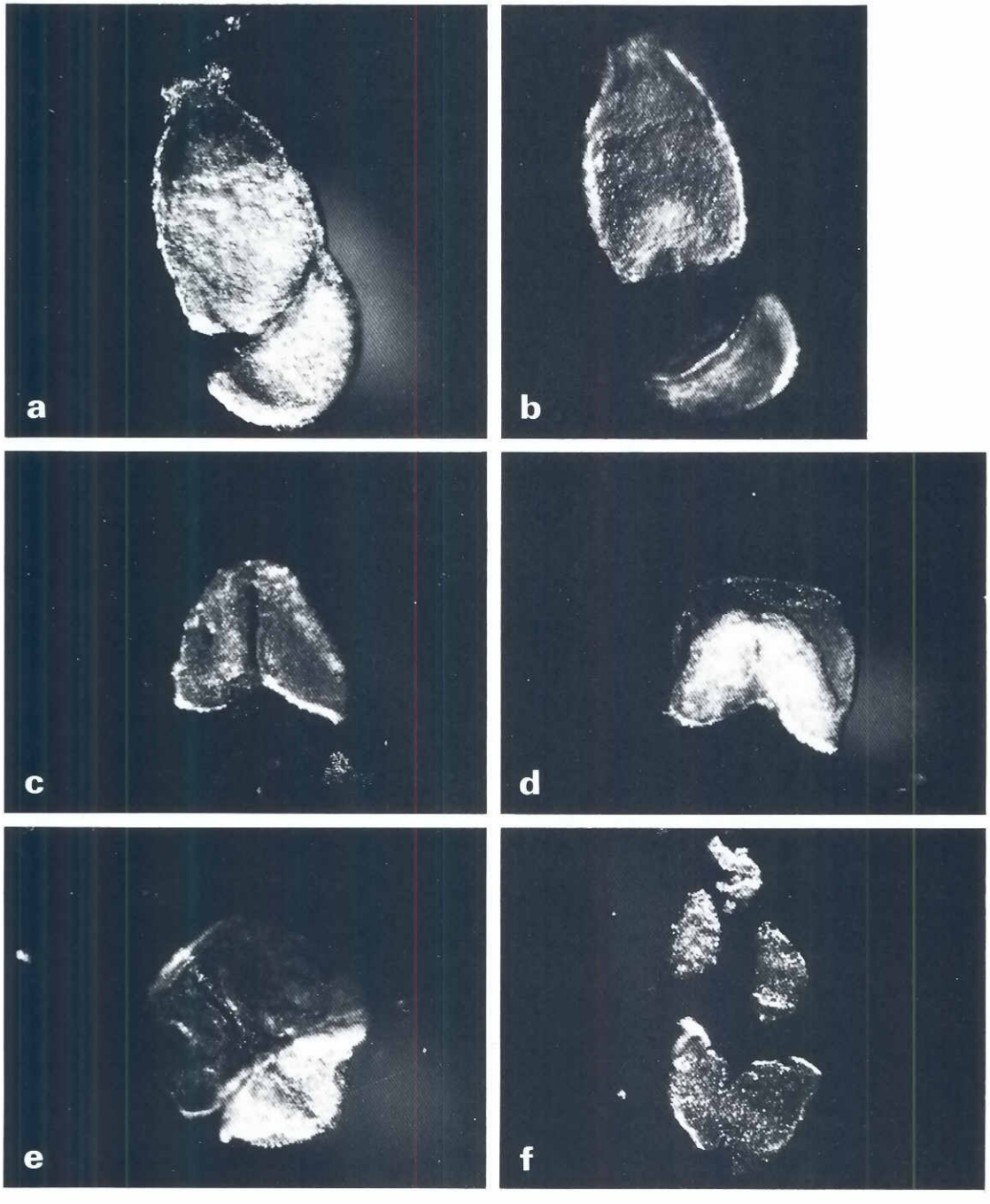

Fig. 3. Separation of germ layers in 9-day egg cylinders (head fold stage). (a and b) *Separation of the embryonic part of the egg cylinder (bottom) from the extraembryonic part.* **(c)** *The embryonic part of the egg cylinder is transformed into a flat shield by a cut through the primitive streak (lower edge).* **(d)** *Spontaneous detachment of the ectoderm after the treatment with enzymes.* **(e)** *The ectoderm turned over before microsurgical removal.* **(f)** *Completely separated germ layers. From the top to the bottom: endoderm, two mesodermal «wings», ectoderm. x55. Figs. 1a and 1c have been reproduced in amodified form from Svajger and Levak-Svajger, 1975, with permission of Springer-Verlag, Berlin.*

The tissue composition and framework of these tumors conforms to the pathological definition of the benign teratoma (for review see Damjanov and Solter, 1974). For the present case the usual terms are embryo-derived teratoma or experimental embryonic teratoma. These tumors are composed of mature tissues in a chaotic arrangement (Fig. 5b). Obviously, in the absence of the body axis and spatially regulated cell and tissue interactions which exist during normal development *in situ* in the amniotic fluid, the embry-

