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The development of 12th to 14th day foetuses following reimplantation of pre- and early-primitive-streak-stage mouse embryos

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SUMMARY

This paper describes a technique for transplanting early postimplantation mouse embryos from their own implantation site to a decidua in another pregnant mouse. Evidence is provided that this procedure is compatible with their continued development. At a low frequency both 6th and 7th day embryos can re-establish themselves and continue apparently normal development and placentation for at least 6–8 days.

INTRODUCTION

Normal pregnancy following the transfer of early mammalian embryos to the oviduct or uterus of suitably primed females is now a routine occurrence in many species including man (Whittingham, 1979; Edwards & Steptoe, 1983). This technique has proved invaluable in a number of diverse studies of mammalian development including the identification of maternal effects (McLaren, 1981), the analysis of cell lineages (Gardner, 1978), and the genetic manipulation of embryos (Seidel, 1983; Gordon, 1983). However, normal implantation and development have only been reported when embryos are transferred before the stage at which they naturally implant in the uterus. Consequently, many profitable avenues of investigation applicable to preimplantation embryos are not feasible at later stages. As a result relatively little is known about the dramatic developmental changes, including the formation of the foetal tissues, which occur soon after implantation.

For this reason attempts have been made to reimplant egg-cylinder and early-primitive-streak-stage embryos. This paper describes the most successful strategy employed to date and provides the first evidence that postimplantation mammalian embryos can continue normal development and form an apparently functional chorioallantoic placenta when transferred from one pregnant female to another.

Key words: mouse embryo, transplantation, implantation, primitive streak stage.

MATERIALS AND METHODS

The basic protocol for the reimplantation experiments, adopted after a series of pilot studies using donor embryos and recipient females of various ages, was the transfer of egg-cylinder-stage embryos into normally pregnant females whose own litters were 24 h less advanced than the donor embryos. In all experiments host and donor embryos were distinguishable by differences in glucose phosphate isomerase (GPI) allozymes and by the presence or absence of retinal pigmentation at the time of analysis.

Animals

In the majority of experiments albino outbred PO strain (Pathology, Oxford) female mice previously mated to PO males, belonging to the same closed colony homozygous for the *Gpi-1*^{a} allele of GPI, were used as recipients. Donor embryos were obtained from matings between PO females and agouti C3H/HeH males (Harwell). These embryos, therefore, were *Gpi-1*^{a}/*Gpi-1*^{b} and carried a dominant pigment marker. In a few experiments reciprocal transfers were performed. In one series of experiments PO donor embryos were transferred to PO recipients which had been mated to agouti male mice carrying a translocation (T(1;2)5Ca) (Harwell) which results in approximately 50 % embryo mortality after implantation due to genomic imbalance (Carter, Lyon & Phillips, 1956). The surviving host embryos from these matings were *Gpi-1*^{a}/*Gpi-1*^{b} and pigmented.

All mice were maintained on a regime of 14 h light/10 h dark with the midpoint of the dark period being 19.00 h. The morning on which a copulation plug was detected was designated the first day of gestation.

Recovery and transfer of embryos

Donor embryos were dissected from the uterus on either the 6th (egg-cylinder formation) or 7th (early-primitive-streak stage) day of gestation. Dissections were undertaken in PBl medium (Whittingham & Wales, 1969) containing 10 % (v/v) foetal calf serum instead of bovine serum albumin. The conceptuses were removed in their entirety so that both the ectoplacental cone and Reichert's membrane remained intact. 6th and 7th day embryos were transferred into 5th and 6th day recipients respectively.

Recipients were anaesthetized by a single intraperitoneal injection of Avertin (0·1 ml 5 g^{-1} body weight). A small midventral incision was made through the skin and body wall of the lower abdomen and the uterus was gently pulled through the opening. Donor embryos were transferred into the implantation sites (Fig. 1) with the aid of a dissecting microscope (Wild) and fibre-optic illumination (Volpi). Initially, a channel was made through the antimesometrial uterine muscle into the centre of the decidua with a sharp 25-Gauge syringe needle (Microlance, Becton-Dickinson). The embryos were inserted in a small volume of PBl + 10 % FCS using a fine hand-drawn siliconized (Repelcote) Pasteur pipette, braked with paraffin oil (Boots U.K., Ltd) and containing a marker air bubble. Each implantation site received a single donor embryo transferred in the appropriate orientation such that the ectoplacental cone was directed mesometrially. A maximum of nine transfers was made to any individual recipient. For 5th day recipients it was necessary to inject 0·3 ml of a 5 % (w/v) solution of Pontamine Sky Blue (Hopkins & Williams) into the tail vein prior to surgery in order to pin point the implantation sites (Psychoyos, 1961).

