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Commentary

Commentary on paper by Leroy C. Stevens (1960)

In 1953, Leroy C. Stevens, a young scientist from the Jackson Laboratory at Bar Harbor Maine, set out to examine the deleterious effect of cigarette paper on mice under the auspices of a grant supported by the tobacco industry. During the course of his research, he noticed that some mice from the 129 inbred strain developed large testicular tumors. Rather than discarding these mice, he bred them and selected a strain (129^{Sv}) with a high incidence of tumors that displayed a large realm of differentiated tissue such as teeth, hairs, muscles or endothelial structures, resembling classical human ovarian teratomas (Stevens and Little, 1954). After discovering that these tumors were of germ cells origin, several colleagues including Barry Pierce from the University of Colorado in Denver, started to transplant pieces of such tumors back into the animal to see for how long these germ cells tumors could be maintained and the diversity of cell types they could form. By transplanting back into the murine peritoneum, Pierce and Stevens obtained fluid ascitic tumors which contained thousands of free-floating structures that strikingly resembled day 5 or 6 mouse embryos, both in term of the appearance of cellular layers and their organization.

In this classic paper published in *Developmental Biology* in 1960 (Stevens, 1960), Stevens tests the developmental capacities of these embryonic structures and compares their morphogenetic potential to that of the embryos themselves. After isolating such embryo-like structures from ascites tumors, he transplanted them singly into the anterior chamber of a mouse eye. A tumor grew, which was isolated, minced and re-transplanted sub-cutaneously. The grafts were observed daily, '*except Sunday*', as specified in the paper. In one graft, Stevens noted a particularly well-developed embryo-like object, which had generated structures ''*unmistakably similar to portions of normal embryo of about 9 days*'', with neuroepithelial cells, amnion, yolk sac epithelium and mesodermal cells. In the discussion, Stevens explains that Pierce and Dixon had previously observed the presence, in ascites tumors, of a central core of embryonal carcinoma (EC) cells surrounded by a layer of visceral yolk sac, which they had referred to as 'granules' (Pierce and Dixon, 1959). In 1959, Stevens further analyzed and described these granules and called them teratomatous '*embryoid bodies*', a well inspired name that survived 60 years of highly competitive research. In the conclusion of the attached 1960 publication, Stevens writes: '*..the embryoid bodies derived from the testicular teratomas of strain 129 mice have similarities in embryonic potency as well as in morphology to normal mouse embryos*'. This seminal finding opened the route towards using EC cells, rapidly replaced by ES cells, to engineer modified mice *via* blastocyst injection (Dewey et al., 1977).

I had the privilege of meeting Leroy Stevens a few times. In 1985, my late friend Peter Hoppe from the Jackson laboratory, a close colleague of Leroy Stevens and one of the few scientists who could successfully and reproducibly cultivate fertilized mouse embryos up to the blastocyst stage in the late 1970's, took me to Leroy's house, which was literally built into the trees in Mount Desert island, Maine. Leroy offered us several drinks and told us about his regrets not to have been more closely associated to all the work that had followed his discovery, yet without any bitterness or resentment. He was a kind, civilized and modest person. His contributions to the emergence of ES cells and, consequently, to the experimental and conceptual revolution that followed, were immense. The surprising and beautiful self-organizing potential of the embryoid bodies Stevens and Pierce described in the 1950s is now being resurrected by the increasing interest of the community of developmental and stem cells biologists to engineer *in vitro* and study various types of pseudo-embryos showing similar self-organizing properties.

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DEVELOPMENTAL BIOLOGY, 2, 285–297 (1960)

Embryonic Potency of Embryoid Bodies Derived from a Transplantable Testicular Teratoma of the Mouse[1]

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Structures morphologically similar to early embryos were observed in human testicular teratomas by Peyron in 1939. Since then, many other workers have recognized embryoid bodies in teratomas of the human testis and ovary (Friedman and Moore, 1946; Dixon and Moore, 1953; Melicow, 1955; Masson, 1956; Simard, 1957; Gaillard, 1955–1958; Cabanne, 1957; and Evans, 1957). These structures, like human blastocysts of about 13–18 days, are composed of ectodermal and endodermal vesicles with mesodermal cells between them. Many are incompletely and irregularly developed; some, however, are remarkably similar to normal embryos. Peyron offered one of his tumors as an atlas of presomite human embryology that was more complete than any other source at that time. Teratomatous embryoid bodies are considered by some to be homologous to early embryos; however, prominent students of germinal tumors have rejected this assumption (Nicholson, 1929; Willis, 1953).

Testicular teratomas occur relatively frequently in inbred strain 129 mice (Stevens and Little, 1954; Stevens and Hummel, 1957). A developmental study (Stevens, 1959) of these primary testicular teratomas of the mouse has shown that the earliest tumors observed (in newborns) are composed of a nest of undifferentiated embryonal cells with ectopic germ cells interspersed. In other tumors of newborns, epithelial vesicles enclose pools of blood and products of cellular degeneration. The epithelium rapidly becomes transformed into two different types. One resembles ectoderm, and the other endoderm.

[1] This investigation was supported by research grant C–2662 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by a grant, E–121, from the American Cancer Society, Inc.

Mesenchymal cells located between the epithelial vesicles resemble embryonic mesoderm. Examination of primary tumors in progressively older mice revealed that the ectoderm-appearing epithelium gives rise to neural tissue; the endoderm-appearing epithelium to respiratory, alimentary, and glandular epithelium; and the mesenchyme to muscle, cartilage, bone, marrow, and adipose tissue. This is interpreted as a demonstration of the formation of germ layers in testicular teratomas.