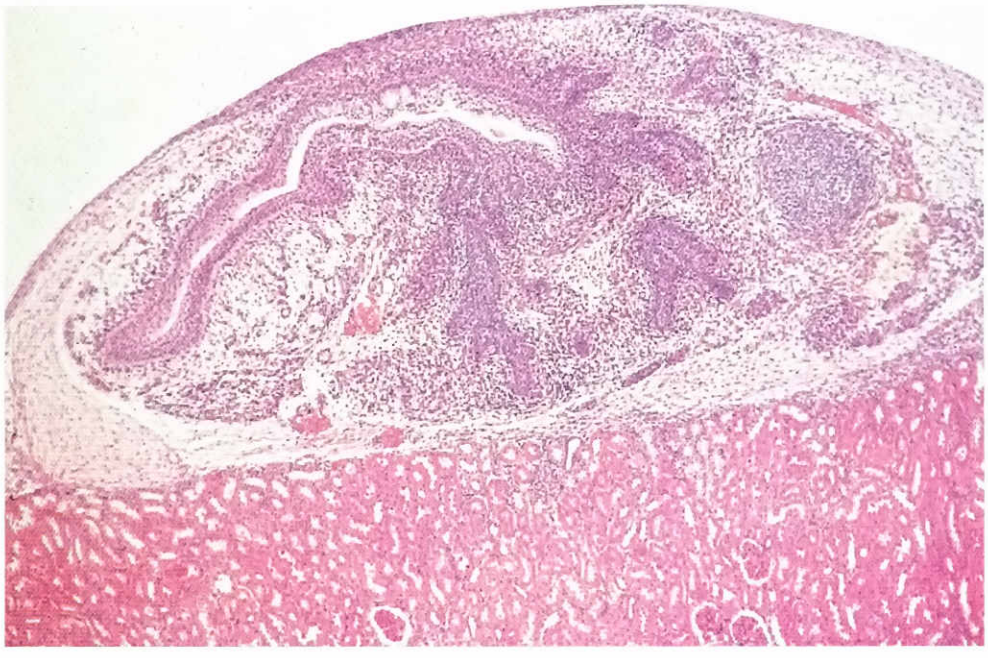

Fig. 4. An example of the position of a renal graft between the capsule and the parenchyma of the kidney. *Note that there is no penetration of the graft into the parenchyma. x12.*

onic cells express their inherent capacities for differentiation missing the environmental conditions indispensable for the organization of the embryonic body. Data relevant to the tissue composition of teratomas derived from whole rat egg cylinders or isolated germ layers are reviewed in detail elsewhere (Skreb and Svajger, 1975; Svajger *et al.*, 1986). Here we will only point to some features which are in line with the main purpose of this review.

Although by definition the benign teratomas are composed of mature tissues in a disorganized arrangement, well expressed organotypic combinations of different tissues regularly occur. The pseudostratified ciliated columnar epithelium with goblet cells is usually associated with the hyaline cartilage as in the respiratory tube *in situ*, and the epithelium of the digestive tract is surrounded by smooth muscle cells. Moreover, in some tumors structures may be found which completely mimic whole organs with all details of their histological structure (Fig. 6 a,b,c,d; Fig. 7). Among them are: a) tooth germs or even a regular row of them in which incisors can be clearly distinguished from molars, b) fingers (toes) with metacarpal (metatarsal) bone and three phalanges with the nail on the distal one, c) thyroid gland, d) thymus colonised by lymphocytes with the adjacent parathyroid gland (origin from the same cell mass of the endodermal epithelium of the third pharyngeal pouch!), e) trachea (epithelium + cartilage) with bronchial tree and lungs, f) esophagus, stomach, small and large intestine with all segmental characteristics including eosinophils and plasmocytes in the lamina propria and three smooth muscle layers, sometimes even with single intramural ganglionic cells, g) urogenital sinus with epithelial buds of the prostate, h) cerebrospinal ganglia, brain ventricles and choroid plexus with an ample subependymal capillary network (Skreb and Svajger, 1975; Svajger *et al.*, 1981, 1986).

These observations strongly indicate that in spite of the atypical mechanisms of morphogenesis, not only do embryonic cells retain their capacity for cell- and tissue-specific differentiation, but also in teratomas mechanisms are not lost which enable cells and tissues to interact to such a degree of specificity that very complex and regionally specific organotypic structures can be built.

The rationale of the design of experiments

Our experiments with the transplantation of isolated germ layers of the rat embryo to an ectopic site were planned with the presumption that the tissue composition of the resulting teratomas reflects the developmental potential of embryonic cells (germ layers) at the moment of isolation. We therefore transplanted whole isolated germ layers, restricted parts of them or combinations of them under the kidney capsule, recovered the resulting teratomas after 15-30 days (period sufficient for differentiation of various tissues) and analyzed them by routine histological methods.

Survey of results

In our previous reviews (Skreb and Svajger, 1975; Svajger *et al.*, 1981, 1986) we summarized the results obtained on rat cylinders belonging to the following stages:

a) Pre-primitive streak stage: 8-day embryos (equivalent to 6-day mouse embryo),
b) Early primitive streak stage: 8.5-day embryos (equivalent to 7-day mouse embryo),
c) Head fold stage: 9-day embryos (equivalent 7.5-8-day mouse embryos).

This is a brief summary of all results and conclusions.

Ectoderm

(1) The embryonic ectoderm isolated at the pre-primitive streak and the early primitive streak stages gave rise to tissue derivatives of all three definitive germ layers. Typical endodermal derivatives (respiratory tube, glands, intestine) were regularly present in teratomes (Fig. 8a,b,c). It was concluded that all cells of the future embryonic body reside within the primitive ectoderm before and at the onset of gastrulation. Analogous results have been obtained with mouse embryos (Diwan and Stevens, 1976).