Identification and evaluation of reimplanted embryos

Recipients were killed on the 12th, 13th or 14th day of gestation (i.e. 6 to 8 days after transfer). The excised uterus was placed in phosphate-buffered saline (PBS) and all implantation sites which had received a donor embryo were dissected. All conceptuses, whether host or donor, normal or abnormal, were recovered. The presence or absence of heartbeat and visceral yolk sac (VYS) circulation was recorded and the approximate developmental stage and degree of normality assessed. Retinal pigmentation was used to distinguish normal host and donor embryos and later their origin was confirmed and the identity of abnormal or retarded

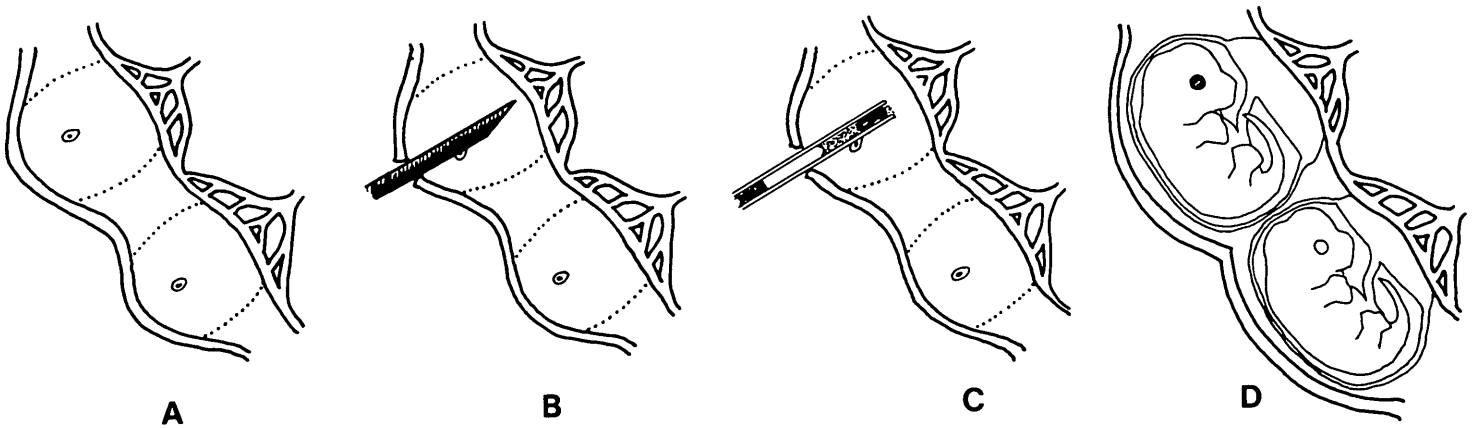


Fig. 1. A diagram of the reimplantation procedure. (A) Median longitudinal section of the host uterus showing two 6th day embryos within their implantation sites. (B) The uterus was held, adjacent to an implantation site, with watchmaker's forceps. A sterile 25G syringe needle was inserted through the uterine wall into the antimesometrial end of the decidua and then withdrawn. (C) A Pasteur pipette containing the donor embryo was inserted into the channel made by the syringe needle. The embryo was orientated with its ectoplacental cone towards the distal tip of the pipette. As the pipette was slowly withdrawn from the decidua the embryo was released by gentle expiration. (D) The recipient uterus 6 or 7 days later showing a donor (black eye) and host embryo (white eye) side by side.

embryos established by GPI electrophoresis (see below). A note was made as to whether foetuses were twinned or single within an implantation site.

