

## Developmental potential of teratocarcinoma stem cells *in utero* following aggregation with cleavage-stage mouse embryos

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### SUMMARY

Embryonal carcinoma cells were aggregated with cleavage stage mouse embryos, cultured briefly, and transferred as morulae to the uteri of pseudopregnant females. When midgestation foetuses were examined, many were morphologically abnormal. The severity of this abnormal development was correlated with the extent of contribution by embryonal carcinoma cells to the foetuses as indicated by GPI (glucose phosphate isomerase) analysis. This was true for all three of the cell lines studied, NG-2, PSA-1, and LT1-2D.

The clear correlation between increasingly abnormal development and more extensive participation by the embryonal carcinoma cells was not observed in control experiments in which embryos of different stages of development were aggregated together. The data therefore suggest that the embryonal carcinoma cells studied here are unable to support normal development in the absence of a substantial number of host embryonic cells. It remains unclear whether this is a consequence of the karyotypic abnormalities of the cells tested, or whether it reflects a characteristic limitation in the ability of embryonal carcinoma cells to independently direct normal development.

When aggregates were allowed to develop to term and the extent of chimaerism was examined in the live-born animals, it was found to be sporadic and limited. This is consistent with the results indicating that large contributions by embryonal carcinoma cells are not compatible with normal development at midgestation. The chimaerism observed in the live-born animals was comparable in both frequency and in tissue distribution to that generally obtained in other studies using either the aggregation or blastocyst injection techniques.

### INTRODUCTION

Embryonal carcinoma cells, the stem cells of mouse teratocarcinomas, express either a neoplastic or a normal embryonic phenotype depending upon their immediate environment. In an extraembryonic environment they develop into malignant tumours that contain a wide variety of differentiated tissues (Klein-smith & Pierce, 1964). In contrast, when placed in an embryonic environment, usually by injection into a mouse blastocyst, these formerly neoplastic cells behave as normal embryonic cells and contribute to the formation of normal

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tissues in a chimaeric mouse (Brinster, 1974, 1975; Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975; Illmensee & Mintz, 1976; Papaioannou, *et al.* 1978; Papaioannou, 1979; Papaioannou, Evans, Gardner & Graham, 1980). Certain embryonal carcinoma cells have even formed functional germ cells in a chimaeric mouse (Mintz & Illmensee, 1975; Illmensee, 1978; Stewart & Mintz, 1981).

These results are of interest not only because embryonal carcinoma cells provide an example of malignant cells whose behaviour is modulated by their environment but also because, potentially, embryonal carcinoma cells offer a means for manipulating the genetic composition of mice. Embryonal carcinoma cells that have been genetically modified *in vitro* have been shown to contribute normal differentiated derivatives to the tissues of chimaeric mice (Dewey, Martin, Martin & Mintz, 1977; Watanabe, Dewey & Mintz, 1978; Illmensee, Hoppe & Croce, 1978; Illmensee & Croce, 1979). Unfortunately, very few chimaeric mice were obtained and the contribution by injected embryonal carcinoma cells was small and sporadic.

If chimaeric mice produced by combining embryonal carcinoma cells with embryonic cells are to be useful for studying genetic alterations *in vivo*, contribution by the embryonal carcinoma cells to the mice must be both more extensive and more consistent. Extensive contribution by the embryonal carcinoma cells could be limited by the technique used to generate the mice and/or by the ability of the cells to direct normal development. At the time these studies were undertaken, injection of embryonal carcinoma cells into blastocysts was the only method that had been used to obtain such chimaeras. These experiments were performed to determine whether a different method of producing chimaeric mice might be used to increase the relative contribution of embryonal carcinoma cells to chimaeras. The approach taken was to aggregate embryonal carcinoma cells with cleavage-stage embryos. The NG-2 embryonal carcinoma cell line was used because this is the genetically modified cell line which has, to date, been found to contribute most extensively to chimaeric mice created by blastocyst injection. In addition, the ability of two other embryonal carcinoma cell lines to contribute to aggregation chimaeras was tested.

#### MATERIALS AND METHODS

##### *Embryonal carcinoma cells*

Three cell lines were used in these studies: NG-2, PSA-1, and LT1-2D. NG-2 is an HPRT deficient subline of PSA-1 (Dewey *et al.* 1977). PSA-1 was isolated from OTT 5568, a teratocarcinoma derived from a white-bellied agouti, 129/Sv embryo (Stevens, 1970). Karyotypic analysis indicates that PSA-1 and NG-2 are both trisomic for chromosome 6 and are XO (Martin *et al.* 1978; Cronmiller & Mintz, 1978). LT1-2D was clonally derived from the spontaneous ovarian tumor LT72484. It has a modal number of 40 chromosomes. Its only

visible chromosomal abnormality is an elongation of one of its chromosomes 8 (N. Takagi, personal communication). All three of these embryonal carcinoma cell lines are homozygous for the *Gpi-1a* allele which codes for the electrophoretically slower form of glucose phosphate isomerase (GPI).

The embryonal carcinoma cells were cultured in DME (Dulbecco's modified Eagle's) medium on mitomycin C-treated feeder layers of mouse embryo fibroblasts as previously described (Martin & Evans, 1975). For aggregation with embryos, the cells were harvested from confluent plates using 0.05 % trypsin with EDTA. The resulting single cell suspension was seeded at  $3 \times 10^7$  cells per 100 mm tissue culture dish and incubated for 30 min. During this time any residual feeder cells attached to the dish, whereas most of the embryonal carcinoma cells remained in suspension. These unattached embryonal carcinoma cells were collected and seeded at  $10^6$  cells per 35 mm bacteriological dish. These were incubated at 37 °C overnight, during which time the cells formed clumps of various sizes. The smallest of these clumps (10–20 cells) were used for aggregations.

