J. Embryol. exp. Morph. **95**, 95–115 (1986)
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Chimaerism of primordial germ cells in the early postimplantation mouse embryo following microsurgical grafting of posterior primitive streak cells *in vitro*

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SUMMARY

A microsurgical grafting technique has been used to introduce primordial germ cell (PGC) precursors into intact primitive-streak-stage mouse embryos *in vitro*. Operated embryos were cultured for 36–40 h and then analysed by a combined histochemical and autoradiographic method. PGC chimaerism occurred in embryos that received grafts of caudal primitive streak cells but not adjacent embryonic endoderm or anterolateral ectoderm/mesoderm cells. Graft-derived PGCs were found to be migrating through the gut endoderm alongside host-derived PGCs in approximately half of the chimaeric embryos whereas in the other 50 % of cases PGCs remained at the site of grafting in association with graft-derived somatic cells. A similar pattern of somatic chimaerism was produced by primitive streak and anterolateral ectoderm/mesoderm grafts: the allantois was colonized predominantly, with, in addition, formation of amnion, surface ectoderm and caudal mesoderm in a few embryos. The majority of embryonic endoderm grafts failed to incorporate into host embryos and formed yolk-sac-like vesicles.

The findings of this study indicate that (a) PGCs originate from the embryonic ectoderm *via* the primitive streak during development of the mouse embryo, and (b) anterolateral ectoderm and mesoderm cells are unable to form PGCs after heterotopic grafting to the posterior primitive streak site. The combined microsurgical and embryo culture methods provide an experimental system for the analysis of PGC development in intact mouse embryos.

INTRODUCTION

The mammalian germ cell lineage has long been a topic of interest for developmental biologists (reviews: Mintz, 1960; Heath, 1978; Eddy, Clark, Gong & Fenderson, 1981; Eddy & Hahnel, 1983; McLaren, 1981; Snow & Monk, 1983). The mechanisms by which germ cells are able to combine extreme cellular specialization with developmental totipotency is a major unsolved problem of embryogenesis. Studies of mouse embryos indicate that female germ cells, like multipotential somatic cells, undergo inactivation of an X-chromosome during

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Key words: germ cells, postimplantation, mouse embryo, chimaera, microsurgery, culture.

early postimplantation development but, thereafter, differ from somatic cells by undergoing reactivation of the inactive X-chromosome soon after colonization of the genital ridges, probably in association with the onset of meiotic prophase (Gartler, Rivest & Cole, 1980; Johnston, 1981; McMahon, Fosten & Monk, 1981; Monk & McLaren, 1981). This finding has raised the possibility that developmental totipotency is a property of mature germ cells acquired during development and not maintained throughout the germ cell lineage. According to this hypothesis, the critical events that may determine the acquisition of developmental totipotency in germ cells must be sought in the period of embryogenesis that precedes colonization of the genital ridges. This early phase of development of the mammalian germ line comprises two main events: separation of primordial germ cells (PGCs) from somatic cells and migration of PGCs to the genital ridges.

Early studies, in which PGCs were recognized on the basis of cytomorphological features (Witschi, 1948) or following the histochemical demonstration of elevated cytoplasmic alkaline phosphatase (AP) activity (Chiquoine, 1954; Mintz & Russell, 1957) demonstrated a posterior location in head-fold-stage embryos. Moreover, a recent experimental study, in which segments of mouse egg cylinder were isolated and cultured *in vitro*, has indicated that PGC precursors may become confined to the caudal end of the embryo as early as day 7 of development (Snow, 1981).

The question of the cell type of origin of PGCs in the early embryo is unresolved. The majority of authors have described PGCs as appearing first in the embryonic endoderm* overlying the caudal end of the primitive streak at the head-fold stage of development (Witschi, 1948; Chiquoine, 1954; Mintz & Russell, 1957). On the other hand, Ożdżenskí (1967) recognized AP staining in cells situated within the posterior end of the primitive streak in pre-head-fold embryos and, only at later stages, in the overlying endoderm (Ożdżenskí, 1967). This finding has been confirmed in recent years by other workers (Tam & Snow, 1981).