(2) At the head fold stage the embryonic ectoderm differentiates

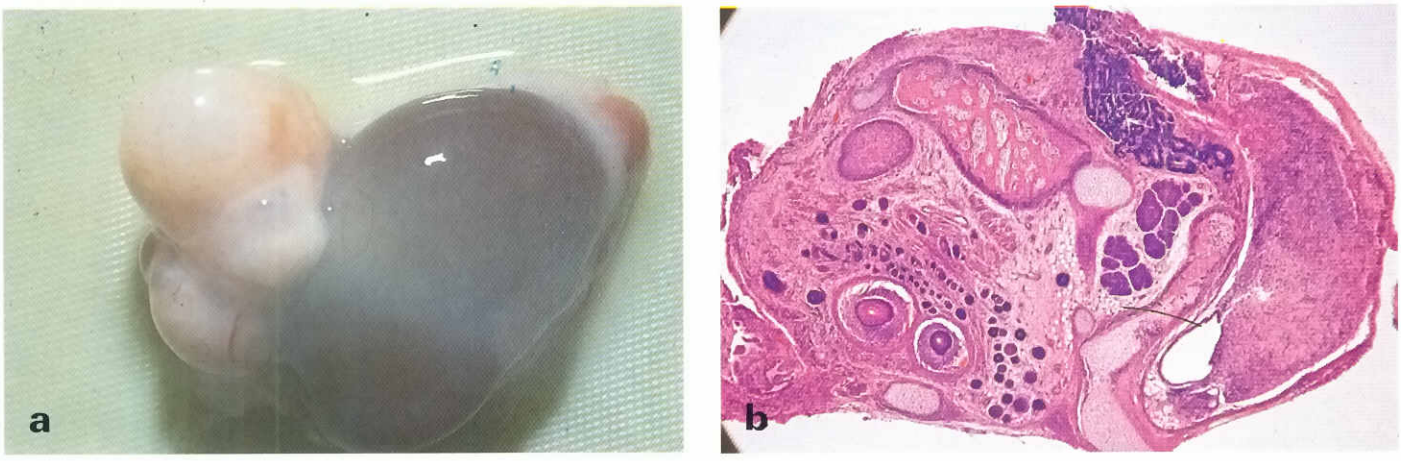

Fig. 5. Experimental embryonic teratoma. (a) *Macroscopic appearance (ectoderm, head fold stage) 15 days after transplantation under the kidney capsule. Natural size.* **(b)** *Typical histological composition of a teratoma (anterior part of the whole egg cylinder at the head fold stage) 15 days after transplantation. From left to right: brain, respiratory tube with glands and cartilage, thymus, membrane bone, epidermis, hairs and two whiskers. x10.*

only into ectodermal and mesodermal tissues. Derivatives of the primitive gut are absent (Fig. 8d). This could mean that the migration of presumptive cells of the definitive endoderm terminates before the end of migration of mesodermal cells.

(3) If at the pre-primitive streak or the early primitive streak stages the primitive ectoderm is cut transversally or longitudinally into two halves, both halves differentiate into tissue derivates of all three definitive germ layers and there is no essential difference in histological composition of teratomas developed from two halves of the same ectoderm. The result is the same when the ectoderm is manipulated in the same way without previous removal of the primitive endoderm.

These results suggest that: a) within the primitive ectoderm areas with restricted developmental capacities are not yet sharply demarcated, and b) cells with endodermal and mesodermal destinations can leave the primary ectoderm in regions other than the usually positioned primitive streak.

(4) At the head fold stage the cup-shaped egg cylinder was cut through the primitive streak and thus transformed into a flat embryonic shield (Fig. 3c). By two parallel longitudinal cuts through the head folds and at various distances laterally from them, the shield was divided into three parts. The median part (the future neural groove) was grafted alone and the two lateral parts (presumed to form the surface ectoderm) were grafted together. Teratomas resulting from both series of grafts contained both the neural tissue and the skin. It may be concluded that at this stage these two major developmental potentialities of the definitive ectoderm are not yet strictly confined to any particular region (Svajger and Levak-Svajger, 1976; Skreb *et al.*, unpublished results). After the incomplete closure of the neural tube in 10-day embryos (10-12 somites) the neuroectoderm has lost the capacity to differentiate into epidermis, even in the open areas of the two neuropores (Svajger *et al.*, 1981).

Mesoderm

The developmental fate of the isolated mesoderm as neural isograft is intriguing. Deprived of the ectoderm and endoderm with which it establishes mutual inductive interactions during normal development *in situ*, it differentiates almost exclusively into the brown adipose tissue (BAT). We tested the developmental capacities of the mesoderm isolated from embryos ranging in stage from the head fold (unsegmented mesodermal «wings») to 12-somite stage (paraaxial unsegmented mesoderm, anterior and posterior five to six somites as one piece, lateral plate, mesoderm of the tail region, and mesenchyme of the head and neck, including the 1st and the 2nd branchial arch). All these grafts differentiated into BAT, with the exception of the posterior somites in which, in addition to the BAT, nodules of cartilage developed (Fig. 8e), and the head and neck mesenchyme (mesectoderm of the neural crest origin) which differentiated almost exclusively into membrane bone. The BAT is also present (often in large amounts) in combined grafts, such as endoderm + mesoderm or those dissected out of the posterior part of the whole egg-cylinder (all three germ layers) at the head fold stage (Svajger and Levak-Svajger, 1974; Fig. 7f). At first sight, the «choice» of the isolated mesoderm to direct its developmental pathway towards BAT is difficult to interpret. A plausible interpretation was recently put forward by Loncar (1991, this issue). The BAT is characterized by a rich capillary network and adrenergic innervation. During normal development *in situ* the development of BAT starts with the penetration of a vascular bud into a particular area of mesenchyme. Also when the graft is placed under the kidney capsule, it is soon reached by a neurovascular outgrowth which originates from the hilus of the kidney which is one of the typical localizations of BAT. The renal capsule itself also has an adrenergic innervation (Kobayashi, 1978). One could therefore suppose that in the absence of conventional inducing tissues the undifferentiated mesenchyme is converted to BAT by ingrowing blood capillaries and adrenergic nerves from the host organ.

