Investigation on the origin of the definitive endoderm in the rat embryo

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SUMMARY

Single germ layers (or combinations of two of them) were isolated from the primitive streak and the head-fold stage rat embryos and grown for 15 days under the kidney capsule of syngeneic adult animals. The resulting teratomas were examined histologically for the presence of mature tissues, with special emphasis on derivatives of the primitive gut.

Ectoderm isolated together with the initial mesodermal wings at the primitive streak stage gave rise to tissue derivatives of all three definitive germ layers. Derivatives of the primitive gut were regularly present in these grafts. At the head-fold stage, isolated ectoderm (including the region of the primitive streak) differentiated into ectodermal and mesodermal derivatives only.

Endoderm isolated at the primitive streak stage did not develop when grafted and was always completely resorbed. At the head-fold stage, however, definitive endoderm differentiated into derivatives of the primitive gut if grafted together with adjacent mesoderm.

These findings indirectly suggest the migration of prospective endodermal cells from the primitive ectoderm, and therefore a general analogy with the course of events during gastrulation in the chick blastoderm.

INTRODUCTION

The origin of endoderm is one of the most intricate problems in the embryology of vertebrates (see Peter (1941) for review of older literature). According to the classical view the endoderm of avian and mammalian embryos arises by delamination from the epiblast (‘Deuterendoderm’) and therefore differs in its origin from the ‘Protendoderm’ of the amphibian embryo, which arises as a consequence of gastrulation (Peter, 1941).

During the last four decades, however, this view has been subjected to substantial revision as far as the chick embryo is concerned. Various experimental techniques, including grafting of dissected parts of the blastoderm on to the chorioallantoic membrane (Rudnick, 1935; Hunt, 1937a) and labelling of selected areas of the epiblast with vital dyes or carbon particles (Hunt, 1937b; Fraser, 1954; Vakaet, 1962; Spratt & Haas, 1965) or with tritiated

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thymidine (Nicolet, 1965; Modak, 1966; Rosenquist, 1966; Nicolet, 1967, 1970; Gallera, 1972) have shown that prospective endodermal cells are localized in the epiblast at the primitive streak stage. These results indicate that the definitive endoderm of the avian embryo arises by morphogenetic movements of superficial cells, and therefore, contrary to the classical view, does not differ essentially in its origin from the endoderm of the amphibian embryo.

There are few experimental data on the origin of the definitive endoderm in mammalian embryos. Grobstein's experiments (1952) indicated that also in the mammalian embryo the primitive ectoderm contains prospective endodermal cells. He mechanically removed the outer cell layer (primitive endoderm) of mouse embryonic shields prior to the appearance of the head process (various egg-cylinder lengths and developmental stages), and grafted pre-cultured clusters of endoderm-deprived shields into the anterior chamber of the eye of adult mice for 30 days. Gut epithelium was present in many of the recovered grafts. The author concluded that 'the inner cell layer, or primitive ectoderm, of the mouse embryonic shield cannot be regarded as a germ layer with capacities sharply limited to ectodermal differentiation'.

Using a more reliable method for the separation of germ layers by pre-treatment with proteolytic enzymes (Levak-Švajger, Švajger & Škrej, 1969), we recently repeated Grobstein's experiment in a more precise form on rats (Levak-Švajger & Švajger, 1971). In renal homografts of ectoderm isolated at the pre-primitive streak stage (two-layered embryonic shield), different mature tissues, derivatives of all three germ layers, developed in most recovered grafts. Derivatives of the primitive gut were present in 14 out of 15 grafts. These results have confirmed Grobstein's conclusion.

The present paper deals with the differentiative capacities of rat embryo germ layers isolated during the next developmental stages: the primitive streak and the head-fold stage. It was hoped to obtain data on the origin of the definitive endoderm in the rat embryo.

**MATERIAL AND METHODS**

The inbred Fischer strain of albino rat was used in the experiments. Gestation was considered to have begun early in the morning when sperm was found in the vaginal smear. Twenty-four hours later the eggs were considered to be 1 day old.