Circumstantial evidence is now accumulating to support the idea that PGCs may segregate from the multipotential embryonic ectoderm and not from the embryonic endoderm adjacent to the primitive streak. For instance, PGCs have been found to resemble migratory mesoderm cells, but not the highly specialized embryonic endoderm cells, at both light and electron microscopic levels (Spiegelman & Bennett, 1973; Clark & Eddy, 1975). X-chromosome inactivation has been shown to occur in PGCs in a manner that is random with respect to the parental origin of the X-chromosome (Johnston, 1981; McMahon, Fosten & Monk, 1981). A similar pattern of X-inactivation is found in embryonic

* Cell lineage relationships in the endoderm of the primitive-streak-stage embryo are complex, possibly involving three distinct cell types: future yolk sac endoderm, future foetal endoderm, and cells that are destined to die during the next 24 h of development (Lawson, Meneses & Pedersen, 1985). For this reason, the term 'embryonic endoderm' will be used in referring to the portion of the endodermal cell layer that overlies the caudal end of the primitive streak.

ectodermal derivatives whereas cells from the endoderm layer of the yolk sac, a derivative of the embryonic endoderm (Gardner & Papaioannou, 1975), undergo preferential paternal X-inactivation (Takagi & Sasaki, 1976; West, Frels, Chapman & Papaioannou, 1977). Studies of mouse chimaeras produced by aggregation of genetically distinct morulae show a positive correlation between the proportion of each genotype that contributes to the adult germ line and to somatic derivatives of the definitive embryo (Ford *et al.* 1975; Falconer & Avery, 1978). These observations suggest that the germ line and embryonic ectoderm share a common stem cell later in development than do either lineage with the embryonic endoderm.

Experimental studies of mouse embryos rendered chimaeric by morula aggregation have shown that individual cleavage-stage blastomeres can contribute daughter cells to both germ line and soma during development (Kelly, 1975). At the blastocyst stage, single cells of the inner cell mass and its derivative the embryonic ectoderm, have been found to produce germ cell chimaerism following injection into host blastocysts (Gardner & Rossant, 1976; Gardner, Lyon, Evans & Burtenshaw, 1985). The potential of embryonic endoderm cells for germ-line colonization has not been tested, however, in blastocyst injection experiments. There is no information available concerning the potential of postimplantation embryonic cells for formation of PGCs.

In recent years, experimental microsurgical methods have become available that permit the introduction of labelled cells into intact early postimplantation mouse embryos (Beddington, 1981; Copp, 1983). Embryos rendered chimaeric by these methods undergo normal development for several days in whole-embryo culture (method of New, Coppola & Terry, 1973) thus making possible studies of cell fate and developmental potential during postimplantation embryogenesis. In the present paper, we describe the application of one of these microsurgical procedures for introducing PGC precursors into embryos at the primitive-streak stage of development. This approach has enabled us to investigate the cell type of origin of PGCs prior to the stage of appearance of the AP marker.

MATERIALS AND METHODS

Source and culture of embryos

Early-allantoic-bud-stage embryos were obtained on day 8 of gestation (day 1 = day of finding a copulation plug) from random bred A/Strong mice and from inbred C3H which were either heterozygous (*C*/*c*) or homozygous (*c*/*c*) at the albino locus. Trophoblast giant cells and Reichert's membrane were removed during dissection but the ectoplacental cone was kept intact. Embryos were dissected, stored and manipulated in alpha-modification of Eagle's Minimal Essential Medium (Stanners, Eliceiri & Green, 1971) containing 10 % foetal calf serum and 0·02 M-Hepes, equilibrated with 5 % CO_2 in air (referred to later as α-MEM).

Embryo culture was by the roller bottle method of New *et al.* (1973) with the following modifications. Up to three embryos were placed in 2·5 ml of undiluted rat serum, prepared by immediate centrifugation of freshly drawn arterial blood (Steele & New, 1974), in 30 ml stoppered glass reagent bottles (Scientific Supplies, UK). Bottles were gassed with 5 % CO_2, 5 % O_2, 90 % N_2, sealed with Vaseline (Boots, UK) and rotated continuously at 37 rev. min^{-1} in

a 38°C incubator. The gas atmosphere was replenished after 12 and 24 h of culture. Rat serum was not changed during the course of an experiment.

Labelling and microsurgical procedures

Donor embryos were labelled, following dissection, by roller culture for 4 h in rat serum containing 5 μCi ml^{-1} methyl-[^{3}H]thymidine, specific activity 2 Ci mmol^{-1} (Radiochemical Centre, Amersham). After labelling, embryos were washed in three changes of α-MEM containing excess cold thymidine ($4{\cdot}7\times10^{-6}$ M) and were dissected further in the same medium.