Endoderm

At all developmental stages examined grafts of the isolated endoderm were completely resorbed. However, when grafted in combination with the mesoderm at the head fold stage, it differentiated into a variety of derivates of the primitive gut (Fig. 8f). At the same stage, the capacities to differentiate into different segments of the definitive gut are already roughly restricted to particular areas of the endoderm. These areas express these capacities when

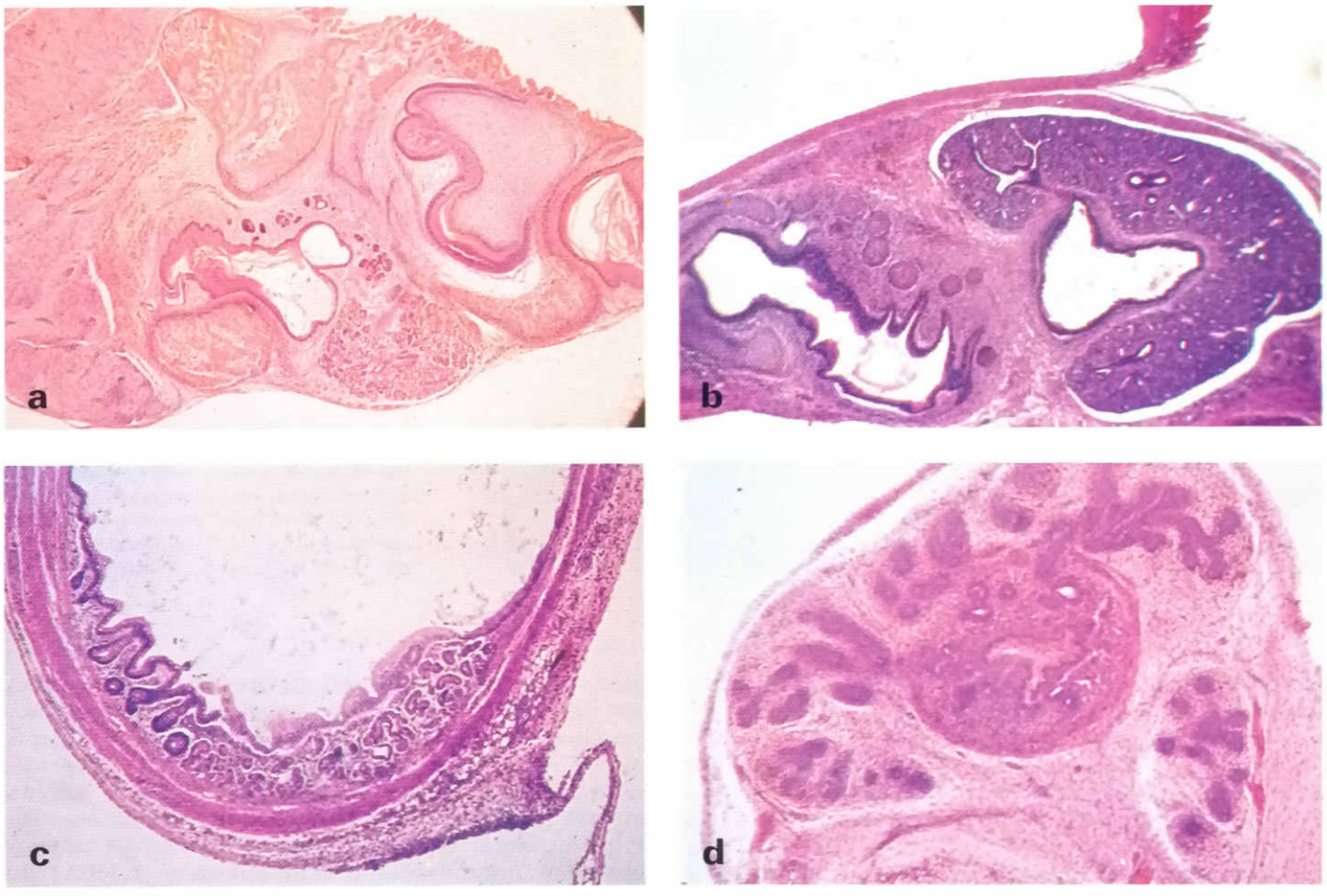

Fig. 6. Organotypic tissue associations in grafts. (a) *Tooth germ. x20.* **(b)** *Trachea, branched bronchi, lung. x20.* **(c)** *Segment-specific differentiation of the primitive gut (characteristic surface epithelium, glands, intestinal villi and smooth muscle layers). From right to left: esophagus, stomach, small intestine. x33.* **(d)** *Urogenital sinus with prostatic buds. x40.*

grafted together with the adjacent mesoderm and ectoderm (Svajger and Levak-Svajger, 1974). Obviously, appropriate epithelial-mesenchymal interactions are needed for the differentiation of the endoderm.

Role of the primitive embryonic endoderm in gastrulation

The primitive embryonic endoderm (the embryonic part of the visceral endoderm) is a transient thin epithelial layer adjacent to the primitive ectoderm. During gastrulation it is replaced by the definitive endoderm and displaced to the yolk sac endoderm. The question arises whether the role of the primitive endoderm is restricted to the formation of extraembryonic structures or whether it also plays a role in the development of the embryo itself (Rossant, 1986).

When the isolated primitive ectoderm or the whole two- layered 8-day rat egg cylinders (pre-primitive streak stage, equivalent to the 6-day mouse embryo) were transplanted under the kidney capsule for 15 or 30 days, tissue derivates of all three definitive germ layers developed in both series. This indicates that at this stage (immediately before the onset of gastrulation) the primitive endoderm neither substantially contributes to the definitive endoderm nor essentially influences the capacity for differentiating the primitive embryonic ectoderm.