#### *Mouse embryos*

Females of the inbred strain SWR/J (Jackson Laboratories, Bar Harbor, Maine), which are albino and homozygous for *Gpi-1b*, were mated either with SWR/J males or with random-bred ICR (Swiss albino) males (Simonsen, Gilroy, California) that had been identified as homozygous for *Gpi-1b*.

To obtain embryos, females were superovulated by injecting 5 i.u. PMS (Pregnant Mare's Serum, Sigma) intraperitoneally followed 48 h later by 5 i.u. HCG (Human Chorionic Gonadotropin, Sigma). Following the injection with HCG, the mice were mated and the females were checked for vaginal plugs the next morning. Noon of the day on which vaginal plugs were found was taken to be day 0.5 of gestation. Two days after identification of vaginal plugs, 8-cell stage embryos were flushed from the oviducts and uteri of pregnant females. The embryos were exposed to 0.5 % pronase (Calbiochem, grade B) in warm phosphate-buffered saline for 2–3 min to remove their zonae pellucida (Mintz, 1962). After exposure to pronase, they were rinsed six times for 5 min each in modified Biggers' standard egg-culture medium (Biggers, Whitten & Whittingham, 1971; Spindle & Pedersen, personal communication) and cultured in drops of the same medium under mineral oil (Sigma, selected batches) until they were used in the aggregations.

#### *Aggregations*

Aggregations were done in modified Hanks' BSS (Spindle & Pedersen, personal communication) containing 0.2 µg/ml phytohaemagglutinin (PHA; Bacto PHA P, Difco; Mintz, Gearhart & Guymont, 1973). After association of the embryonal carcinoma cells with one or two embryos in warm PHA-containing medium, the aggregates were rinsed three times in Eagle's medium (as modified by Spindle & Pedersen, 1973) containing 10 % foetal calf serum (Hy Clone Inc.,

Logan, Utah) and placed in drops of the same medium under mineral oil at 37°C in 5 % CO<sub>2</sub> in air. It was found that the embryonal carcinoma cells do not survive well in medium without serum and that the embryos are more viable *in utero* if cultured in the Eagle's medium rather than in DME medium. The aggregates were cultured overnight to allow them to compact and form morulae.

#### *Transfer to pseudopregnant females*

Random-bred CD-1 mice from Charles River were used for foster mothers. Before use, these mice were inoculated with Sendai vaccine (Microbiological Associates, Los Angeles) and were quarantined for 2 weeks. One day after the SWR/J females were mated, CD-1 females in oestrus were mated with vasectomized males of proven sterility. This 1-day time lag compensated for the slower development of the aggregate embryos due to *in vitro* culture. Compacted aggregates were surgically transferred to the uteri of foster mothers 2 days after the detection of a vaginal plug.

The development of some aggregates was evaluated after approximately 8 days *in utero* (10½ days gestation). Other aggregates were allowed to develop to term and samples of fifteen tissues were collected from each of the resulting animals. Presence of embryonal carcinoma cell derivatives was detected by the presence in tissue homogenates of the GPI-1A isozyme. GPI assays were performed as previously described (Fujii & Martin, 1980).

### RESULTS

#### *Development of aggregates containing NG-2 cells and single SWR/J embryos*

In earlier experiments, splitting cleavage-stage embryos and arranging their blastomeres around the outside of embryonal carcinoma cell clumps resulted in aggregate blastocysts with inner cell masses that appeared to be composed solely of embryonal carcinoma cells (Fujii & Martin, 1980). This achieved our original goal which was to maximize the contribution of the embryonal carcinoma cells to the inner cell masses of aggregate blastocysts. However, these aggregate blastocysts implanted at a very low frequency when transferred to the uteri of pseudopregnant females. To decrease the amount of stress to which the embryos were subjected, intact rather than split embryos were employed in the following experiments.

Small clumps of NG-2 embryonal carcinoma cells, corresponding in size to one 8-cell-stage blastomere and containing approximately ten to twenty embryonal carcinoma cells, were aggregated each with one 8-cell-stage SWR/J embryo in the presence of PHA. After culture overnight, they were transferred to the uterus of a pseudopregnant female. 8 days later (approximately 10½ days gestation), the females were sacrificed and the developing foetuses were removed for examination and GPI analysis. It was immediately apparent that many of the foetuses were not developing normally. In these and subsequent experiments,

several abnormal phenotypes, ranging from grossly abnormal to slightly deformed or retarded, were observed. These could be grouped into six categories (Fig. 1).

*Group 1.* Members of this group most typically appeared as lumps on the yolk sac. In a few cases, development proceeded further, however it did so in a disorganized manner which resulted in a bizarre assortment of structures grouped together on the yolk sac. In Fig. 1, group 1, for example, a beating heart tube, deformed brain vesicles, and an allantoic stalk are shown haphazardly associated together. This group represents the most abnormal of the structures to develop from implanted aggregates.

*Group 2.* These were the most retarded and primitive of the structures that could be recognized as 'foetuses'. They were small and flat, roughly resembling embryos at 7½ days development. Enclosed in the amnion, a head process with crude cephalic structures was usually visible at one end while an allantoic stalk projected from the other.

*Group 3.* Foetuses belonging to group 3 resembled 8-day foetuses and were distinguished from those in group 4 by a lesser degree of body closure. The body walls of group-3 foetuses were contiguous with the yolk sac along most of their length. When enclosed in the amnion, they exhibited extreme lordosis. After dissection from the amnion, well-developed somites were visible, arranged on either side of an open neural groove.

*Group 4.* The foetuses in this group crudely resembled 8½- to 9-day foetuses. They had not yet acquired a kyphosis curve and their neural tubes were not completely closed. The midgut appeared more or less closed and the heart had visibly begun to develop, protruding from the ventral side.