Donor cell clumps were prepared by the following procedure (Fig. 1). Hand-held fine glass needles were used to isolate the part of the wall of the egg cylinder that contained the posterior end of the primitive streak (defined as the proximal third of the distance from the root of the allantois to the tip of the egg cylinder) together with the allantois and posterior quarter of the amnion (Fig. 1B,C). The edges of this large fragment of egg cylinder (Fig. 1D) were trimmed by the same method to yield a 'minimal primitive streak fragment' (Fig. 1E,F). The minimal fragment comprised, on its concave surface, cells of the primitive streak in continuity with allantois and amnion and, on its convex surface, the embryonic endoderm* (Fig. 1G). These two layers were separated by proteolytic enzyme digestion of the intervening extracellular matrix materials followed by manual dissection: minimal fragments were incubated for 4 min at 4°C in a solution of 0·5 % trypsin and 2·5 % pancreatin (Difco) in calcium- and magnesium-free Tyrode's solution, pH 7·7 (Levak-Svajger, Svajger & Skreb, 1969). After incubation and thorough washing in α-MEM containing excess cold thymidine, the cell layers were peeled apart using hand-held fine glass needles (Fig. 1H). This procedure resulted, in the majority of cases in a clean separation. Any fragments that failed to yield unequivocally coherent cell layers following separation were not used for grafting. Contaminating cells could not be identified in serial histological preparations of representative separated cell layers (not illustrated). Cell counts of such preparations revealed that separated primitive streak layers (including allantois and amnion) contained a mean of $924{\cdot}5 \pm 206{\cdot}0$ cells whereas separated endoderm layers contained $301{\cdot}8 \pm 46{\cdot}9$ cells. Isolated primitive streak and endoderm cell layers were dissected further into 5–8 and 4–6 clumps of cells, respectively, each of which was used for grafting into a single host embryo. By calculation, primitive streak clumps contained 100–200 cells, and endoderm clumps contained 50–75 cells.

Grafts were also performed using ectoderm and mesoderm from an anterolateral region of the embryo (Fig. 1). Isolation of the fragment of egg cylinder, and removal of the overlying endoderm (which was discarded), were performed by the method described above. No attempt was made to separate the ectoderm and mesoderm in these fragments. Lateral ectoderm/mesoderm cell clumps for grafting contained a similar number of cells to primitive streak grafts.

Host embryos were the unlabelled litter mates of donor embryos. Each embryo received a graft of a single donor cell clump by the method described in Fig. 2.

Analysis following culture

Operated and control embryos were cultured until they reached the stage of onset (36–40 h culture) or completion (48 h culture) of axial rotation. Embryos cultured for 36–40 h were fixed, keeping extraembryonic membranes intact, in 95 % ethanol at 4°C for 24 h. Following fixation, embryos were dehydrated, and embedded in paraffin wax (m.p. 56°C). Serial sections, thickness 6 μm, were stained to demonstrate alkaline phosphatase activity using naphthol AS-MX phosphate as substrate (Sigma Diagnostics Procedure No. 85; Ackerman, 1962). Coverslips were mounted with u.v.-inert aqueous mounting medium (Hopkin and Williams, UK) and all AP-positive cells were photographed at ×10–16 using a Zeiss photomicroscope to show their position in the embryo. Coverslips were then removed by soaking slides in distilled water. Slides were treated with 5 % trichloroacetic acid at 4°C for 30 min to remove unincorporated [^{3}H]thymidine, and were coated with autoradiographic stripping film (Kodak AR10). Exposure times ranged from 6 to 10 weeks and were determined separately for each batch of slides by periodic inspection of sections of labelled control embryos. After developing, autoradiograms

* See footnote p. 96.