Grossly normal donor foetuses, together with their placentae, were fixed in formol–acetic–alcohol and processed for routine histology. Foetuses were removed briefly from 70% alcohol during dehydration, lightly blotted and weighed. In addition their crown–rump lengths were measured and their somite numbers counted. Subsequently, serial histological sections (7 μm) were prepared and stained with haemalum and eosin. Twelfth to 14th day PO embryos and 13th and 14th day (C3H/HeH×PO) embryos were treated similarly and used as controls for assessing the developmental normality of reimplanted embryos.

Electrophoresis

The VYS, or part of it, from any putative donor embryo, including all embryos which were developmentally retarded or resorbing, was frozen in a small volume of PBS. Grossly abnormal conceptuses and membrane vesicles were frozen intact. Electrophoresis was carried out using Titan III plates (Helena Laboratories) according to the protocol described by McLaren & Buehr (1981), except that the concentration of the tris–glycine bridge buffer was reduced to 0·025%. The plates were stained using the method of Eicher & Washburn (1978).

RESULTS

Transfer of 7th day embryos into 6th day recipients

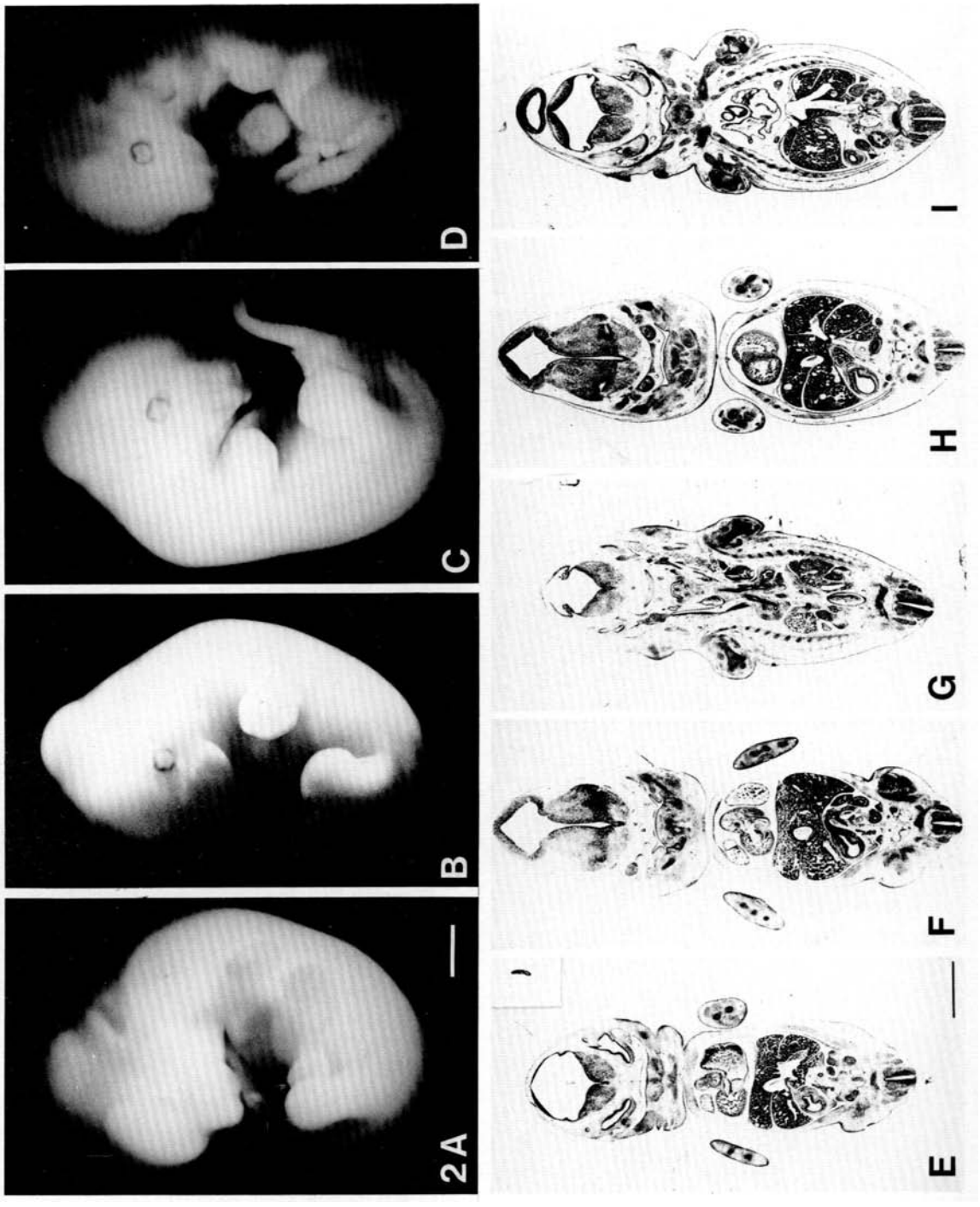
The incidence of recovery and the extent of development of donor embryos are given in Table 1. In all, eight donor embryos formed grossly normal foetuses complete with placenta, vigorous heartbeat and VYS circulation. The first of these to be recovered was misclassified, due to a failure to detect retinal pigmentation on the 12th day of gestation, and discarded as a normal host embryo. Subsequently, GPI analysis showed it to have been donor in origin. The remaining seven reimplanted conceptuses were available for more rigorous scrutiny. A comparison of their weights, crown–rump lengths and somite numbers is shown in Table 2. In all cases the initial 24 h advantage of donor embryos was not maintained but instead their gross morphology resembled the developmental stage of their more advanced host littermates. However, there is no evidence that this retardation with respect to their chronological age increased between the 12th and 14th days of gestation and it may, therefore, have occurred soon after transfer. Reimplanted embryos which were twinned with host embryos in a single implantation site, and invariably had what have been called 'fused placentae' (McLaren & Michie, 1959*a*), tended to be smaller than those which developed alone (Table 2).