*Group 5.* Foetuses in this group were clearly retarded although they often appeared normal in other respects. They had the appearance of 9½-day foetuses including the typical kyphosis curve of later developmental stages, clearly developed mandibular arches, somites, apparently normal hearts, and the beginning of small limb buds.

*Group 6.* In this category were placed foetuses that were very nearly normal. Usually their only defect was a misshapen, sometimes stunted head. Otherwise, they often appeared to be normally formed 10½-day foetuses. They had well-formed limb buds, apparently normal hearts, and clearly defined somites. In a few cases, this group may have included samples that were damaged during dissection.

The degree of normalcy of development was described by classification of each foetus either as normal or as a member of one of the above groups. The degree of chimaerism of each foetus was then estimated by eye from GPI assays according to the relative intensity of each of the GPI isozyme bands (Fig. 2). When midgestation foetuses developed from NG-2 cells aggregated with single cleavage-stage SWR/J embryos were examined using these criteria, it was found that although the number of foetuses containing embryonal carcinoma cell

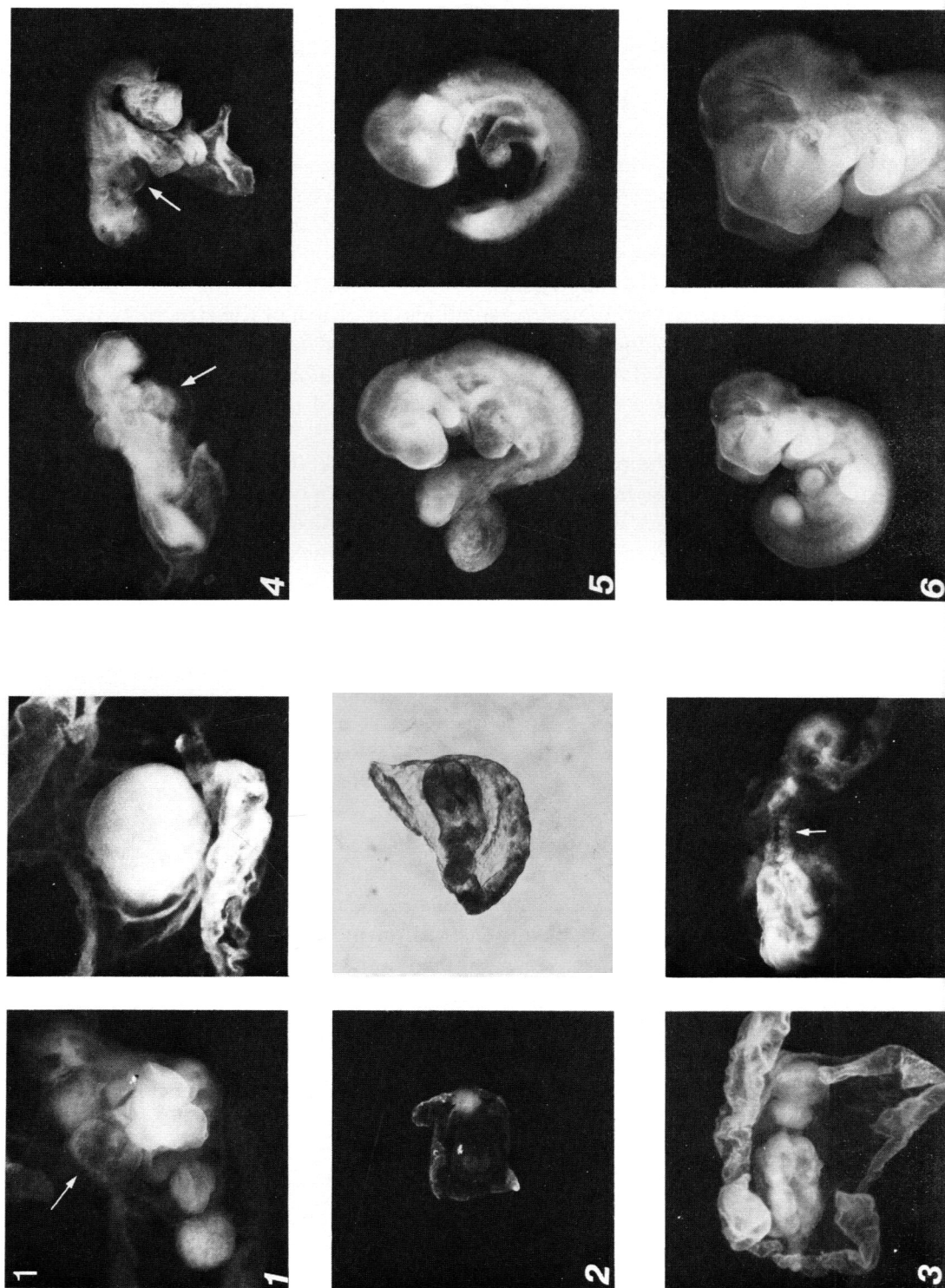


Fig. 1

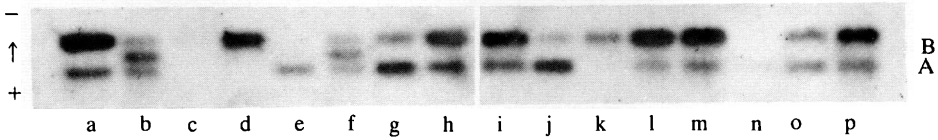


Fig. 2. Examples of GPI isozyme ratios.