Pregnant females were anaesthetized with ether on gestation days 8½ (at 3 p.m. on the 9th day) and 9 (at 8 a.m. on the 10th day). The entire egg-cylinders (embryonic shield + extra-embryonic membranes + ectoplacental cone) were isolated with watchmaker's forceps in sterile Tyrode's saline. Only egg-cylinders belonging to stages 13 and 15 (Nicholas, 1962) were selected for the experiment. These stages correspond to stages 12 and 14 of Witschi (see New, 1966).
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The egg-cylinders were treated as in our previous experiments with stage 12 egg-cylinders (Levak-Švajger & Švajger, 1971). The ectoplacental cone and the Reichert’s membrane were removed. The embryonic shields with their extra-embryonic parts were treated with a mixture of 0.5% trypsin (crystallized, lyophilized, Worthington) and 2.5% pancreatin (Difco) in calcium- and magnesium-free Tyrode’s saline at 4°C (Levak-Švajger et al., 1969). After 20–30 min enzymic reactions were stopped with Tyrode’s saline to which a few drops of rat serum were added. The egg-cylinders were then twice rinsed in pure saline and transferred to saline–rat serum mixture in which they were further manipulated with electrolytically sharpened and polished tungsten needles. First, a circular cut was made through the outer cell layer (endoderm) at the level of the amnion. The embryonic endoderm was then everted over the
FIGURE 2
Manipulation of the head-fold-stage egg-cylinder. (A–C) External aspect. (D–F) Cross-section. (See text.)
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underlying layers of the embryonic shield. The denuded, cup-shaped inner cell layer (ectoderm) was partially (about one-third of the surface) covered with mesoderm expanding bilaterally from the primitive streak. The embryonic ectoderm with the incomplete mesoderm was separated from the extra-embryonic part of the egg-cylinder by a transverse cut and grafted under the kidney capsule (series I). The endoderm was grafted as a separate series (II).

Treatment of stage 15 egg-cylinders (head-fold) (Fig. 2)

These embryos were treated in a somewhat different way from the previous ones. After isolation of the egg-cylinders from the uterus, the ectoplacental cone and Reichert’s membrane were removed. The embryonic shield was separated from the extra-embryonic part of the egg-cylinder by an oblique cut immediately below the insertion of the amnion. A second oblique cut was made through the entire wall of the embryonic shield, running from its free edge (near the posterior end of the primitive streak) to its top-end (the region of Hensen’s node). The originally cup-shaped embryo was thus transformed into an almost flat shield. Now the primitive streak and the neural groove were not continuous in line as they are in the chick blastoderm, but diverged from Hensen’s node forming a blunt angle. The embryonic shields were then treated with enzymes for 30 min and rinsed in saline–rat serum mixture as described before. During this treatment the ectoderm had detached spontaneously from the underlying mesoderm, except for the region of the primitive streak, where a complete separation was achieved with needles. The endoderm remained loosely adherent to the overlying mesoderm. Two grafts were prepared from each shield:

(a) The entire ectoderm including the region of the primitive streak (series III). The presence of the primitive streak, which contained cells with developmental capacities other than ectodermal, was presumed to assure the continuity of cell migration after grafting.

(b) The incomplete endoderm + mesoderm (series IV). The caudal (posterior) part of the endoderm–mesoderm combination, immediately underlying the primitive streak in situ, was cut off and discarded. The combined graft was thus restricted to the region in which there was no continuity with cells migrating through the primitive streak.

All operations were performed under a dissecting microscope, using magnifications of ×25–100.