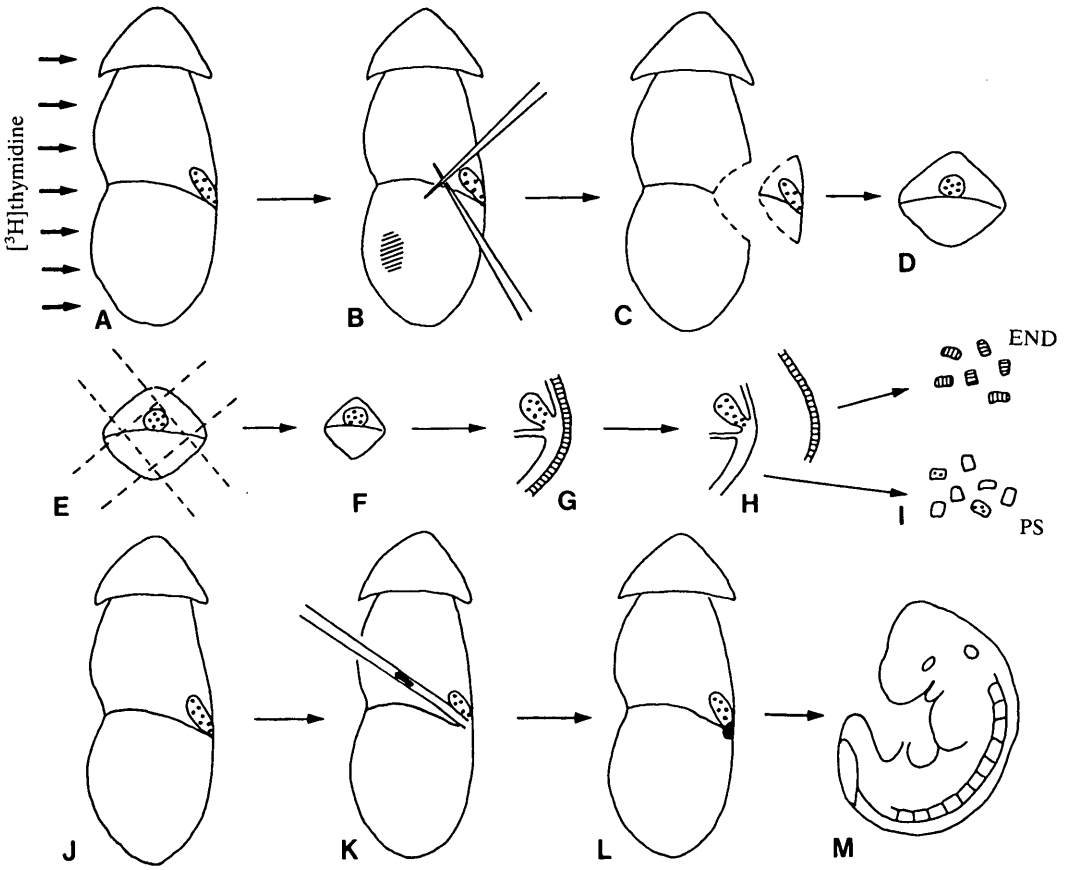


Fig. 1. Diagrammatic representation of the design of the experiment. Embryos at the allantoic bud stage of development were selected from litters dissected on day 8 of gestation and assigned at random to either donor or host embryo groups.

(A) Donor embryos were labelled by culture for 4 h in rat serum containing 5 μCi ml^{-1} [^{3}H]thymidine.

(B,C) Hand-held fine glass needles were used to isolate the posterior end of the primitive streak together with allantois and root of the amnion. Diagonal hatching indicates site of origin of LEM cells.

(D) Frontal view of posterior primitive streak region after isolation from the egg cylinder.

(E,F) Hand-held fine glass needles were used to trim the primitive streak region (along the dashed lines) to produce a 'minimal primitive streak fragment'.