GPI isozyme ratios were estimated by eye. The amount of GPI-1A isozyme present was taken as an indication of the extent of chimerism. *c*, *d*, and *k* represent samples in which only the GPI-1B isozyme was present (all B). In *a*, *h*, *i*, *l*, *m*, and *p* the GPI-1B isozyme was present in greater quantities than the GPI-1A isozyme ( $B > A$ ) whereas in *o* the two isozymes appeared to be present in roughly equal amounts ( $B = A$ ). *g*, *j*, and *n* are samples in which the GPI-1A isozyme was present in greater quantities than the GPI-1B isozyme ( $B < A$ ). In *e* only the GPI-1A isozyme was detectable (all A). *b* and *f* illustrate the pattern obtained from samples that are heterozygous for the two GPI isozymes, in this case, blood samples from foster mothers.

derivatives (frequency of chimaerism) was high (79 %), all but one of these chimaeric foetuses were abnormal (Table 1).

#### *Development of embryo controls*

Control experiments without embryonal carcinoma cells were conducted to determine whether the abnormal and/or retarded development was a consequence of the procedures employed (Table 1; Fig. 3). SWR/J embryos were mock processed through the entire aggregation procedure to test the effects on development of the culture process, pronase, culture media, PHA, and experimental manipulations. Of these, only 2 out of 34 (6 %) showed any developmental defect at 10½ days of gestation and these foetuses displayed the more advanced, only slightly retarded phenotypes. This demonstrates that the abnormalities observed in the experimental samples were not due to the techniques employed. Furthermore, Table 1 shows that the implantation frequency of mock-processed embryos (28 %) is similar to that of the experimental aggregates, indicating that the relatively low frequency of implantation obtained in our experiments is probably due to less than optimal conditions of embryo culture or transfer to the uterus rather than the properties of the experimental aggregates.

Additional control experiments were performed in view of the hypothesis that embryonal carcinoma cells correspond to embryonic ectoderm cells of the peri-implantation embryo (reviewed by Martin, 1978). Since embryonal carcinoma cells are presumably at a more advanced stage of development than the 8-cell

Fig. 1. Abnormal phenotypes found at midgestation.

Abnormal phenotypes found at midgestation arranged from the least normal in the upper left corner (A) to the most normal in the lower right corner (F). Numbers (1–6) refer to the classification group. Arrows mark a beating heart tube in the group 1 sample, the developing somites of the group 3 foetus, and the developing hearts of group 4 foetuses. Magnification  $\times 25$  for groups 1–5,  $\times 12$  for group 6.

Table 1. *Frequency of implantation, chimerism, and normal development of embryonal carcinoma cell – embryo aggregates transferred to females that subsequently became pregnant*

Type of aggregation	No. implanted		No. chimaeric		No. normal	
	No. transferred		No. implanted		No. chimaeric	
NG-2 +						
1 SWR 8-cell	19/62	31 %	15/19	79 %	1/15	7 %
NG-2 +						
2(SWR × ICR) 8-cell	21/60	35 %	13/21	62 %	4/13	31 %
NG-2 +						
2(SWR × ICR) 8-cell						
(term)	25/190*	13 %	7/24*†	29 %	7/7	100 %
PSA-1 +						
1 SWR 8-cell	33/98	34 %	19/33	58 %	8/19	42 %
LT1-2D +						
1 SWR 8-cell	30/115	26 %	12/30	40 %	2/12	17 %
mock processed						
8-cell	34/120	28 %	—	—		
8-cell +						
8-cell	7/19	37 %	4/7	57 %	4/4	100 %
ICM +						
8-cell	21/74	28 %	15/21	71 %	12/15	80 %
Embryonic Ectoderm +						
8-cell	17/101	17 %	5/17	29 %	1/5	20 %

\* For this category the number of animals that were born is substituted for the number implanted, although it is probable that this is an underestimate of the number implanted.

† One of the twenty-five animals born was not assayed.

stage embryos with which they were aggregated, it seemed possible that the abnormal development observed might be the consequence of stage differences between the partners of the aggregates. To determine if this was the case, cells from strain 129 embryos at various stages of development were aggregated with single 8-cell SWR/J embryos. Embryos of the 129 strain were used because they are closely related to (and more readily available than) the 129/SvS1 embryo from which the NG-2 cell line was derived.

When single 8-cell 129 embryos were aggregated with single SWR/J 8-cell embryos, development was completely normal in all cases. When single inner cell

Fig. 3. Control embryo–embryo aggregations: Normalcy of development versus extent of contribution.

Normalcy of development, as estimated by classification in one of the phenotypic groups described in the Results (N, normal, and 6 through 1), was plotted against the extent of contribution of the 129 cells as estimated by the relative amount of Gpi-1a isozyme present. 'All B' indicates that no 129 derivatives were present whereas 'all A' indicates that only 129 derivatives were present. Each symbol represents one mid-gestation sample.