Grafting procedure and examination of grafts

Each specimen prepared as described above was transferred by means of a braking pipette under the capsule of the right kidney of an adult male rat of the same strain, about 3 months old. Fifteen days following transfer the recipient animals were killed. The grafts were isolated from the host tissue, fixed in Zenker's fluid, embedded in paraffin wax, serially sectioned and stained
Table 1. Incidence of mature tissues in renal homografts of rat embryo germ layers

<table>
<thead>
<tr>
<th>Series ...</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage of the embryo (Nicholas) ...</td>
<td>13 (primitive streak)</td>
<td>15 (head-fold)</td>
<td></td>
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<tr>
<td>Type of graft ...</td>
<td>Ecto + meso</td>
<td>Endo</td>
<td>Ecto</td>
<td>Endo + meso</td>
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<tr>
<td>No. transferred</td>
<td>17</td>
<td>17</td>
<td>11</td>
<td>11</td>
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<tr>
<td>No. differentiated</td>
<td>15</td>
<td>—</td>
<td>11</td>
<td>10</td>
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<tr>
<td>Skin</td>
<td>—</td>
<td>—</td>
<td>11</td>
<td>—</td>
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<tr>
<td>Neural tissue</td>
<td>15</td>
<td>—</td>
<td>11</td>
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<tr>
<td>Derivatives of the primitive gut</td>
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<tr>
<td>Pharynx oesophagus</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>5</td>
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<tr>
<td>Respiratory tube</td>
<td>15</td>
<td>—</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Glands</td>
<td>14</td>
<td>—</td>
<td>—</td>
<td>8</td>
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<td>Thymus</td>
<td>2</td>
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<tr>
<td>Thyroid</td>
<td>3</td>
<td>—</td>
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<td>2</td>
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<tr>
<td>Intestine</td>
<td>8</td>
<td>—</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>—</td>
<td>2</td>
<td>10</td>
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<td>Adipose tissue</td>
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<td>White</td>
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<tr>
<td>Brown</td>
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<td>6</td>
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<td>6</td>
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<td>Heart</td>
<td>11</td>
<td>—</td>
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with hemalum and eosin. The sections were examined histologically for the presence of mature tissues, with special emphasis on derivatives of primitive gut.

RESULTS

The results of histological examination of grafts belonging to all four experimental series are listed in Table 1. The incidence of survival and differentiation into mature tissues was very high in series I, III and IV. On the contrary, all grafts of isolated endoderm (series II) were completely resorbed. All successful grafts were tumorous masses of different sizes, composed of mature tissues, with no signs of any immunological reaction. The general appearance of these grafts did not differ from those of whole embryonic shields transferred under the kidney capsule (Škreb, Švajger & Levak-Švajger, 1971). With only a few exceptions, the grafts were loosely adherent to both
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the kidney capsule and the kidney parenchyma. They were well vascularized from both sides.

With regard to their histological structure, the grafts were typical teratomas. Neural tissue, skeletal muscle, bone and cartilage were present in chaotic mixtures. On the other hand, epithelial tissues most frequently appeared in organotypic association with tissues of mesenchymal origin. Oesophageal and intestinal epithelia formed tubes or cysts surrounded by characteristic muscular layers, which sometimes contained small intra-mural ganglia. Ciliated columnar epithelium of the respiratory tube was closely associated with cartilage, which often formed a complete or incomplete annulus. Different epithelial appendages (hairs, sebaceous glands, serous or mucous glands, thyroid, thymus) were often found in continuity or in close proximity to their original surface epithelium. Cysts of different sizes, filled with an amorphous, probably mucous material, were a common finding. They were lined by simple, flattened epithelium, usually indefinable as to its origin.

The pattern of histological differentiation was different in each particular series of grafts. Only tissues of mesodermal origin were present in all three successful series of grafts (I, III and IV), and cartilage was the most constant representative.

With respect to the differentiation of ectodermal and endodermal tissues, the most prominent features were the following:

(a) Series I. Neural tissue and derivatives of the primitive gut (including foregut, midgut and hindgut) developed in all grafts (Figs. 3, 4). Epidermis and its derivatives were absent.