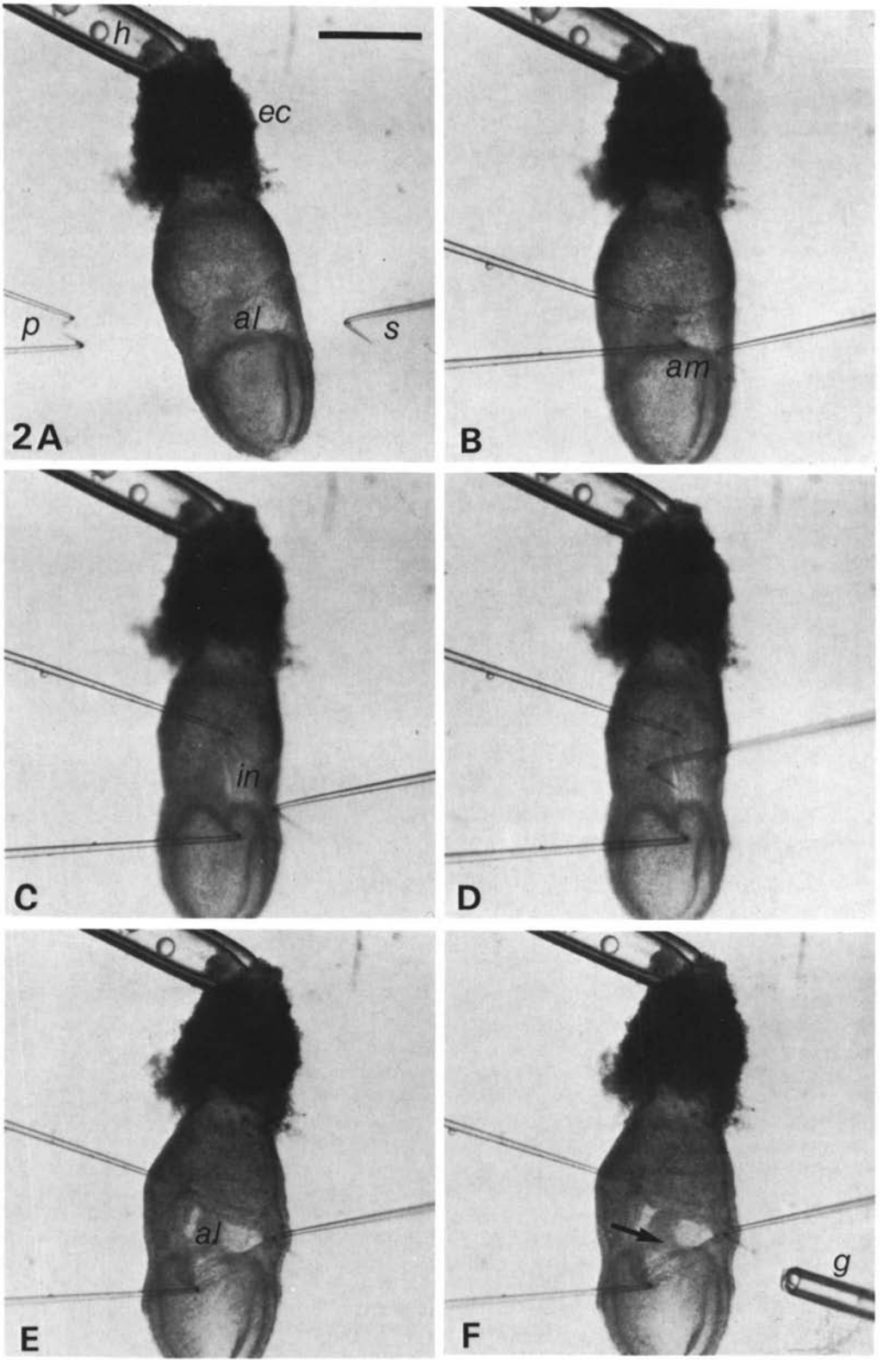
(G) Diagrammatic side view of the minimal fragment (enlarged from F) to show the position of PS (primitive streak, allantois and root of amnion) and END cell layers.

(H) PS and END cell layers were separated by a combination of proteolytic enzyme digestion of intervening extracellular matrix material and dissection with fine glass needles.

(I) PS and END cell layers were subdivided by manual dissection to yield cell clumps for grafting.

(J,K) Unlabelled host embryos received grafts of donor cell clumps by the method shown in Fig. 2.

(L,M) Operated embryos were cultured for 36–40 h to the stage of axial rotation and analysed by sequential histochemical and autoradiographic procedure described in Materials and Methods. Dotted regions represent allantois, cross hatching represents embryonic endoderm.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were mounted in u.v.-inert medium and inspected for the presence of silver grains over AP-positive (PGCs) and AP-negative (somatic) cells. Due to the very low background labelling observed, cells with four or more grains over their nuclei were considered labelled.