Teratomatous embryoid bodies of the mouse were first observed in a retroperitoneal metastatic growth of testicular origin (Stevens, 1959). They exhibited the inversion of the primary germ layers typical of early mouse embryos and were composed of two layers of epithelia: the outer resembling endoderm; the inner, ectoderm. When a highly pleomorphic transplantable testicular teratoma of the mouse (402A VI) is maintained as an ascites tumor, thousands of free-floating embryoid bodies similar to mouse embryos 5 and 6 days of age (Figs. 1–3) are contained in the peritoneal fluid (Stevens, 1959). They are composed of a layer of endoderm which invests morphologically undifferentiated embryonal cells. The morphologically undifferentiated cells may assume an epithelial arrangement resembling ectoderm (Fig. 4), or they may exist as a compact mass within the enveloping endoderm.

The aim of this investigation was to test the developmental capacity of these embryoid bodies. It will be shown that like normal embryos they give rise to a wide variety of tissues. It is concluded that embryoid bodies derived from a transplantable testicular teratoma of the mouse are similar in histogenetic potency as well as in morphology to normal mouse embryos.

MATERIALS AND METHODS

Two sublines (402A III and 402A VI) of a transplantable teratoma derived from the testis of a strain 129 mouse were employed. These tumors have been maintained by serial transplantation for

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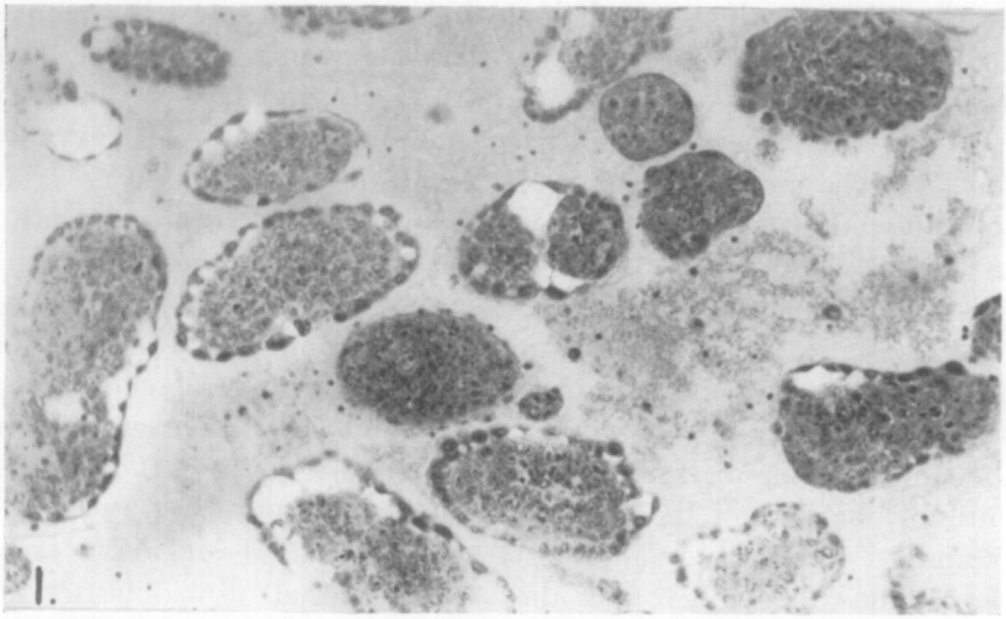
FIG. 1. Free-floating embryoid bodies in ascitic fluid. Magnification: × 150.
FIG. 2. Normal 5-day-old mouse embryo (× 150).
FIG. 3. Normal 6-day-old mouse embryo (× 150).
FIG. 4. Embryoid body showing ectodermal epithelium formation (arrow) (× 350).
FIG. 5. Free-floating embryoid bodies in ascitic fluid (× 35).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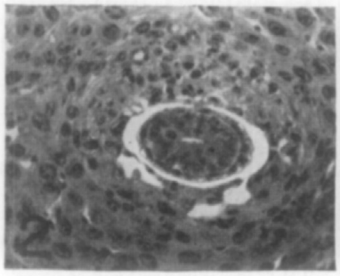
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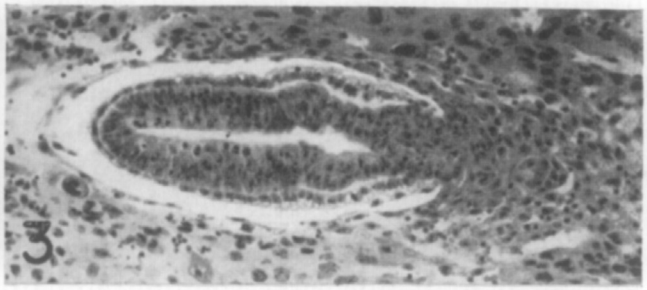
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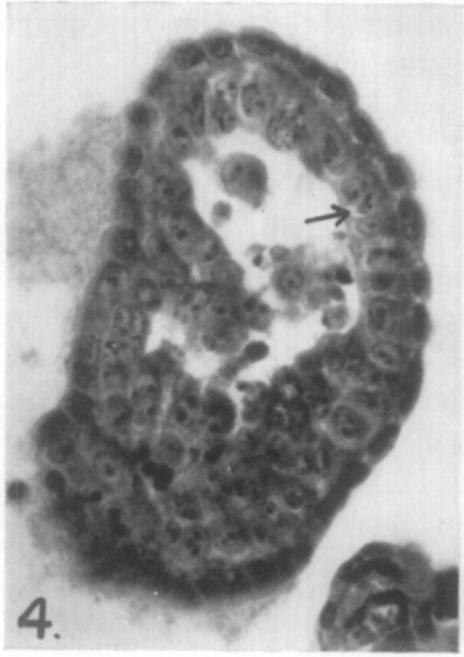
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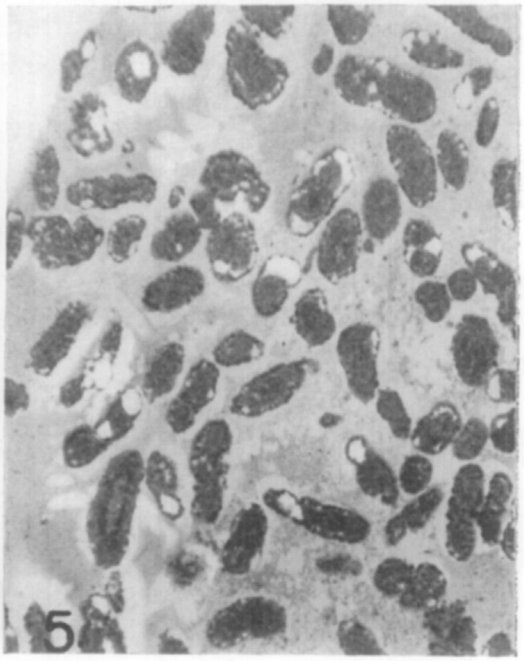
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nearly six years; their morphological characteristics have been previously described (Stevens, 1958). The behavior of 402A VI as an ascitic tumor has been investigated by Pierce and Dixon (1959a, b).