Examination of histological sections revealed that five of the seven recognized reimplanted foetuses were indistinguishable from controls (Fig. 2). All foetal organs appeared normal both in size and morphology. This was true also of their placentae which contained abundant nucleated red blood cells in the labyrinthine foetal capillaries. The two remaining reimplanted foetuses that were studied were not wholly normal. In one the abnormality was trivial in so far as only the right eye was slightly defective. The optic cup appeared normal and lens induction had occurred but the overlying surface epithelium remained thickened, possibly due to some mechanical constraint *in utero* interfering with ocular growth. The contralateral eye was normal and in all other respects this embryo resembled those

Table 1. *Recovery and developmental status of donor embryos after 6, 7 or 8 days*

Nature of experiment	No. transferred	No. total* resorption	No. mole	No. abnormal host only	No. normal host only	No. donor membrane vesicle	No. grossly abnormal donor	No. advanced resorbing donor†	No. grossly normal donor
7th → 6th day	160	27 (16·9 %)	38 (23·8 %)	14 (8·8 %)	53 (33·1 %)	6 (3·8 %)	13 (8·1 %)	8 (5 %)	1‡ (0·6 %)
7th → 6th day§	109	17 (15·6 %)	31 (28·4 %)	8 (7·3 %)	31 (28·4 %)	4 (3·7 %)	9 (8·3 %)	2 (1·8 %)	7 (6·4 %)
6th → 5th day	29	0	10 (34·5 %)	3 (10·3 %)	14 (48·3 %)	1 (3·5 %)	0	0	1 (3·5 %)
7th → 6th day T(1;2)5Ca × PO	77	3 (3·9 %)	35 (45·5 %)	7 (9·1 %)	17 (22·1 %)	14 (18·2 %)	0	0	1 (1·3 %)

* This category represents recipients which underwent complete resorption such that the uterus resembled that of a non-pregnant female.
† These embryos appeared grossly normal but retarded or resorbing. The most retarded had 25 somites and the most advanced had 54 somites.
‡ This embryo was misclassified as host in origin and discarded before electrophoretic analysis, which revealed it to be donor in origin.
§ Results following the recovery of the first normal unequivocally donor embryo.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described above (Figs 2F & 2G). The seventh embryo had an abnormal heart which was manifest as an enlarged pericardial cavity and reduced thickening of the myocardium (Fig. 2D). Presumably, as a consequence of cardiac malfunction there was noticeable oedema in other tissues.