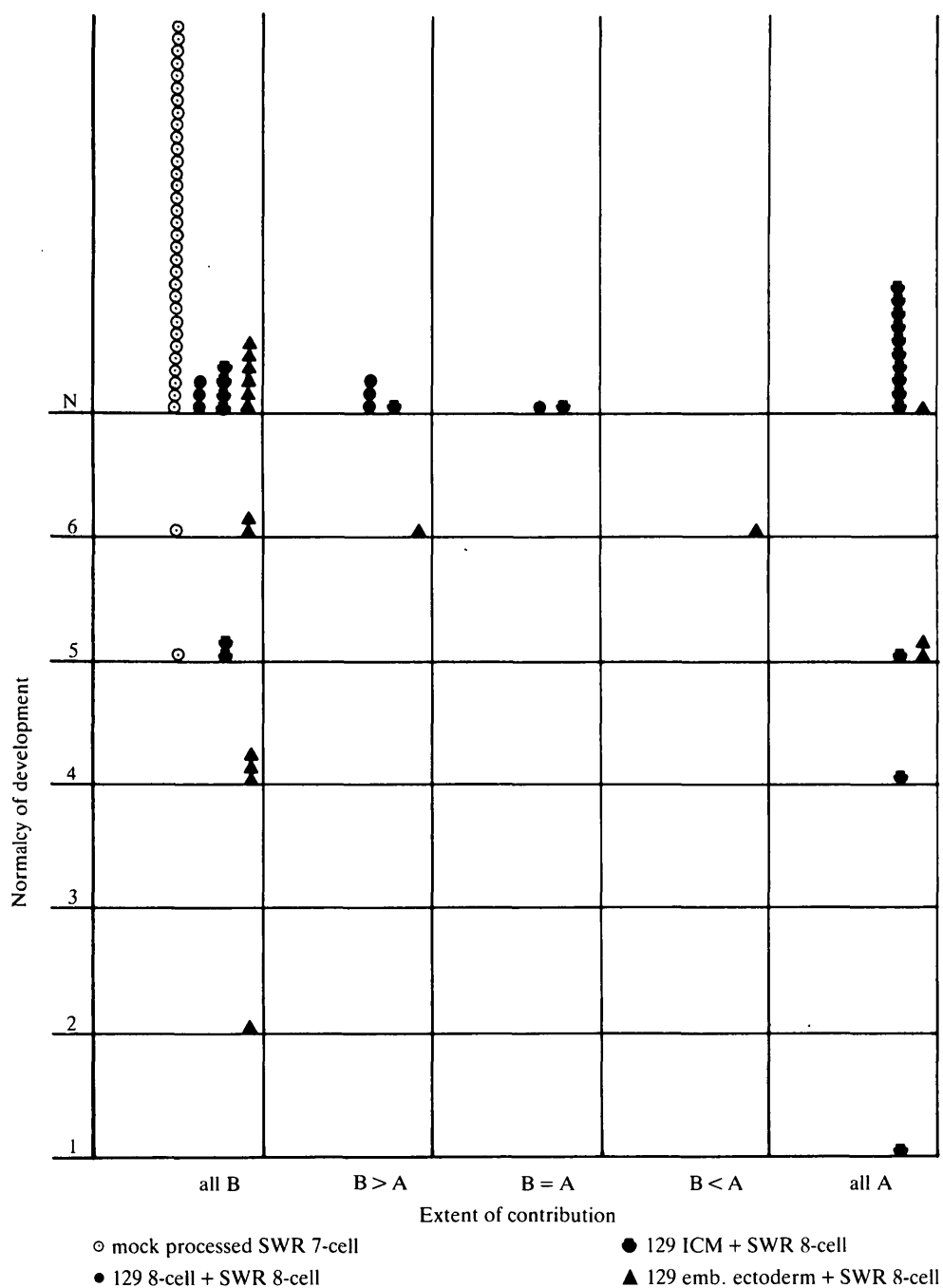


Fig. 3

masses isolated from 129 blastocysts were aggregated with single SWR/J 8-cell embryos, 16 out of 21 (76 %) were normal (Fig. 3). In order to obtain embryonic ectoderm, isolated inner cell masses were cultured for 2 days and their outer layer of endoderm was removed by immunosurgery (Solter & Knowles, 1975). When these were aggregated with single SWR/J 8-cell embryos, only 17 out of 101