When the egg cylinders are isolated and grafted 1.5 or 1 day before the appearance of the primitive streak (7- and 7.5 day rat embryos, equivalent to 5- and 5.5- day mouse embryos respectively), the results are different (unpublished data). Because of the small size of the cylinders it was very difficult to remove the primitive endoderm and the incidence of successful grafts was low. The preliminary results were the following: a) Whole two-layered egg cylinders grafted for three weeks developed into teratomas composed of tissue derivatives of all three definitive germ layers, b) halves of transversely cut whole egg cylinders also differentiated into tissues of all three germ layers, although the repertoire of tissues was considerably reduced, with the exception of neural tissue and cartilage, c) the isolated whole embryonic ectoderm was regularly resorbed. Because it was twice as large as the halves of the whole egg cylinder, the deficient critical cell mass could hardly be taken into consideration as a cause for this failure. One might conclude that immediately before the onset of gastrulation the absence of the primitive endoderm can not affect the capacity of the

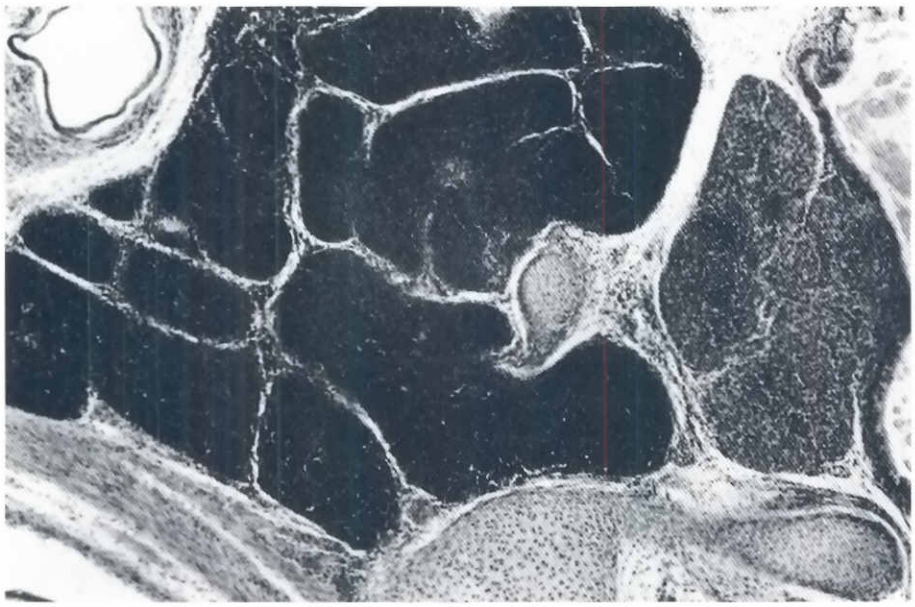

Fig. 7. Thymus and parathyroid gland within a teratoma. *An example of highly specific tissue and cell association even when they originate from the same small area (the endodermal epithelium of the 3rd pharyngeal pouch to which the parathyroid gland is still attached). x80.*

primitive ectoderm to undergo morphogenetic changes and differentiation, but one day before it is indispensable for «preparing» the primitive ectoderm for its further development.

It has been previously shown that in the early chick blastoderm the primitive endoderm (hypoblast) induces the formation of the primitive streak in the competent primitive ectoderm (epiblast) thus determining the anterio-posterior axis of the blastoderm (Azar and Eyal-Giladi, 1981; Eyal-Giladi 1984). This role of the primitive endoderm can not be considered to play any role in the transplantation experiments because the early egg cylinder transferred on an ectopic site develops in a disorganized way, without any axis formation (see the next section). The fact that even in these experimental conditions the primitive ectoderm needs the support of the primitive endoderm before gastrulation in order to be able to develop autonomously later on, indicates that an interaction other than an induction of the primitive streak is in question. However, these are no arguments which could support any hypothesis on the nature of this influence.

Course of development and morphogenesis of the ectoderm as a renal isograft

In the course of normal gastrulation *in situ* in both the chick and mouse embryo, temporally and spatially coordinated movements of individual cells of the primitive ectoderm occur. These cells migrate in streams using the primitive streak and the Hensen's node as the passageway to their final destinations (Solursh and Revel, 1978). In the region of the primitive streak a disruption of the basement membrane occurs (Mitrani, 1982; Sanders, 1984) and cells migrate downwards by a mechanism analogous to the one at work during gastrulation in amphibia, *i.e.* by acquiring a bottle-like shape prior to the detachment (Tam and Meier, 1982). Finally, they incorporate into the definitive endoderm and mesoderm.

A coordinated movement of cells is not possible in a renal isograft. Moreover, the entire basal surface of the ectoderm is deprived of its basement membrane due to the previous digestion with enzymes. If the absence of the basement membrane is a prerequisite for epithelial cells to migrate downwards and to become mesenchyme-like, then, in renal isografts, the whole basal surface of the ectoderm could be considered as a potential analogue of the primitive streak. So it is. We traced the morphogenetic events in grafts of the pre-primitive streak- and the head fold-stage embryos at various intervals after transplantation (1,2,3,5,7 and 12 days, Svajger *et al.*, 1981, 1986). During the first 24 hours the basal surface of the ectoderm usually does not change (Fig. 9a). After 48 and 72 hours considerable changes occur. Most frequently they consist of the breaking up of a portion of the coherent epithelial structure of the ectoderm and its conversion into an unorganized, mesenchyme-like cell mass (Fig. 9 b,c). Subsequently rosette-like, cystic or tubular structures as well as compact clusters of cells form within this mesenchyme (Fig. 9 d,e). They probably represent rudiments of epithelial structures which will develop in teratomas. Another form of cell migration from the basal surface of the ectoderm occurred less frequently, usually when the ectoderm was isolated at the head fold stage. It is characterized by the protrusion of groups of closely packed cells, often in the form of tongue-like projections (Fig. 9 f), reminiscent of the initial migration of neural crest cells *in situ* (Morris and Thorogood, 1978).