Solid tumors were homogenized in saline and injected intraperitoneally. The ascitic fluid resulting from these injections was serially transplanted. The tumor 402A VI was used at the twenty-seventh to thirty-first transplant generations as ascites tumors, and 402A III at the ninth. Thousands of free-floating embryoid bodies, homogeneous in size (Fig. 5), were observed in the ascitic fluid. They were washed in tissue culture medium, and, with the aid of a stereoscopic microscope and a micropipette, single embryoid bodies were grafted into the anterior chambers of the eyes of mature strain 129 mice. The anterior chamber of the eye was used as the transplant site since it permitted direct observation of the grafts during and after operations. Since the embryoid bodies are composed of neoplastic cells, the resulting growths were large enough to be minced and grafted subcutaneously via trocar. Transplantable tumor sublines were established from the intraocular grafts.

Sixteen mice received grafts of single embryoid bodies from the same donor. The intraocular grafts were observed daily, except Sunday, with the aid of a stereoscopic microscope.

Of 68 intraocular grafts, 49 were recovered. Twenty-five of these were fixed in Vandegrift's solution and prepared for histological examination at various intervals after grafting, and 24 were transplanted subcutaneously to strain 129 weaning-age animals.

OBSERVATIONS

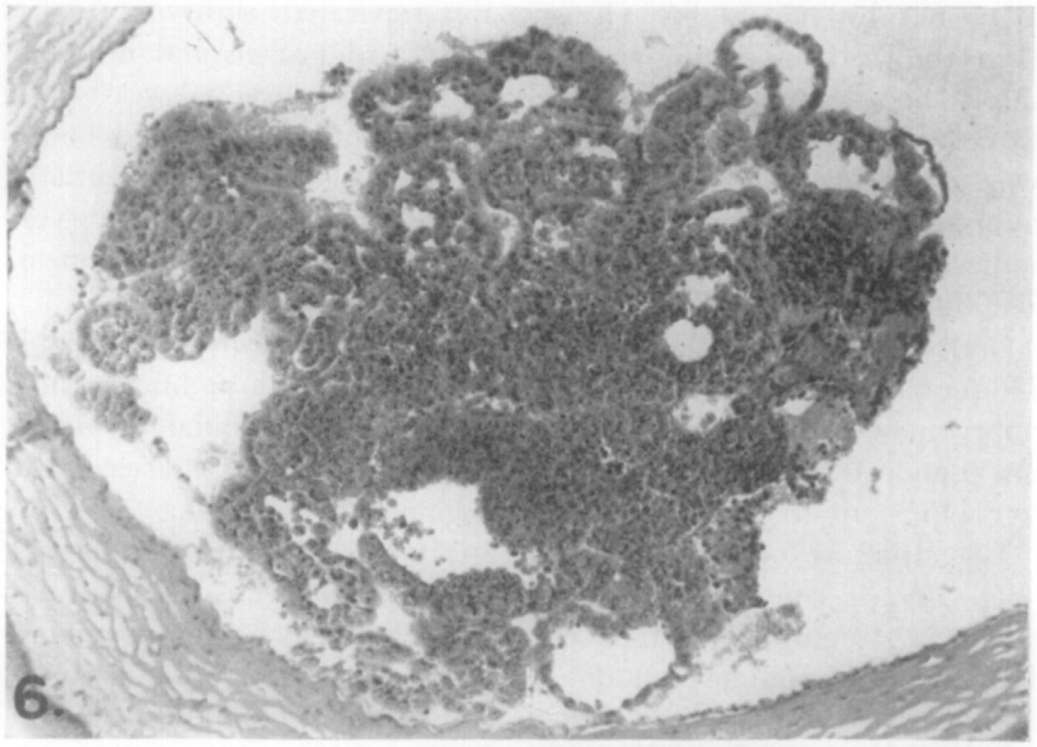
A. Intraocular Grafts of Single Embryoid Bodies

During the first week, the grafts enlarged, became vascularized, and were composed of both cystic and solid portions (Fig. 6). The solid portions of the growths were composed mainly of undifferentiated embryonal cells.