Embryos cultured for 48 h were inspected for heart beat, yolk sac circulation and gross malformations, and then were dissected from their extraembryonic membranes in α-MEM. Crown–rump length was measured in the living state using an eyepiece graticule and somites were counted. Embryos were washed in PBS and dissolved in 0·2 N-NaOH at 37 °C; total protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Ożdżenski (1967) and Tam & Snow (1981) described PGCs, recognized on the basis of their high AP activity, as first appearing during development of the mouse embryo at the caudal end of the primitive streak and base of the allantois on day 8 of gestation. In a preliminary histochemical study we confirmed the presence of AP-positive cells at this site in presomite embryos with head folds, but not in embryos at earlier developmental stages (data not shown). The aim of the grafting

Fig. 2. Photomicrographs to show the sequential phases of the grafting procedure. Unlabelled allantoic bud stage embryos were placed individually, or in groups of two or three, together with an equal number of prelabelled donor cell clumps, in drops of α-MEM containing excess cold thymidine under heavy liquid paraffin (Boots, UK) in 90 mm plastic tissue culture dishes. Grafts were performed under ×40 magnification on the stage of a Zeiss dissecting microscope.

(A) A holding pipette (*h*) with flame-polished tip (external diameter 120 μm), controlled by a right-handed Prior micromanipulator, is introduced into the drop of medium from the left side. The embryo is held steady by suction applied to the ectoplacental cone (*ec*) using an Agla micrometer syringe pump. The embryo is positioned so that its posterior aspect lies on the surface of the culture dish. The outline of the allantoic bud (*al*) can be seen through the wall of the exocoelom. A pair of sharp glass needles (*p*) with subterminal right-angled bends are introduced into the drop from the left side and a single right-angled needle (*s*) is introduced from the right side. Both paired and single needles are controlled by Leitz micromanipulator units.

(B) The convergent tips of the paired needles are apposed and lowered onto the anterior (uppermost) surface of the embryo just superior to the insertion of the amnion (*am*) so that the tips pierce the wall of the exocoelom. The embryo is steadied to prevent rotation using the single needle.

(C) The tips of the paired needles are separated to create an incision (*in*) in the wall of the exocoelom parallel to the long axis of the egg cylinder.

(D) The tip of the single right-angled needle is inserted through the incision in the wall of the exocoelom.

(E) The single needle is retracted so that the incision is converted to a triangular window. The allantoic bud (*al*) is now clearly visible on the posterior wall of the egg cylinder.

(F) The egg cylinder is lowered by means of the holding pipette and right-angled needles so that its posterior surface is flattened slightly on the culture dish. A hand-held mouth-controlled grafting pipette (*g*), external diameter approximately 50 μm, containing heavy liquid paraffin for braking purposes, is used to pick up the donor cell clump, create a space at the junction of the allantois with the root of the amnion (arrow) and deposit the graft therein. In cases where the donor cell clump becomes dislodged immediately after grafting, or is imperfectly positioned, the graft can be retrieved using the grafting pipette and repositioned. Following grafting, the embryo is lifted from the culture surface and microinstruments are removed. The window in the wall of the egg cylinder closes rapidly and no evidence could be discerned after 1 h of culture.

Scale bar, 200 μm.

experiment was, therefore, to determine directly the PGC-forming capacity of the cell types comprising the posterior end of the embryo at a developmental stage preceding the appearance of AP staining in PGCs. Grafts were performed at the earliest stage in which it was possible to locate accurately the caudal end of the primitive streak in living embryos: this proved to be the early allantoic bud stage.

The following cell types were assayed for PGC-forming ability following grafting into host embryos: ectoderm and mesoderm cells from the caudal end of the primitive streak (PS), overlying embryonic endoderm (END) and lateral ectoderm/mesoderm (LEM). PS and END together form the caudal end of the definitive embryonic region at the allantoic bud stage of development and are therefore most likely to contain precursors of the PGC lineage. LEM cells were isolated from a region far from the site of first appearance of PGCs. The potential of LEM cells for formation of PGCs was determined by heterotopic grafting into the base of the allantoic bud.

The sequential histochemical and autoradiographic method of analysis enabled individual cells in grafted embryos to be identified as belonging to either PGC or somatic lineages (AP-positive or -negative respectively) and as derived from either donor or host components of the graft combination (labelled or unlabelled respectively).