(b) Series III. Skin and neural tissue developed in all grafts (Figs. 5, 6). A rudimentary gut was present in only 2 out of 11 grafts.

(c) Series IV. The grafts completely lacked ectodermal derivatives. Endodermal epithelia were present in all grafts, but they were restricted to derivatives of the foregut (Figs. 7, 8).

DISCUSSION

Reliability of methods

The method which we have proposed for the separation of germ layers was a simple modification of the common enzymic technique for ‘splitting off’ of epithelia from the underlying mesenchyme at the level of the basement membrane (Levak-Švajger et al. 1969). A basement membrane exists between ectoderm and endoderm and between each of these and mesoderm in the two-layered and the three-layered mouse embryonic shield respectively (Pierce, 1966). Treatment of embryonic shields with enzymes results in a decrease of mutual adhesiveness of germ layers to such a degree that subsequent mechanical separation by needles can be accomplished with relative ease.

The treatment with enzymes of head-fold egg-cylinders (stage 15) which
Figs. 3 and 4. Details of the histological structure of teratomas obtained from ectoderm+mesoderm isolated at the primitive streak stage. ca, cartilage; he, heart muscle; in, intestine; ph, pharynx; re, respiratory tube; thy, thyroid.
have been transformed into flat shields brings about a spontaneous and ‘clean’ detachment of ectoderm from the underlying mesoderm. This is most probably due to the cell-intrinsic deformation tendency of neurectoderm which is involved in neural groove formation at this stage.

Prior to neurulation (stage 13), however, a spontaneous deformation and detachment of ectoderm did not occur and therefore no advantage could be taken of transforming the egg-cylinder into a flat shield before treating it with enzymes. The embryonic endoderm is very thin and transparent. Any defect in it which could arise during mechanical manipulation appears as a large hole. Any eventual ‘contamination’ of endoderm with adherent mesodermal cells could be easily detected by careful inspection during manipulation.

It has been previously demonstrated that, after transfer under the kidney capsule, the rat embryonic shields are in an environment which is suitable for their further growth and histological differentiation (Skreb et al. 1971). The great diversity of differentiation and organ-specific association of tissues in the resulting teratomas is unlikely to have been non-specifically induced by the host tissue. The results of the present experiment suggest that migration of cells through the primitive streak and Hensen’s node continues after transfer of the ectoderm under the kidney capsule. However, because these cell movements occur in an environment which is very different spatially, cell groups establish contacts and inductive interactions by chance rather than by topographically and chronologically ordered displacements.

In our present experiment, different tissue derivatives, representative of particular definitive germ layers, frequently developed in grafts. Their presence varied regularly in relation to the original germ-layer composition of the graft. It is therefore very probable that the final histological composition of the grafts could be regarded as an expression of the developmental capacities of the embryonic material at the moment of transfer.

In comparison with the chick blastoderm explanted in vitro, the rat embryonic shield transplanted under the kidney capsule is subjected to much greater environmental changes, and therefore greater disturbance of normal development is to be expected. However, when areas of the chick neurectoderm and mesoderm were cultivated as intra-coelomic grafts (i.e. in environmental conditions similar to those in rat renal homografts), they formed neural structures showing regional characteristics which were generally in accordance with the prospective significance of the excised areas (Hara, 1961). Bearing this in mind, one may postulate that the developmental capacities realized in renal homografts of isolated rat embryo germ layers correspond at least roughly to their developmental significance in normal intra-uterine development.

*Developmental capacities of the ectoderm at the primitive streak stage*

At this stage ectoderm was isolated and grafted together with incipient mesodermal wings. A complete removal of this ‘pioneer’ mesoderm is not
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possible. Moreover, the removed mesoderm would have been replaced very soon after grafting by newly migrated cells. At this stage migration of cells through the primitive streak has just begun. The presence of the primitive streak and the incipient mesodermal wings explains the differentiation of mesodermal derivatives in these grafts.