In many cases as the embryoid bodies enlarged, constrictions appeared that resulted in the formation of several embryoid bodies

FIG. 6. Four-day-old intraocular growth resulting from a single embryoid body. Magnification: × 120.

FIGS. 7 and 8. Branches or buds of growths derived from grafts of single embryoid bodies (× 160).



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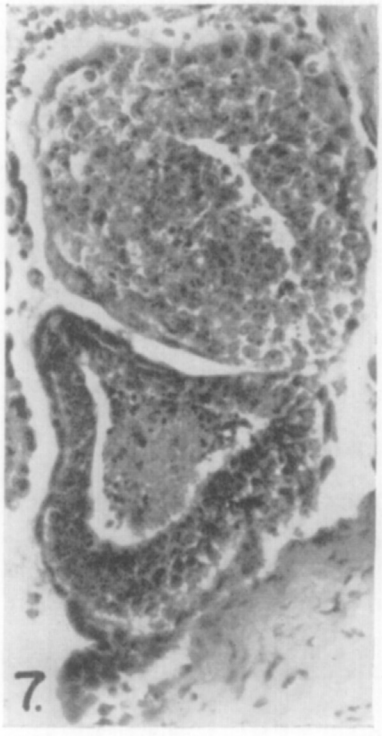

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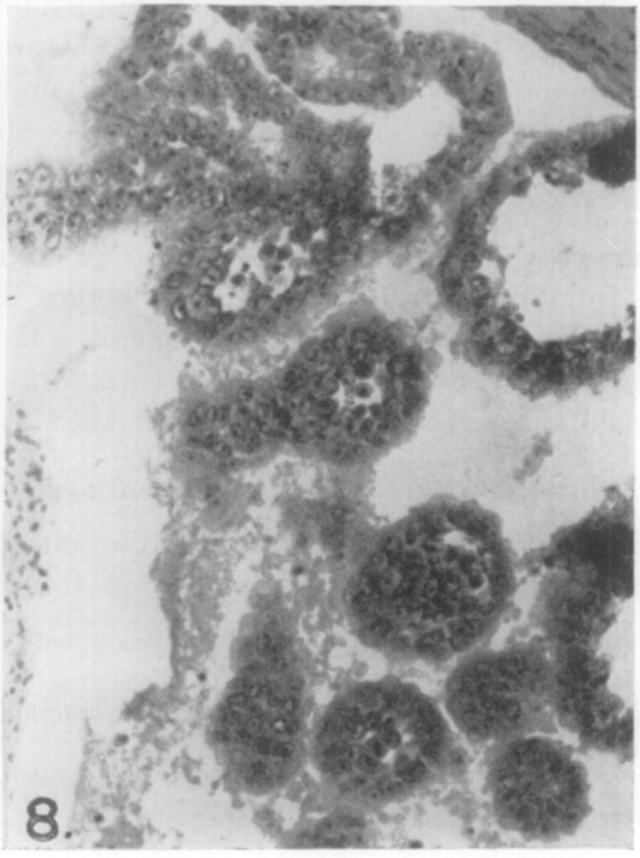

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connected by thin stalks. Occasionally separated embryoid bodies were observed, indicating that the stalks had broken and new embryoid bodies similar in size and shape to the original grafts had formed. Histologically the buds or branches of the embryoid bodies were composed of endodermal epithelium enclosing undifferentiated embryonal cells (Figs. 7, 8). Frequently, intraperitoneal embryoid bodies showed constrictions indicating fission (Fig. 9). It appears that the embryoid bodies can reproduce by budding.

Grafts fixed during the second to fourth weeks were more highly differentiated than younger grafts. They contained undifferentiated embryonal cells, primary ectoderm and endoderm, cuboidal, columnar, and pseudostratified ciliated epithelia, neuroepithelium, muscle, cartilage (Figs. 10–15), and trophoblastic giant cells.

The structure of one intraocular graft was of particular interest (Fig. 16). It contained several formations that were unmistakably similar in structure to portions of normal embryos of approximately 9 days. These formations contained folded neuroepithelium continuous laterally with flattened to cuboidal epithelium resembling amnion, and were enclosed by yolk sac epithelium. Mesodermal cells occupied the area between the folded neuroepithelium and yolk sac epithelium, giving the appearance of condensations of mesenchymal cells. An epithelium reminiscent of coelomic epithelium was also represented, but positive identification is not claimed. Figures 16 and 17 illustrate the similarity of these embryoid formations to the posterior region of a normal 9-day mouse embryo.

Intraocular grafts of embryoid bodies derived from subline 402A III were not as highly differentiated as those from subline 402A VI, reflecting a corresponding difference in morphology of these tumors maintained in solid form (Stevens, 1958).