Transfer of 6th day embryos into 5th day recipients

This regime was compatible with continued development of the transferred embryo but the incidence of successful reimplantation was reduced (Table 1). The single foetus recovered after 8 days was entirely normal and all measured parameters were commensurate with control conceptuses (Table 2).

The effect of increased host embryo mortality

It was confirmed from inspection of implantation sites that litters produced from mating normal females to males heterozygous for the T(1;2)5Ca translocation suffer approximately 50 % mortality by mid-gestation. About half the embryos in these litters appear retarded on the 7th day although they may survive in varying degrees of disorganization up to recognizable early somite stages. It was anticipated that the transfer of normal 7th day embryos to such recipients would increase the incidence of reimplantation due to the absence of host competition in 50 % of implantation sites. Unfortunately, the results show that there is no benefit to reimplantation but rather an increase in the frequency of resorption sites or moles (Table 1). Only one donor embryo appeared grossly normal after 7 days and it was somewhat smaller and less advanced than its littermates (Table 2).

DISCUSSION

Implantation in the mouse begins on the 5th day of gestation and by the 7th day the uterine epithelium of the crypt has disappeared and the trophoblast has eroded the decidual tissue as far as the maternal blood vessels. This results in the primary and secondary trophoblast giant cells of the conceptus lying in intimate contact with the endometrium (Snell & Stevens, 1966; Potts, 1969). At this point, well hidden from direct observation, the embryo embarks on the complex process of gastrulation. This gross rearrangement of the embryo essentially lays the foundation, providing both the architecture and the building material, for organogenesis and subsequent foetal development. It is during gastrulation that one would like to have direct access to the embryo, without forfeiting the possibility of its continued development, in order to determine the origin of particular foetal tissues and their early lineage relationships one with another.

Fig. 2. Control embryos and reimplanted embryos recovered 7 days after transfer. (A) Control PO embryo on the 13th day of gestation (Bar = 1 mm). (B,C) Normal reimplanted embryos. (D) Reimplanted embryo with an abnormal heart. (E) Frontal section of a control 13th day PO embryo. Inset: Sagittal section of a 7th day (C3H/Heh × PO) embryo at the time of transfer. (F,G) Frontal sections of the reimplanted embryo with an abnormal eye. All other organ systems appear normal. (H,I) Frontal sections of embryo depicted in Fig. C.

Table 2. *A comparison of the development of donor embryos and control embryos of host and donor genotype*

Reimplanted embryos						Control embryos*			
Nature of experiment	Age of recipient at time of recovery	Single or twinned	Crown–rump length (mm)	Weight (mg)	Somite number	Age and genotype	Crown–rump length (mm)	Weight (mg)	Somite number
7th → 6th day	12th day	Twin**	6	27·4	42	12th day PO	6·45 ± 0·55†	29·6 ± 3·2	41 ± 1·4
7th → 6th day	12th day	Single	6·5	31·8	45	13th day PO	7·8 ± 0·8	68·8 ± 16·7	50·3 ± 1·8
7th → 6th day	13th day	Single	10	93·0	54	14th day PO	9·97 ± 0·28	131·7 ± 9·36	58 ± 1·4
7th → 6th day	13th day‡	Twin	9	64·2	54				
7th → 6th day	13th day§	Single	9	102·4	57	13th day C3H/HeH × PO	8·8 ± 0·35	68·1 ± 6·65	53·8 ± 1·99
7th → 6th day	13th day	Single	9·5	86·4	55				
7th → 6th day	14th day	Twin	9·8	N.D.	60	14th day C3H/HeH × PO	10·2 ± 0·42	139·7 ± 6·7	60·2 ± 1·03
6th → 5th day	13th day	Single**	9·5	80·2	60				
7th → 6th day T(1;2)5Ca × PO	13th day	Single**	8	46·8	52				

* Each class of control embryo comprises 10 embryos obtained from 2 separate litters.
† The standard deviations are given for all measured parameters.
‡ This embryo had an abnormal heart (see text).
§ This embryo had a defective eye (see text).
** These were unpigmented PO donor embryos. All others were C3H/HeH × PO.