It remains to explain the presence of the endodermal epithelial formations (gut and its derivatives) in these grafts, as well as in those of isolated pre-primitive streak ectoderm (Levak-Švajger & Švajger, 1971). The alternatives put forward by Grobstein (1952) are: (a) the presence of prospective endodermal cells within the ectoderm, (b) the regenerative capacity of the ectoderm (see also Rudnick, 1935), and (c) the full plasticity of the ectoderm at this stage. There is no decisive support for any of these alternatives. If we favour the first one, i.e. an ectodermal origin of the definitive embryonic endoderm, this is mainly by analogy with the mechanism of endoderm formation in the chick blastoderm as demonstrated by Nicolet (1967, 1970). Experimental evidence already suggests that primary induction involves the same type of embryonic tissue interaction in birds and in mammals (Waddington, 1936; Törö, 1938). On the other hand, it is possible that the mechanism of the germ-layer formation could be modified in some details as an adaptation to the entypic development of the mouse and rat embryo (Bonnevie, 1950).

The idea of the ectodermal contribution to the definitive endoderm in the mouse and rat embryo is not new. Sobotta (1903) asserts that the mouse embryonic ectoderm before gastrulation ‘nicht allein Ektoderm ist sondern dass es Teilen aller drei Keimblätter den Ursprung gibt, dem gesamten Ektoderm und Mesoderm und Teilen des Entoderms’. It was suggested that the head process contributed to the lining of the gut as it seemed to be continuous with the endoderm on histological sections through the mouse embryonic shield (Jolly & Férester-Tadié, 1936; Snell & Stevens, 1966). Consistent with this view is the designation of the ectoderm before gastrulation as the primary (Widakowich, 1910; Huber, 1915) or primitive ectoderm (Grobstein, 1952) in contrast to the secondary or definitive ectoderm after gastrulation.

Some previous results from our laboratory strongly suggest a remarkable change in the ultrastructural and enzymic pattern of the rat and mouse embryonic endodermal cells after mesoderm formation. At the two-layered stage, both embryonic and extra-embryonic endoderm are composed of cuboidal cells with abundant microvilli, pinocytotic vacuoles and lysosomes, and a high

Figures 5–8

Details of the histological structure of teratomas obtained from ectoderm (Figs. 5, 6) and endoderm + mesoderm (Figs. 7, 8) isolated at the head-fold stage. b.a.t., Brown adipose tissue; br, brain; ca, cartilage; chp, choroid plexus; oe, oesophagus; re, respiratory tube; s, skin.
activity of acid phosphatase and nonspecific esterase. After mesoderm formation these characteristics become restricted to the extra-embryonic endoderm, whose cells also show the longest phase of DNA synthesis. The cells of the embryonic endoderm are at this stage flattened, depleted of microvilli, vacuoles and lysosomes, and devoid of hydrolytic enzyme activity (Rodé, Damjanov & Škreb, 1968; Solter, Damjanov & Škreb, 1970, 1973; Solter, Škreb & Damjanov, 1971). These findings suggest a highly developed absorptive (i.e. nutritive) function of the entire endoderm before gastrulation. After gastrulation, the differentiated absorptive cells are replaced by (or have dedifferentiated into?) the flattened cells destined to differentiate into gut epithelium.

The present results indicate that the histogenetic capacity of ectoderm with incipient mesoderm at the primitive streak stage does not differ significantly from the capacity of ectoderm alone, isolated at the pre-primitive streak stage (Levak-Švajger & Švajger, 1971). The prospective endodermal cells supposedly either still reside within the ectodermal layer, or are just leaving it through the primitive streak.

The surprising fact that skin did not develop in grafts of ectoderm taken from the pre-primitive streak and the primitive streak stages (Levak-Švajger & Švajger, 1971, and the present experiment) is not relevant to the problem of endoderm formation and therefore will be discussed elsewhere.