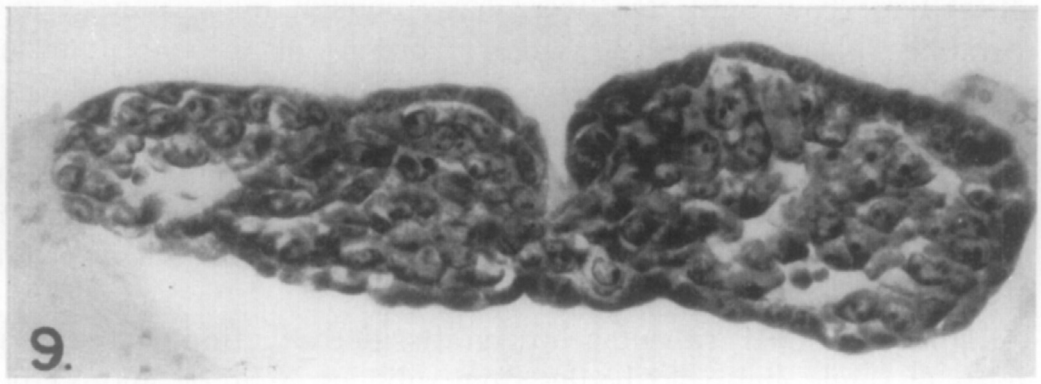
B. Subcutaneous Transplants of Intraocular Grafts

Twenty-four embryoid bodies from one donor (3008) were grafted singly into the anterior chamber of the eye, and 16 of these were

FIG. 9. Intraperitoneal embryoid body with constriction suggesting fission. Magnification: × 350.

FIG. 10. Twenty-three-day growth in anterior chamber of eye derived from single embryoid body (× 35).

FIG. 11. Early ectodermal (*EC*) and endodermal (*EN*) vesicles in graft derived from single embryoid body (× 160).



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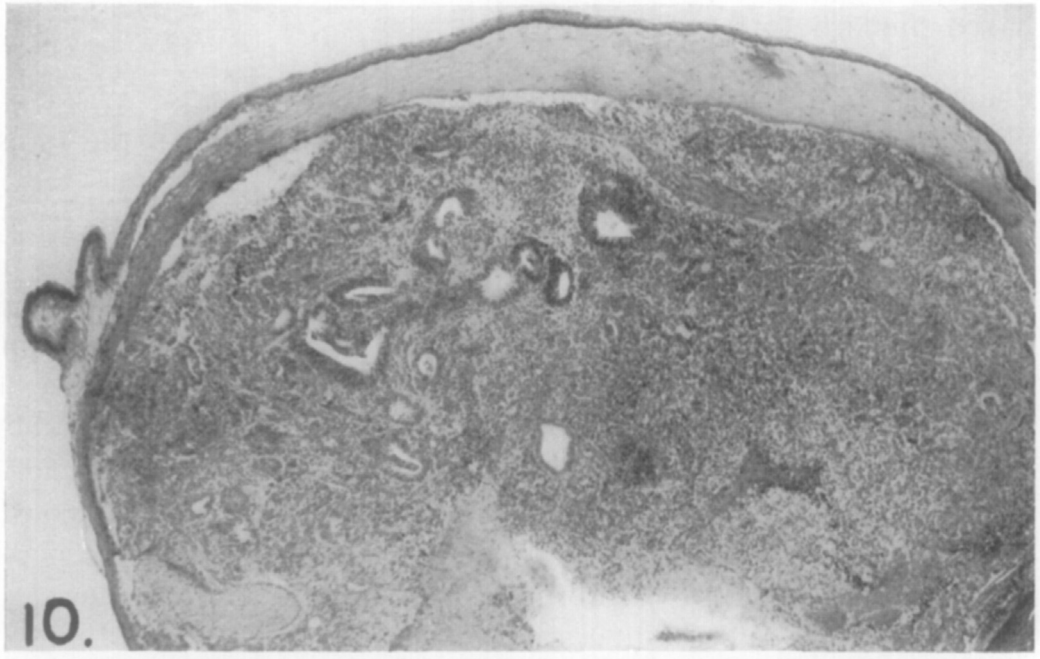

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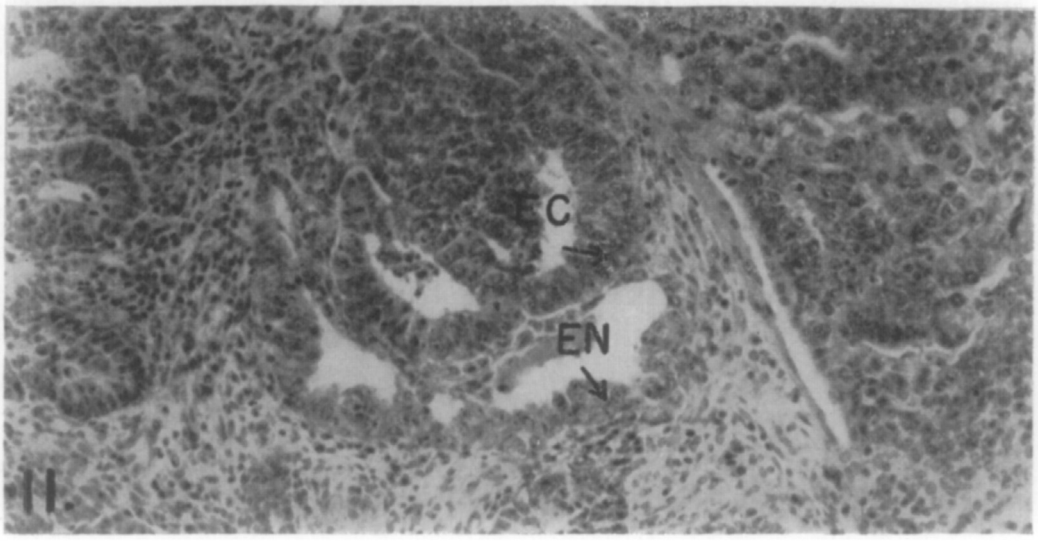

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allowed to grow until they were large enough to be minced and regrafted subcutaneously via trocar. Portions of the subcutaneous grafts were fixed and prepared for histological examination after approximately 3 weeks' growth, and other portions were retransplanted and maintained as sublines of the original tumor. The subcutaneous grafts were similar to each other in histologic composition, containing both immature and adult tissues including neural tissue, epithelia, muscle, and cartilage.