Starting from the initial mesenchyme-like cell masses and epitheloid structures the differentiation of tissue components of the future teratomas continues during the next two weeks (Svajger *et al.*, 1986). Five days after transfer the graft is enlarged and vascularized. The initial formation of epidermis and myotubes occurs but the predominant component is the immature neural tissue with areas of massive cell necrosis. After 7 days the differentiation of the neural tissue and the epidermis is advanced and the first chondrogenic blastemas appear. Two days later the epidermis shows initial keratinization and hair bud formation and the first centers of ossification appear. Twelve days after transfer the lobules of the immature adipose tissue appear while other

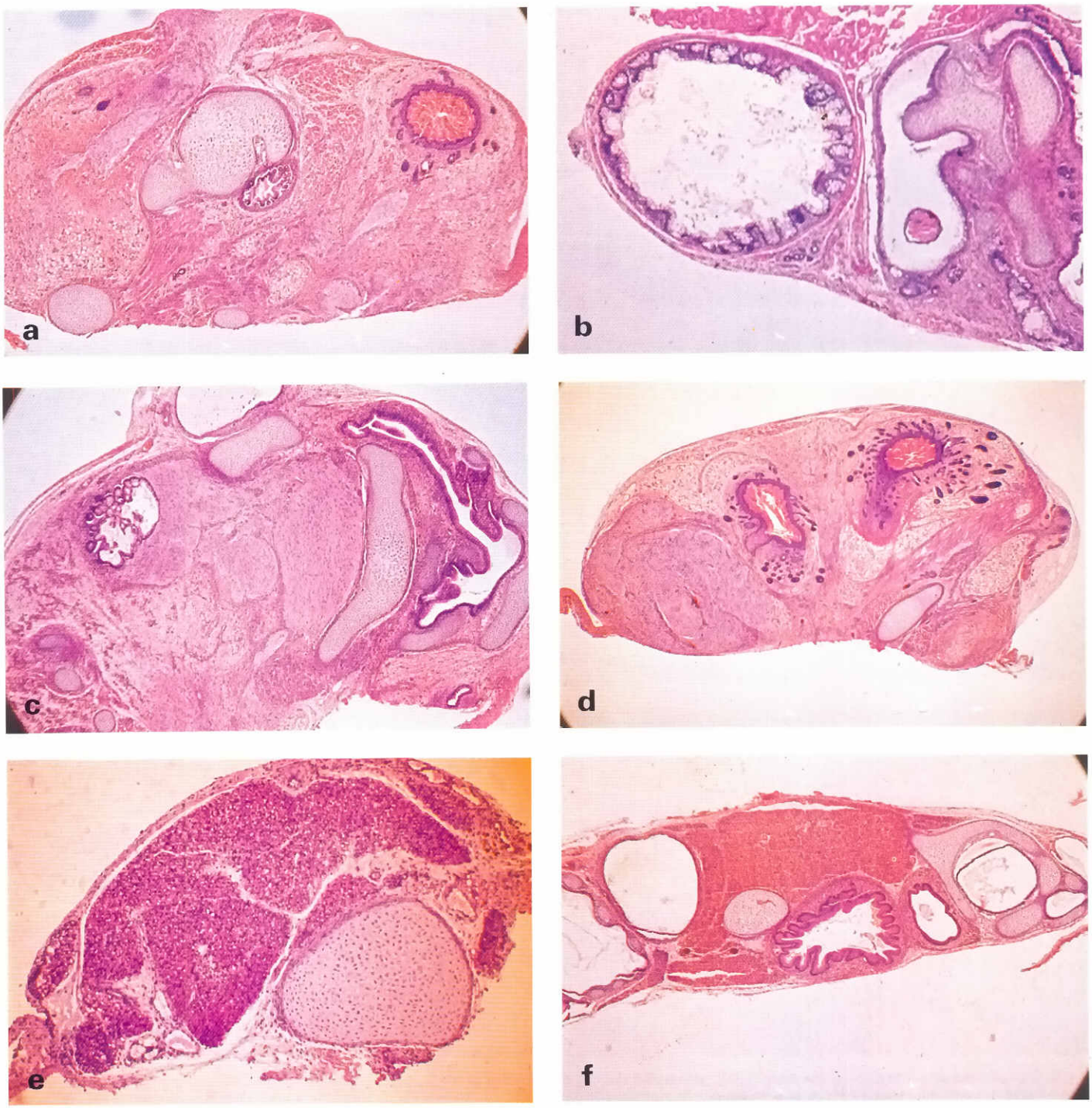

Fig. 8. Differentiation of germ layers in renal homografts. (a, b, c) *Ectoderm isolated at the pre-primitive streak and the early primitive streak stages, 15 days after transplantation. Tissue derivates of all three germ layers are present in teratomas: ectodermal (brain, epidermis), endodermal (respiratory tube, intestine) and mesodermal (cartilage, striated muscle).* **(d)** *Ectoderm isolated at the head fold stage, 20 days after transplantation. Only ectodermal (brain, epidermis with hairs) and mesodermal (cartilage, skeletal muscle, connective and adipose tissue) tissues are present.* **(e)** *Mesoderm isolated at the head fold stage, 15 days after transplantation. Brown adipose tissue and a nodule of cartilage.* **(f)** *Endoderm with the adjacent mesoderm isolated at the head fold stage, 15 days after transplantation. The tumor consists of the esophagus, cystically dilated respiratory tube, cartilage and brown adipose tissue. x15(d), x20(a,c), x25(b), x30(f), x50(e).*