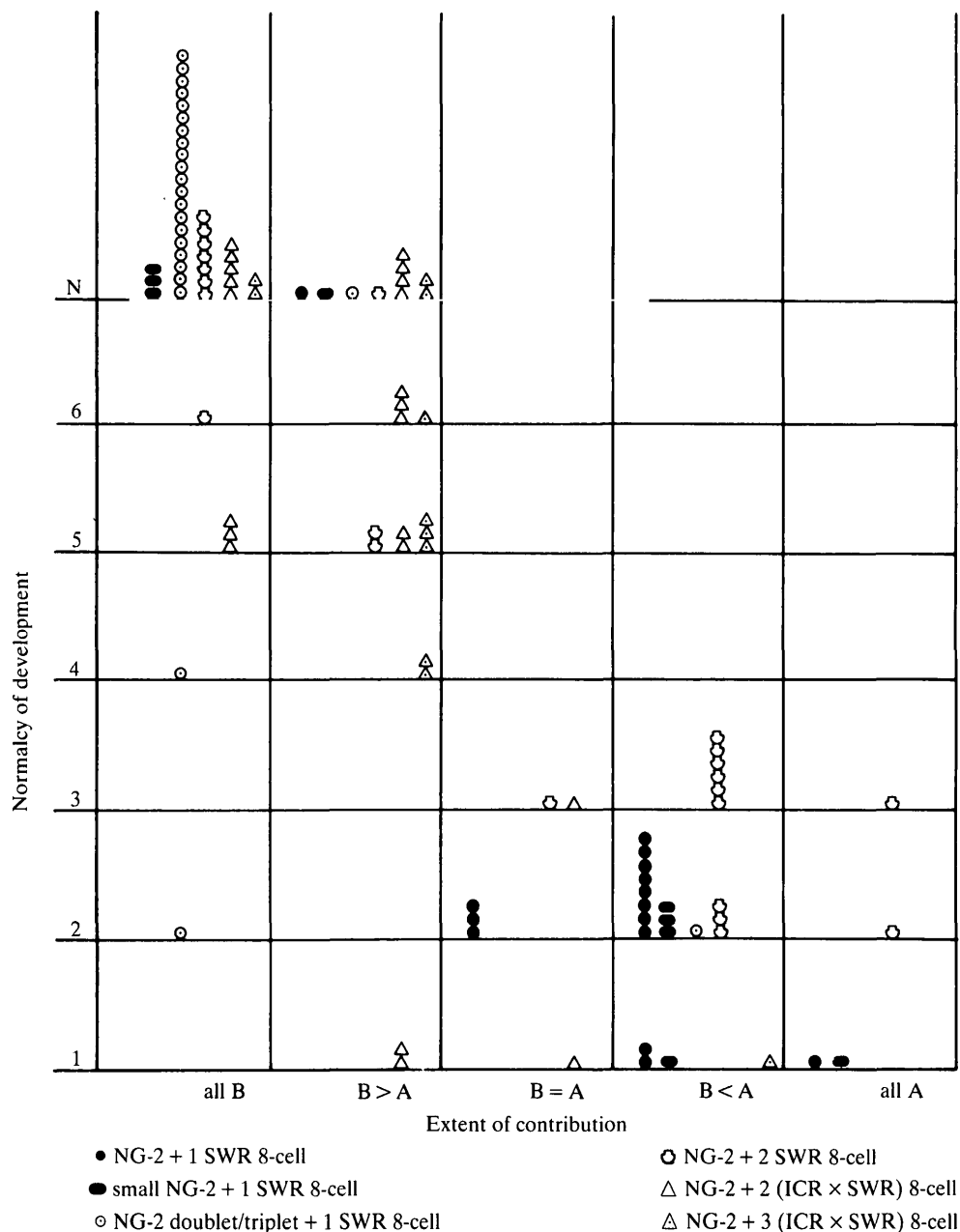


Fig. 4. NG-2 variations: Normalcy of development versus extent of contribution. Parameters measured as in Fig. 3.

(17 %) implanted, possibly reflecting the stress and damage caused by the greater number of experimental manipulations to which this group was subjected. Of the seventeen foetuses that developed only seven (41 %) were normal (Fig. 3). Although the sample size is small, this suggests that the frequency of normal development decreases as the difference between embryonic stages increases. Of the ten abnormal foetuses, six did not contain any derivatives of the strain 129 embryonic ectoderms, raising the possibility that the earlier presence of embryonic ectoderm cells in aggregates might disturb development even when they are subsequently absent from the foetal cell population.

When normalcy of development is compared with extent of chimaerism of the embryo-embryo aggregates (Fig. 3) no apparent correlation between these two parameters is seen. On the contrary, the majority of the abnormal foetuses (15/17) were not chimaeric.

#### *Effect of decreasing NG-2 contribution in chimaeras*

Although it was found that some control aggregates with normal embryonic cells displayed abnormal development, the degree of abnormality of foetuses developed from aggregates containing NG-2 cells was much more severe (Fig. 4). Furthermore, the abnormal foetuses contained extensive embryonal carcinoma cell contributions, in most cases equal to or exceeding that of the SWR/J embryonic cells. This suggested that decreasing the level of NG-2 contribution to each aggregate might result in more normal development. This was, in fact, the case when, in the following experiments, the extent of NG-2 contribution in each aggregate was decreased.

The first approach was to use fewer NG-2 cells. Aggregation with smaller NG-2 clumps of five to ten cells did not appreciably decrease NG-2 contribution to or improve development of the foetuses. Therefore doublets or triplets of cells were used. NG-2 participation in the resulting foetuses was decreased to such a great extent that the frequency of chimaerism fell to 8 %. Since this low frequency of chimaerism was unacceptable, a different approach was taken: the number of embryonic cells in the aggregates was increased.

NG-2 clumps of the original size (ten to twenty cells) were aggregated with two SWR/J 8-cell embryos. Contribution by the NG-2 cells to the foetuses decreased slightly and, concomitantly, development improved somewhat. At this time, the poor mating performance of the SWR/J males caused us to begin using random-bred ICR males which had been shown to be homozygous for the *Gpi-1b* allele. Embryos obtained by mating SWR/J females with ICR males contributed much more extensively to chimaeric foetuses than inbred embryos. This decreased the extent of embryonal carcinoma cell contribution, while maintaining a relatively high frequency of chimaerism (62 %), and was accompanied by a substantial improvement in development (Table 1). Attempts to develop this trend further by using three instead of two embryos did not significantly improve development. Aggregations of NG-2 cells with one SWR/J  $\times$  ICR embryo also resulted

Table 2. *Distribution of NG-2 derivatives in the tissues of chimaeric mice: Aggregation compared with blastocyst injection.*

The number over each column refers to the mouse from which the tissues were collected. The extent of embryonal carcinoma cell contribution was visually estimated from GPI assays. These results are compared with the results of blastocyst injection experiments using NG-2 as previously described by Dewey *et al.* (1977).

Tissue	1 (♂)	2 (♂)	3 (♂)	4 (♂)	5 (♀)	6 (♂)	7 (♂)	blastocyst injection comparison*
1. blood		++						5
2. liver	+++	+++		++				15, 30, 5, 10
3. spleen								10, 10, 15, 5, 5
4. pancreas		++		++				5, 5
5. gut (stomach)	+	+	++	++	+			40, 40, 20
6. kidney		+	++	++	++			50, 20, 40, 5
7. bladder	++	+	++	++				not analyzed
8. gonads	+							25, 15, 20
9. reproductive tract	+		+	++	+			20, 25, 20, 15
10. thymus			+					30, 10
11. heart	+++	+++	+++	+++	+	+++	++	55, 50, 60, 20
12. lungs	+	+	++	++				5, 10, 10
13. salivary glands								10
14. brain								10, 15, 10, 5, 15, 15, 10
15. muscle						+	+	25, 40, 20
16. pigment						+		5, 5

+ &lt;20 %

++ 20 %–50 %

+++ ≥50 %

\* Each number represents % embryonal carcinoma cell contribution to that tissue in one mouse as visually estimated from GPI analyses. From Dewey *et al.* 1977.

in less severely abnormal foetuses that were also less extensively chimaeric (data not shown). The most likely explanation for the improved development of aggregates containing SWR/J  $\times$  ICR embryos is that the hybrid vigor of the embryos enabled them to grow better in the aggregates and thereby to prevent the NG-2 cells from dominating in the developing chimaeric foetus.