Eight additional subcutaneous grafts derived from intraocular grafts of single embryoid bodies from sublines 402A III and VI behaved similarly to the grafts described above.

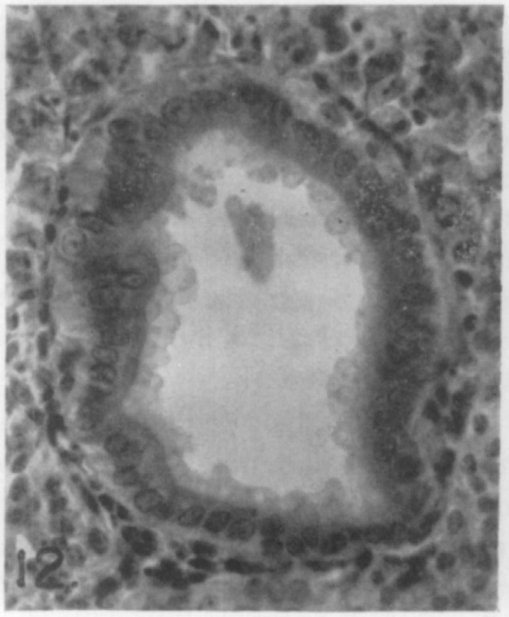
DISCUSSION

Pierce and Dixon (1959a, b) described the behavior of the transplantable testicular teratoma (402A VI) of strain 129 origin during many transplant generations as an ascitic tumor. During the first five transplant generations, numerous free-floating large cysts ranging up to 7 mm in diameter were observed in the ascitic fluid. They were composed of visceral yolk sac cells, mesenchymal cells, hematopoietic and immature nucleated red blood cells, embryonic epithelial cells often resembling neural tubes, early neuroglia and squamous cells. We have also observed these cysts and have found muscle in addition. Pierce and Dixon (1959a) transplanted the cysts subcutaneously and observed their histogenetic capacities. They suggested the possibility that the cysts are "fetiform derivatives of teratocarcinoma."

In addition to these large cysts, Pierce and Dixon described minute "granules" containing a central core of embryonal carcinoma invested with a layer of visceral yolk sac. We have designated these "granules" as embryoid bodies (Stevens, 1959), the subject of this article.

Pierce and Dixon (1959a) traced logical transitions from embryoid bodies (granules) to the large cysts, indicating a common origin. We have confirmed Pierce and Dixon's finding that large cysts, formed during the first few transplant generations fail to appear in later generations, even though enormous numbers of embryoid bodies are present. The embryoid bodies referred to in this article were derived from late transplant generations, when the cysts no longer develop.