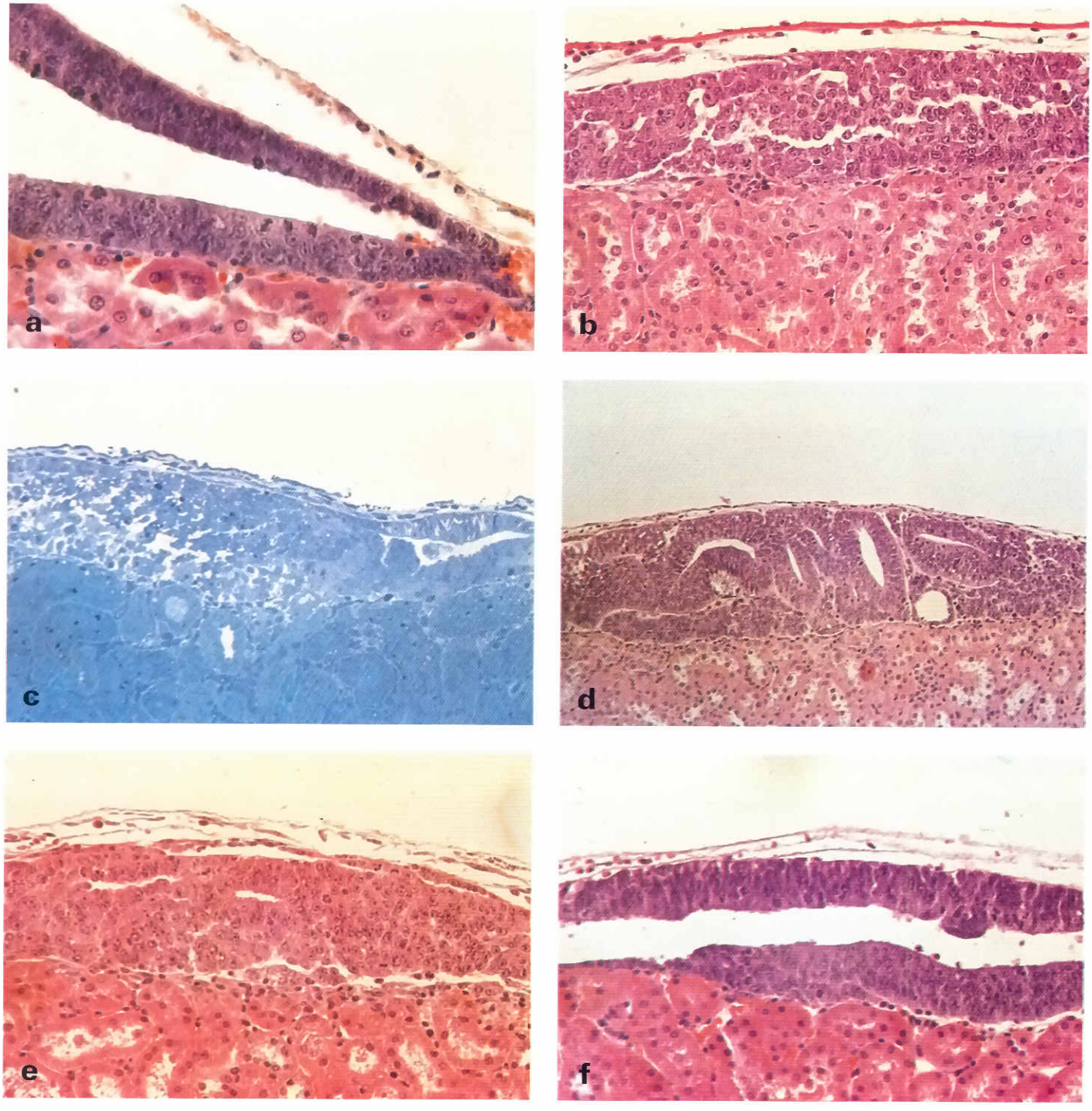

Fig. 9. Morphogenetic features of the embryonic ectoderm during the first three days after transplantation. *The ectoderm isolated at the pre-gastrulation* **(c)** *and at the head fold* **(a, b, d, e, f)** *stage, 12 hours (a), 1(f), 2(b),(c),(e) and 3 (d) days after transplantation. Note the breaking of the epithelium into a mesenchyme-like cell mass (b) (c), formation of cystic epithelial structures (d)(e), condensation of cells into clusters (e) and outgrowth of cells from the basal surface of the ectoderm adjacent to the kidney parenchyma (f). x20 (d), x25(b)(e), x50(a)(f), x120(f).*

tissues continue to develop (growth and maturation). These processes progress during the following days: the grafts enlarge, organotypic cell and tissue combinations occur and the complex structure of teratomas is established.