The results of this series of experiments show that as the contribution of the embryonal carcinoma cells to each foetus decreases, normalcy of development increases (Cramer's contingency coefficient, 0.78;  $\chi^2$ ,  $P < 0.005$ ).

#### *Development to term of NG-2 – embryo aggregates*

Since many of the extensively chimaeric foetuses were dying *in utero*, it is not surprising that in the aggregates that were allowed to develop to term, chimaerism was sporadic and limited. Twenty-five animals, representing 13 % (25/190) of the aggregates transferred to females that subsequently became pregnant, developed to term. One of these animals died and was not retrieved. When the remaining twenty-four animals were screened at 15 to 39 days of age, seven (29 %) contained NG-2 cell derivatives in their tissues (Table 2). One contained NG-2 derived agouti hairs in his coat. GPI analysis revealed the presence of NG-2 derivatives in one to eight tissues of the other mice. Embryonal carcinoma cell derivatives were detected most often in the heart and gut (stomach) and most extensively in the heart, liver, and pancreas. Only one of the twenty-four animals had a tumour. As evaluated by GPI analysis, this tumour contained both embryo and embryonal carcinoma derivatives in roughly equal amounts.

#### *Development of aggregates containing PSA-1 or LT1-2D cells*

Since the NG-2 cells are HPRT deficient, it is possible that their apparent inability to independently form a normal foetus is a consequence of either their HPRT deficiency or the mutagenesis and selection processes that were involved in their isolation. To determine whether this is the case, aggregations were performed with two other cell lines, PSA-1 and LT1-2D, neither of which is HPRT deficient. PSA-1 was chosen because it is the cell line from which NG-2 was derived. Although it has the same karyotype as NG-2, it has not been subjected to either mutagenesis or HAT selection. LT1-2D was chosen because it has a nearly normal karyotype, its only chromosomal abnormality being an elongation of one of its chromosomes 8. Since the purpose of these experiments was to determine whether foetuses composed largely of PSA-1 or LT1-2D derivatives would develop abnormally, aggregations were performed with one SWR/J 8-cell embryo. It was in this combination that NG-2 contributed most extensively to foetuses and participated most often in abnormal development.

The results of these experiments indicate that, in aggregations with both PSA-1 and LT1-2D, the degree of abnormal development is correlated with the extent of contribution by the embryonal carcinoma cells (Fig. 5). (For the combined

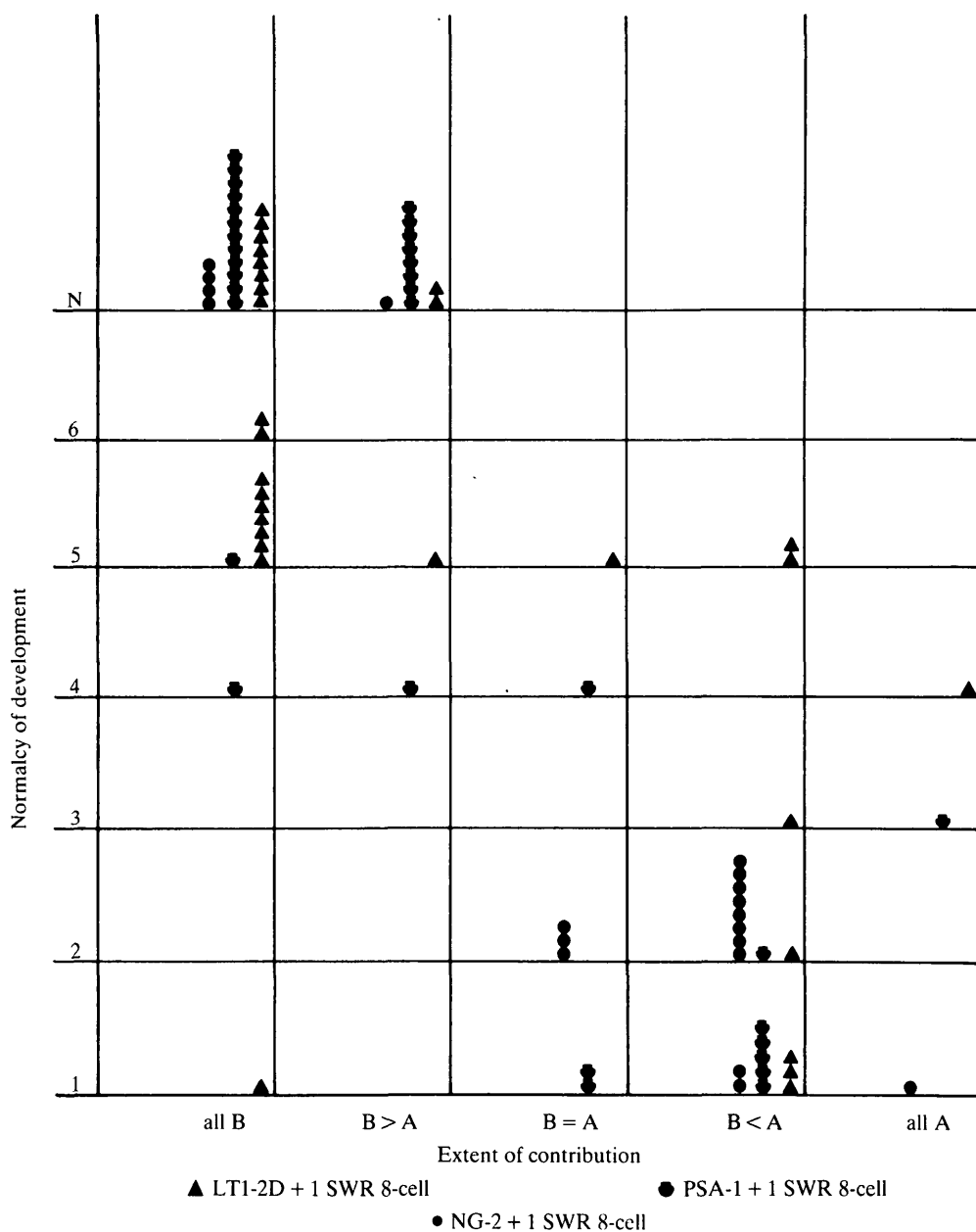


Fig. 5. NG-2, PSA-1, and LT1-2D comparison: Normalcy of development versus extent of contribution.