FIGS. 12–15. Ciliated epithelium, neuroepithelium, cardiac muscle, and cartilage derived from intraocular graft of single embryoid body, respectively.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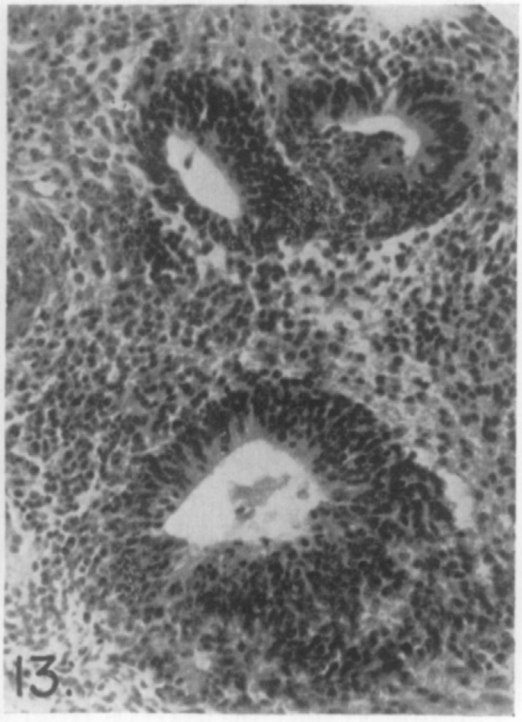
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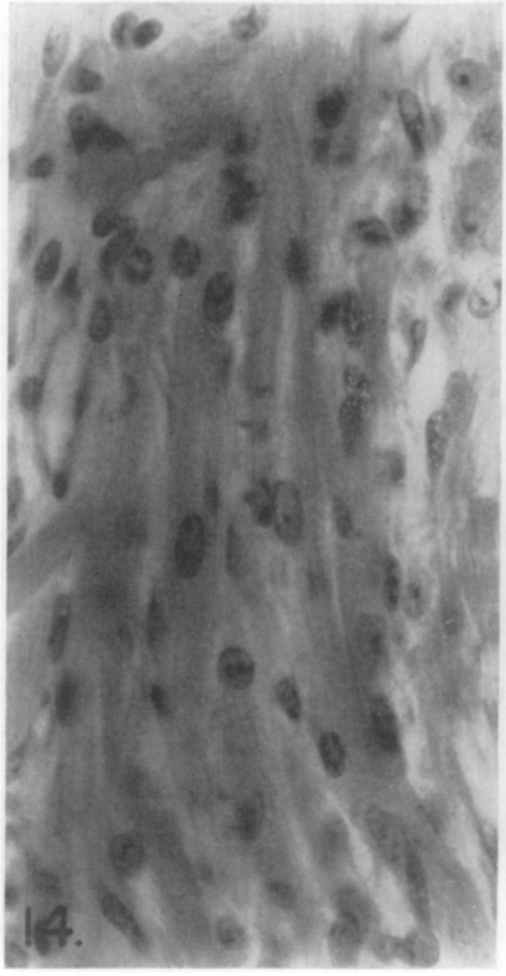
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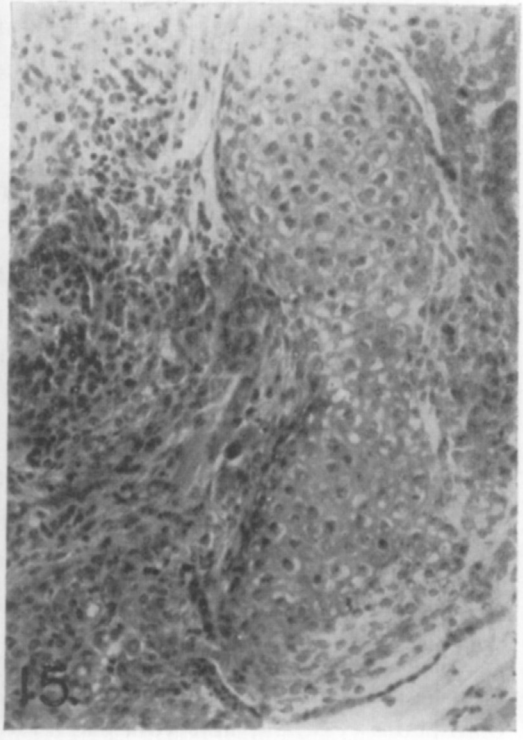
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The embryoid bodies used in the experiments described here bear an unmistakable morphological resemblance to normal mouse embryos 5–6 days of gestation. They are uniform in size (0.1–0.2 mm in diameter) and structure, being composed of endodermal cells and other primitive embryonal cells having ectodermal and mesodermal potencies. When grafted singly into the anterior chamber of the eyes of mature mice, they develop into large growths composed of a variety of tissues. These results are in accord with recent studies on early primary teratomas of strain 129 mice which demonstrated the formation and subsequent differentiation of the three germ layers in these tumors (Stevens, 1959). Furthermore, they demonstrate that the embryoid bodies derived from the transplantable teratomas in ascitic form are similar in embryonic potency as well as morphology to normal mouse embryos. It is stressed that these embryoid bodies are composed of truly neoplastic cells, since they give rise to tumors that grow progressively when serially transplanted.

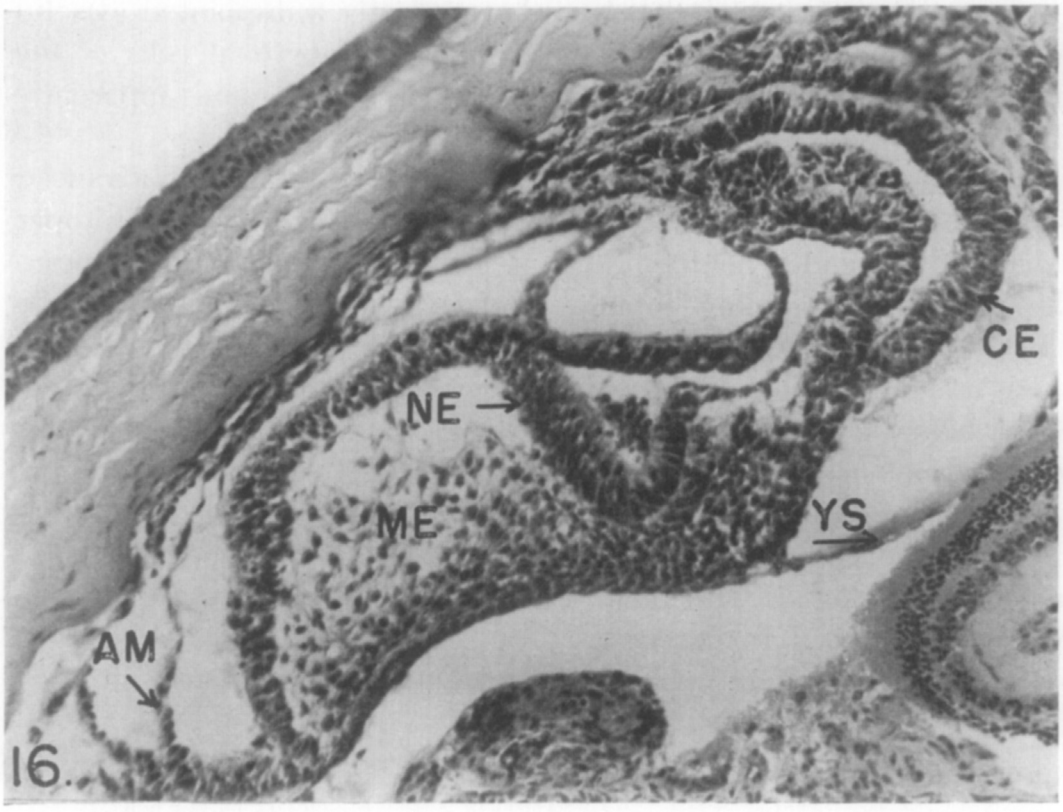
Pierce and Dixon (1959a) have suggested that the embryoid bodies (granules) may be derived from aggregates of cells overlying necrotic portions of solid intraperitoneal implants which break off and seed the peritoneal space. Our observations show that they may also arise as buds from pre-existing embryoid bodies. Peyron has suggested that embryoid bodies in human testicular teratomas may divide symmetrically to form daughter structures like themselves. He compared this process with the division of the armadillo blastocyst into four identical embryos.