**Developmental capacities of the ectoderm at the head-fold stage**

It has been shown that in the chick blastoderm, the migration of the prospective endodermal cells starts very early in development (at the short primitive streak stage) and occurs in the anterior part of the primitive streak. As soon as the head process appears, this region gives rise to somite mesoderm only (Nicolet, 1967). Similarly, in our experiment ectoderm isolated and grafted at the head-fold stage gives rise only to mesodermal and ectodermal tissues. The presence of gut in 2 out of 15 grafts may be ascribed either to the presence of some residual prospective endodermal cells within the primitive streak at the moment of grafting, or to the unsuccessful removal of the endoderm in these specimens.

**Developmental capacities of the endoderm**

The failure of endoderm isolated at the pre-primitive streak (Levak-Švajger & Švajger, 1971) and at the primitive streak stage (present experiment) to differentiate as renal homografts is not surprising. Even at the head-fold stage isolated endoderm does not differentiate but is completely resorbed after transfer under the kidney capsule (Levak-Švajger et al. 1969). At the same developmental stage, endoderm differentiates into typical endodermal epithelia if grafted together with adherent mesoderm (present experiment). Obviously
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an appropriate epithelio-mesenchymal interaction is needed for histological
differentiation of endodermal organs.

Unfortunately, any information on the developmental capacities of the
primary (pre-gastrulation) embryonic endoderm is lacking. This problem might
be approached by grafting pre-gastrulation or early gastrulation endoderm
in combination with post-gastrulation mesoderm. But this could not be regarded
as a decisive experiment, for it has been shown that yolk-sac endoderm of the
13- to 14-day-old rat embryo could differentiate into intestine-like structures
and other mature tissues when grafted into the mother’s omentum (Payne &
Payne, 1961).

The absence of intestine in series IV grafts could be explained by the removal
of the posterior part of the endoderm (underlying the primitive streak) prior
to transplantation.

Conclusion: The possible mechanism of formation
of the definitive endoderm

Neither the present investigation nor the previous one (Levak-Svajger &
Svajger, 1971) have provided direct evidence of an ectodermal origin of the
definitive endoderm in the rat embryo. However, the results of our experiments
strongly suggest an analogy with the course of events during gastrulation in
the chick blastoderm. In the pre-primitive streak stage, the inner cell layer
(the primary or primitive ectoderm) seems to contain prospective cells of all
three definitive germ layers. During the primitive streak stage, the prospective
endodermal and mesodermal cells migrate from the primary ectoderm and
form the definitive endoderm (or at least a part of it) and mesoderm. At the
head-fold stage the ectoderm already lacks prospective endodermal cells, but
it still contains some prospective mesodermal cells, which are able to leave
their original position and to differentiate into mesodermal tissues. At this
stage, endoderm is already formed as a definitive germ layer able to differentiate
into gut and its derivatives if combined with mesoderm. The ectoderm as a
whole does not become the definitive germ layer until after the end of
migration of the mesodermal cells through the primitive streak. This is in
agreement with the claim that ‘it is only legitimate to speak of the separate
germ layers when their segregation from one another is complete’ (De Beer,
1958).

ZUSAMMENFASSUNG

Isolierte Keimblätter (oder Kombinationen zweier von ihnen) von Rattenembryonen auf
den Stadien des Primitivstreifens und des Kopffortsatzes wurden 15 Tage unter der Nieren-
kapsel erwachsender syngegetischer Tiere gezüchtet. Die resultierenden Teratome wurden
histologisch untersucht, mit besonderer Berücksichtigung der reifen Geweben endodermaler
Herkunft.

Das auf dem Stadium des Primitivstreifens zusammen mit der Anlage des Mesoderms isolierte Ektoderm differenzierte sich in die Derivate aller dreien Keimblätter. Die
verschiedenen Abschnitte des Primitivdarms waren in diesen Transplantaten ein regelmässiger Befund.

Auf dem Stadium des Kopffortsatzes, dagegen, differenzierte sich das Ektoderm nur in die ektodermalen und mesodermalen Geweben.


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