Egg cylinders, or their component parts, will continue to grow and differentiate in extrauterine sites although morphogenesis and consequently tissue associations are seldom normal in such circumstances (Grobstein, 1952; Diwan & Stevens, 1976; Skreb & Svajger, 1975; Svajger, Levak-Svajger, Kostovic-Knezevic & Bradamante, 1981). Therefore ectopic grafts are of only limited use in establishing the sequence of events in the embryo itself. Postimplantation culture techniques (New, 1978), restricted mainly to the rat, can support consistently normal growth and development of whole embryos, excluding the parietal yolk sac, over relatively short periods (24–48 h depending on the stage at explantation). The most prolonged development sustained *in vitro* has been the growth of rat embryos from the onset of gastrulation to the 30- to 40-somite stage. However, development under the conditions used was seldom entirely normal over this period and those embryos which survived suffered considerable retardation in growth (Buckley, Steele & New, 1978). In general, the limited duration of guaranteed normal development in culture has allowed only crude estimation to be made of tissue fate and potency in the egg cylinder (Beddington, 1981, 1982, 1983).

Clearly, these problems would be overcome if postimplantation embryos could be manipulated *in vitro* and subsequently returned to the uterus to continue normal development. With such an end in mind it is at least encouraging that 6th and 7th day embryos can resume normal growth and development, and undergo apparently effective placentation, after transfer from their own decidua into one in a different pregnant female. Manipulation would, of course, involve puncturing Reichert's membrane and although the importance of an intact Reichert's membrane for reimplantation has not been tested it is known that injecting through this membrane *in vivo* is compatible with normal development (Weissman, Papaioannou & Gardner, 1978; Jaenisch, 1980).

The extensive development and growth seen in the few successful reimplants (Table 2; Fig. 2) surpasses that achieved *in vitro*. This is probably attributable to their ability to form a functional placenta since it is accepted that growth in culture declines after the stage at which it is thought that the placenta first contributes to nutritional and respiratory exchange (New, 1978). However, no reimplanted embryos have yet been born (0/98 transfers; unpublished observations) although it is still unclear whether this is due to placental failure in the final week of pregnancy or complications during parturition itself.

Although the results demonstrate the feasibility of reimplantation the frequency of successful operations is too low for reimplantation to be seen as an immediately useful procedure. One can only speculate as to why so few reimplant. Obviously, much depends on the accuracy of transfer and certainly practice alone led to an increased rate of donor embryo recovery. Some embryos may not remain within the host decidua whilst others may be located sufficiently eccentrically that mechanical or other influences prevent their normal development and placentation. The high frequency of resorptions, host as well as donor, may be caused by undue damage to either embryo or decidua during transfer. Certain

physiological elements may also play a role. For example, earlier studies have demonstrated that twinning and fused placentae lead to an increase in foetal mortality and decrease in foetal weight (McLaren & Michie, 1959*a*). Similarly, more than eight implantations in a uterine horn results in an elevated midgestation death rate (McLaren & Michie, 1959*b*). Thus, the artificial increase in litter size together with the higher probability of twinning introduced by the reimplantation technique may contribute to the failure of some donor embryos. There is some indication that donor embryos which develop as twins with the resident host embryo do not fare as well as those whose host embryo resorbs (Table 2).

The attempt to overcome the possible disadvantages of overcrowding by the introduction of lethal genomic imbalance into half the host embryos via the T(1;2)5Ca translocation was a conspicuous failure. However, although the affected host embryos start to deteriorate at the egg-cylinder stage they may survive, albeit in an abnormal or disorganized fashion, until the early somite stage. It is possible that resorption of the host embryo at this more advanced stage may jeopardize the development of any adjacent donor embryo. Alternatively, the implantation sites induced by the mutant embryos may not be wholly normal. Certainly trophectoderm-vesicle-induced decidua, which appear histologically normal, (Gardner, 1972) do not support extensive development of transferred postimplantation embryos, even if the contralateral uterine horn contains normally developing host embryos (unpublished observations). Unfortunately, therefore, it still remains to find a means of increasing the frequency of successful reimplantation so that the feasibility of producing viable postimplantation mouse chimaeras can be assessed.

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