The term *parthenogenesis* (a modification of sexual reproduction, usually defined as involving an unfertilized ovum), used by many pathologists to explain the origin of teratomatous embryoid bodies, is inappropriate. Our observations and those of Pierce and Dixon based on teratomatous embryoid bodies of the mouse demonstrate that a form of asexual multiplication is involved.

We have no evidence that embryoid bodies may be precursors of primary teratomas. They have not been observed in early primary testicular tumors of strain 129 mice, and only rarely in the progres-

FIG. 16. Intraocular embryoid body containing structures resembling folded neuroepithelium (*NE*), amnion (*AM*), embryonic mesenchyme (*ME*), coelomic epithelium ? (*CE*), and yolk sac (*YS*). To the left is the host's cornea, to the right, retina. Magnification: × 160.

FIG. 17. Cross section through posterior level of normal 9-day-old mouse embryo (× 160). Compare with Fig. 16.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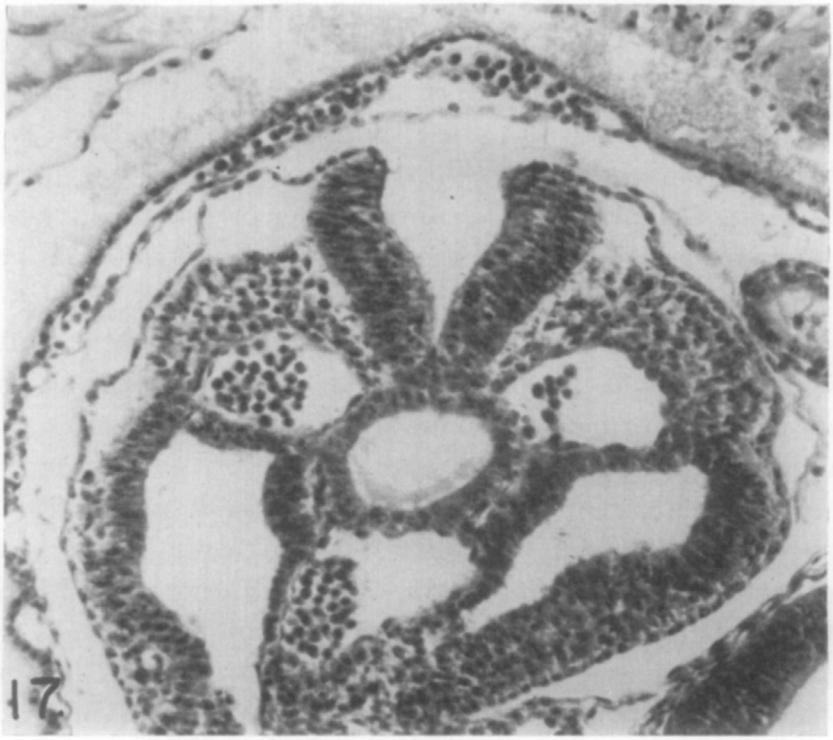
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sively growing teratomas that have been serially transplanted as solid tumors for many years. The undifferentiated embryonal cells of the transplantable tumors give rise to embryoid bodies, and apparently the ascitic fluid favors this development.

Embryoid bodies in one intraocular graft were in a more advanced stage than we have observed previously, containing, in their proper relationships: folded neuroepithelium, amnion, condensations of mesodermal cells resembling somites, and yolk sac. Pierce and Dixon (1960) observed teratomatous embryoid bodies almost identical to these in subcutaneous grafts of large cysts of subline 402A VI origin. They believed these advanced embryoid bodies to be homologous with normal mouse embryos, and we agree with this interpretation.

The findings reported here lend further support to the interpretation that the embryoid bodies resembling 6-day mouse embryos are actually homologous with them. Furthermore, our observations are in accord with Peyron's descriptions of trophoblast, amnio-ectodermal vesicles, endoderm, and mesoderm in human teratomatous embryoid bodies.

SUMMARY

Testicular teratomas occur relatively frequently in inbred strain 129 mice. Occasionally they grow progressively and survive serial transplantation indefinitely. One such tumor has been maintained for approximately six years, and it still retains its highly pleomorphic nature. When established as an ascitic tumor, this teratoma is capable of producing thousands of free-floating formations that resemble 5- and 6-day mouse embryos. The aim of this investigation was to test the histogenetic capacities of these embryoid bodies. Single embryoid bodies were transplanted into the anterior chamber of the eyes of mature strain 129 mice. Growths resulting from these intraocular grafts were examined histologically and some were retransplanted subcutaneously to form solid tumor sublines. Both the intraocular and subcutaneous grafts contained many types of tissues, including a variety of epithelia; neural tissue; cartilage with peripheral ossification; smooth, cardiac, and voluntary muscle; trophoblastic giant cells; layers of ectoderm and endoderm; and undifferentiated embryonal cells. It is concluded that the embryoid bodies derived from the testicular teratomas of strain 129 mice have similarities in embryonic potency as well as in morphology to normal mouse embryos.

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