Parameters measured as in Fig. 3.

PSA-1 and LT1-2D data the Cramer's contingency coefficient is 0.68;  $\chi^2$ ,  $P < 0.005$ .) 42 % of the chimaeric foetuses with PSA-1 contributions were normal, as compared with 31 % normal chimeras with NG-2 contributions (Table 1). Since the sample sizes were small, it is not clear whether this difference reflects

a change in developmental potential that might have occurred during the derivation of NG-2 from PSA1. If so, this could be the consequence of mutagenesis and selection. However, the detrimental effects of such procedures are not the sole cause of abnormal development since PSA-1 as well as LT1-2D cells produced abnormal chimaeras. When compared with PSA-1, LT1-2D did not contribute as often to midgestation foetuses, and fewer LT1-2D aggregates developed normally.

#### DISCUSSION

The results described here demonstrate that the aggregation technique can be used to generate chimaeric mice containing embryonal carcinoma-derived tissue contributions. If only live-born animals are considered, the technique of aggregating embryonal carcinoma cells with 8-cell embryos can be used to obtain chimaerism that is similar in both frequency and tissue distribution to that obtained when the same NG-2 embryonal carcinoma cells are injected into blastocysts (Table 2; Dewey, *et al.*, 1977). These data are consistent with the recent findings of Stewart (1982), who has also created aggregation chimeras with NG-2 cells. Aggregation is thus an alternative method to blastocyst injection for producing chimaeric mice.

Examination of midgestation foetuses revealed that using the aggregation technique it is possible to obtain animals in which the embryonal carcinoma cells predominate in the developing foetus. However, the data indicate that at least for the cell lines employed in this study, development at midgestation is often abnormal, and the degree of abnormality is correlated with the extent of contribution by the embryonal carcinoma cells to the developing foetuses. Such results suggest that the frequency and extent of contribution by certain embryonal carcinoma cells is limited in live-born animals because foetuses with large contributions do not survive to term.

Control experiments with mock processed embryos and embryo-embryo combinations indicate that the abnormalities observed in the aggregation experiments were not caused by the culture conditions, reagents, or experimental manipulations *per se*. The abnormal development of control aggregates between 8-cell embryos and ICM or embryonic ectoderm cells suggests that development may be perturbed when embryo cells of significantly different developmental stages are combined. Thus the considerable difference in developmental stage between embryonal carcinoma cells and 8-cell-stage embryos might play some role in the abnormal development of the aggregates.

Further consideration of the data, however, suggests that the abnormalities observed in the foetuses developed from the experimental aggregates were largely due to limitations in the developmental potential of the embryonal carcinoma cells studied. Although each of the embryonic cell types tested, including 129 embryonic ectoderm, was capable of giving rise to normal 10½-day

foetuses that appeared, within the limits of sensitivity of the GPI assay, to be composed entirely of derivatives of that cell type, this was not the case for any of the embryonal carcinoma cells we tested. This suggests that the embryonal carcinoma cells tested here do not have the same developmental potential as various normal embryonic cell types, including embryonic ectoderm. This is supported by the observation that the degree of abnormal development in the experimental aggregates was strongly correlated with the extent of embryonal carcinoma cell contribution. In contrast, in control experiments there was no correlation between the abnormalities observed and contributions by any one embryonic cell type or strain.

It is not possible from our results to determine whether the chromosomal abnormalities of the three cell lines tested were responsible for the observed abnormal development or whether embryonal carcinoma cells in general are limited in their ability to independently support normal development. In this context, however, it is important to note that Rossant & McBurney (1982) have recently shown that abnormal development *in utero* at midgestation is not restricted to chimaeras formed either with aneuploid cells or by the aggregation technique. They observed many severely abnormal foetuses at midgestation following injection of a karyotypically normal embryonal carcinoma cell line into blastocysts.

It thus remains to be determined whether any embryonal carcinoma cells do, in fact, have the ability to independently support normal development. At present there exist a number of cell lines that may have such potential, including the METT-1 embryonal carcinoma cell line, which has been shown to be diploid and capable of forming functional germ cells in chimaeric mice (Stewart & Mintz, 1981), as well as embryonal carcinoma-like cell lines derived directly from embryos (Evans & Kaufman, 1981; Martin, 1981).

To fully explore the developmental potential of a given embryonal carcinoma cell line, experiments must be performed in which the cells under investigation are combined with embryonic cells in such a way that they predominate in the developing foetus. The data described here demonstrate that the aggregation technique can be used to obtain extensive contributions to developing foetuses by embryonal carcinoma cells with reasonably high frequency. It is not yet clear whether such contributions are greater than those obtained by the blastocyst injection technique. Whichever method is ultimately used, the answer to the question of whether any embryonal carcinoma cell lines have the capacity to direct normal development in the absence of a substantial number of normal embryonic cells should lead to a better understanding of the relationship between embryonal carcinoma cells and the embryonic cells from which they arise.

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