Colonization of PGC lineage by grafted cells

Donor-derived PGCs were found in embryos that received grafts of PS cells (Table 1) but were not seen following grafting of END (Table 2) or LEM cells

Table 1. *Incidence of chimaerism of PGCs and somatic tissues in embryos that received grafts of PS cells*

		Germ cells			Somatic cells							
					Incorporated						Unincorporated	
Embryo no.	Mouse strain	Number unlabelled	Number labelled	Position of labelled PGCs	GUT	MES	SE	ALL	AMN	YS	VES	NI
1	A/Str	38	0	—				+				
2		52	8	ALL				+				
3		16	0	—				+				
4		45	2	ALL				+				
5		43	4	GUT(2),ALL(2)				+				
6		21	0	—				+				
7		72	6	GUT(4),ALL(2)				+				
8		81	0	—				+	+			
9		29	1	ALL				+	+			
10	C3H	197	7	GUT(2),SE(5)			+	+	+		+	
11		168	1	GUT	+	+		+				
12		258	1	ALL				+				

Abbreviations: GUT, gut endoderm; MES, caudal embryonic mesoderm; SE, surface ectoderm; ALL, allantois; AMN, amnion; YS, yolk sac; VES, unincorporated cellular vesicle; NI, no graft identified.

(Table 3). Among nonoperated embryos, labelled PGCs were seen in labelled but not in unlabelled controls (Table 4; Fig. 4).

Labelled PGCs were located in the gut endoderm (Fig. 3A,B), surface ectoderm (Fig. 3C,D) and allantois (Fig. 3E,F) of colonized host embryos. Typically, labelled PGCs in the gut endoderm were separated spatially from the somatic

Table 2. *Incidence of chimaerism of PGCs and somatic tissues in embryos that received grafts of END cells*

		Germ cells			Somatic cells							
					Incorporated						Unincorporated	
Embryo no.	Mouse strain	Number unlabelled	Number labelled	Position of labelled PGCs	GUT	MES	SE	ALL	AMN	YS	VES	NI
13	A/Str	62	0	—							+	
14		36	0	—							+	
15		49	0	—							+*	
16		44	0	—							+	
17		87	0	—						+†		
18		44	0	—							+	
19		71	0	—							+	
20		50	0	—							+	
21		73	0	—							+	
22		55	0	—							+	
23		117	0	—								+
24	C3H	216	0	—							+	
25		145	0	—							+	
26		273	0	—							+	

* Two separate unincorporated vesicles identified.
† Endoderm of yolk sac colonized.
Abbreviations as for Table 1.

Table 3. *Incidence of chimaerism of PGCs and somatic tissues in embryos that received grafts of LEM cells*

		Germ cells			Somatic cells							
					Incorporated						Unincorporated	
Embryo no.	Mouse strain	Number unlabelled	Number labelled	Position of labelled PGCs	GUT	MES	SE	ALL	AMN	YS	VES	NI
27	A/Str	143	0	—					+			
28		22	0	—		+		+				
29		48	0	—				+	+			
30		43	0	—								+
31		55	0	—								+
32		30	0	—								+
33	C3H	137	0	—						+*		
34		227	0	—								+
35		195	0	—								+

* Mesoderm of yolk sac colonized.
Abbreviations as for Table 1.

Table 4. *Incidence of labelling of PGCs in nonoperated labelled and unlabelled control embryos*

Embryo no.	Mouse strain	Germ cells: Number unlabelled	Germ cells: Number labelled
Unlabelled embryos			
36	A/Str	187	0
37		29*	0
38		124	0
39		108	0
40	C3H	174	0
41		180	0
Labelled embryos			
42	A/Str	3	59
43		4	53
44		8	98
45	C3H	18	65
46		31	68

* Embryo had caudal agenesis.

component of the graft (only one labelled PGC was adjacent to labelled somatic cells) and in all cases were closely associated with numerous unlabelled PGCs in the gut (Fig. 3A,B). It appears that labelled PGCs in the gut are participating in the migratory stream of host-derived PGCs that is destined to populate the genital ridges. By contrast, labelled PGCs in the allantois and surface ectoderm, in all

Fig. 3. Photomicrographs of embryos cultured for 36–40 h from the allantoic bud stage. Sections stained to demonstrate AP activity.

(A) Transverse section through mid-trunk region of embryo No. 7 which received a graft of PS cells. A group of PGCs, stained heavily for AP activity, is visible in the gut endoderm (*g*). Neural tube (*nt*) and somitic mesoderm (*sm*) are also stained for AP, although less heavily than the PGCs. Scale bar, 50 μm.