In analyzing the course of development and morphogenetic events which take place in renal grafts of the embryonic ectoderm many questions arise which are difficult or impossible to answer. Speculations about the mosaic or regulative type of development,

cell mixing cell population kinetics etc. (see Svajger *et al.*, 1981; Beddington, 1986) are beyond the scope of this review, especially if one keeps in mind that even for normal development *in situ* «we have practically no idea of what is really going on in cells of the blastoderm when they move, invaginate, induce or are induced, interact, become determined and begin to differentiate», that «we have no idea how positional signaling is accomplished or how cells record and remember their positional value» (Wolpert, 1969) and that the final composition of the tumor is largely dependent on the «survival of fittest cells in conditions that are never quite ideal» (Waymouth, 1974). One can only assert that the primary ectoderm of the pre-gastrulation rat embryo can give rise to properly differentiated tissues and even to elaborate organ-specific tissue associations without the involvement of coordinated morphogenetic movements of cells through the primitive streak. In connection with this fact the following is worth mentioning:

a) The breaking up of the embryonic epithelia to form mesenchyme occurs in various experimental conditions but also during normal development *in situ* (see Svajger *et al.*, 1986, p.6). In the mouse embryo small focal disruptions of the epithelial organization of the lateral ectoderm were observed at the gastrulation stage («polyinvagination», Poelmann, 1980), and later on, during neurulation, in areas of «surface ectodermal placodes». One could therefore assume that the entire ectoderm is able to deposit cells into the mesodermal compartment (Smits-van Prooie, 1986).

b) Supposing that the mesenchyme-like cells which arise by disruption of the ectoderm in renal isografts have been or are being committed in some way and at some time, then their involvement in the formation of various tissues and organ-like tissue associations is reminiscent of tissue-specific aggregation of cells in mixed cell suspensions *in vitro*. A similar mechanism is at work within the tail rudiment of the rat and mouse embryo where the secondary neural tube, the notochord and the tail gut develop from the mass of undifferentiated mesenchyme of the tail bud (Kostovic-Knezevic *et al.*, 1991, this issue).

Reliability of the method

The reliability of results obtained by grafting early embryos to an ectopic site is the crucial problem of the method. It can be divided into few categories: a) «cleanliness» of the separation of germ layers, b) damaging of the embryonic tissue during manipulation, c) cell loss after transplantation and d) influence of the host tissue upon graft.

«Cleanliness» of the separation of germ layers.

The differences in thickness, transparency and texture of particular layers permits an easy detection under the dissecting microscope of any «contamination» of one germ layer with the cells of the adjacent one. At the head fold stage the first step of the separation occurs spontaneously after the treatment with enzymes. Due to the inherent tendency of cells in the axial region of the ectoderm to rearrange their cytoskeleton (the onset of neurulation) the ectoderm begins to invaginate and partly detaches from the underlying mesoderm. The very thin embryonic endoderm shows the tendency to roll up over the mesoderm. The separation has only to be finalized by gentle manipulation with needles.

Damaging of the embryonic tissue

Although exposure to enzymes is to some extent harmful to the components and properties of the cell surface (Waymouth, 1974), the cells are able to restore their basement membranes and other pericellular materials (Osman and Ruch, 1981) and to retain a high percentage viability (Dziadek, 1981). In the light of evidence for rapid wound healing in rat embryos (Smedley and Stanisstreet, 1984) and of an efficient regulation of extensive random cell loss in the mouse embryo (Snow and Tam, 1979), the surgical trauma during separation and transfer is not likely to influence the developmental capacity of the ectoderm.

Cell loss after transplantation

It is very probable that some cells die during the first few days after transplantation while an optimal vascularization of the graft is not yet achieved. A massive cell death is obvious in the rapidly growing neural tissue 5 days after transplantation. However, this could only influence the final size of teratomas and the diversity of its tissue composition but not the results of an experimental series as a whole.

Influences of the host tissue

In all experiments which include the isolation of the embryo, its manipulation and transfer to an atypical site, the embryo is subject to various types of exogenous influences (Svajger *et al.*, 1986). In this connection, the most important seems to be the influence of the immediate environment within the host tissue. This influence can not be absolutely excluded but it is highly improbable that it is of a classical inductive type. Indirect evidence for this statement is the fact that a wide range of tissue differentiation is also achieved in other, differently structured host tissues such as the anterior chamber of the eye, the testis and the cheek pouch of the golden hamster. The great diversity of tissue differentiation and organotypic tissue association in teratomas is unlikely to be induced by the host tissue. The presence of tissue derivatives of particular germ layers varies regularly in relation to the original germ-layer composition of the graft. It is therefore most probable that the final composition of the graft mostly reflects their initial developmental capacities.

The influences of the host which are evident are non-specific and do not belong to the category of the embryonic induction in the usual sense of the word. These are: a) the influence of vascularization and adrenergic innervation on the differentiation of the brown adipose tissue and b) the influence of the male sex hormone of the host on the differentiation of the derivatives of the urogenital sinus (the prostate).

Concluding remarks

The method of separation of germ layers of the early postimplantation rodent embryo is a useful method for analyzing the essential events taking place during gastrulation. This is an indirect method which does not permit clonal analysis by tracing the migration and the ultimate fate of single cells. However, the main information obtained by using this method – that all the fetal primordia originate from cells which were localised in the primitive ectoderm before gastrulation – is consistent with the results obtained by more direct methods on the chick blastoderm (Vakaet, 1962; Rosenquist, 1966; Nicolet, 1971; Veini and Hara, 1975; Fontaine and Le Douarin, 1977; Stern and Canning, 1990). It is also consistent with the indirect or direct evidence of the ectodermal origin of the definitive endoderm in the mouse embryo obtained by various methods: a) the morphological observations of the continuity of the Hensen's node derived cells with the epithelium of the primitive gut (Jolly and Férester-Tadié, 1936; Jurand, 1974;

Poelmann, 1981), b) the injection of single primitive ectoderm cells into the blastocyst (Gardner and Rossant, 1979), c) tracing of the fate of labeled single ectodermal and endodermal cells (Beddington, 1983).

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