(B) High-magnification view of PGCs outlined in (A) showing labelling (15 grains over the nucleus) of a single PGC (arrow). Other PGCs and neighbouring somatic cells are unlabelled. Scale bar, 10 μm.

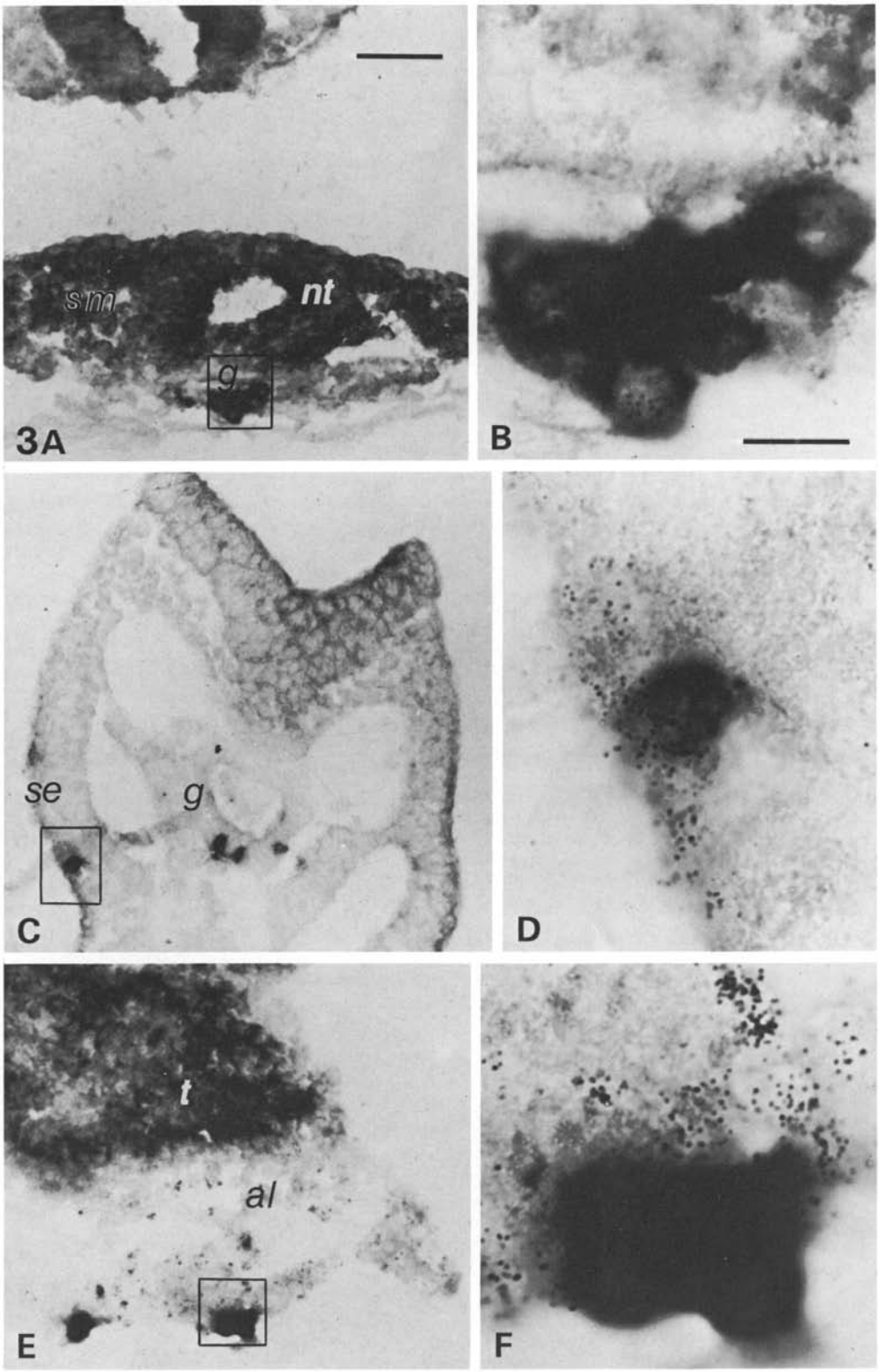
(C) Transverse section through posterior neuropore region of embryo No. 10 which received a graft of PS cells. PGCs are visible in the gut endoderm (*g*) and surface ectoderm (*se*, outlined). Magnification as in (A).

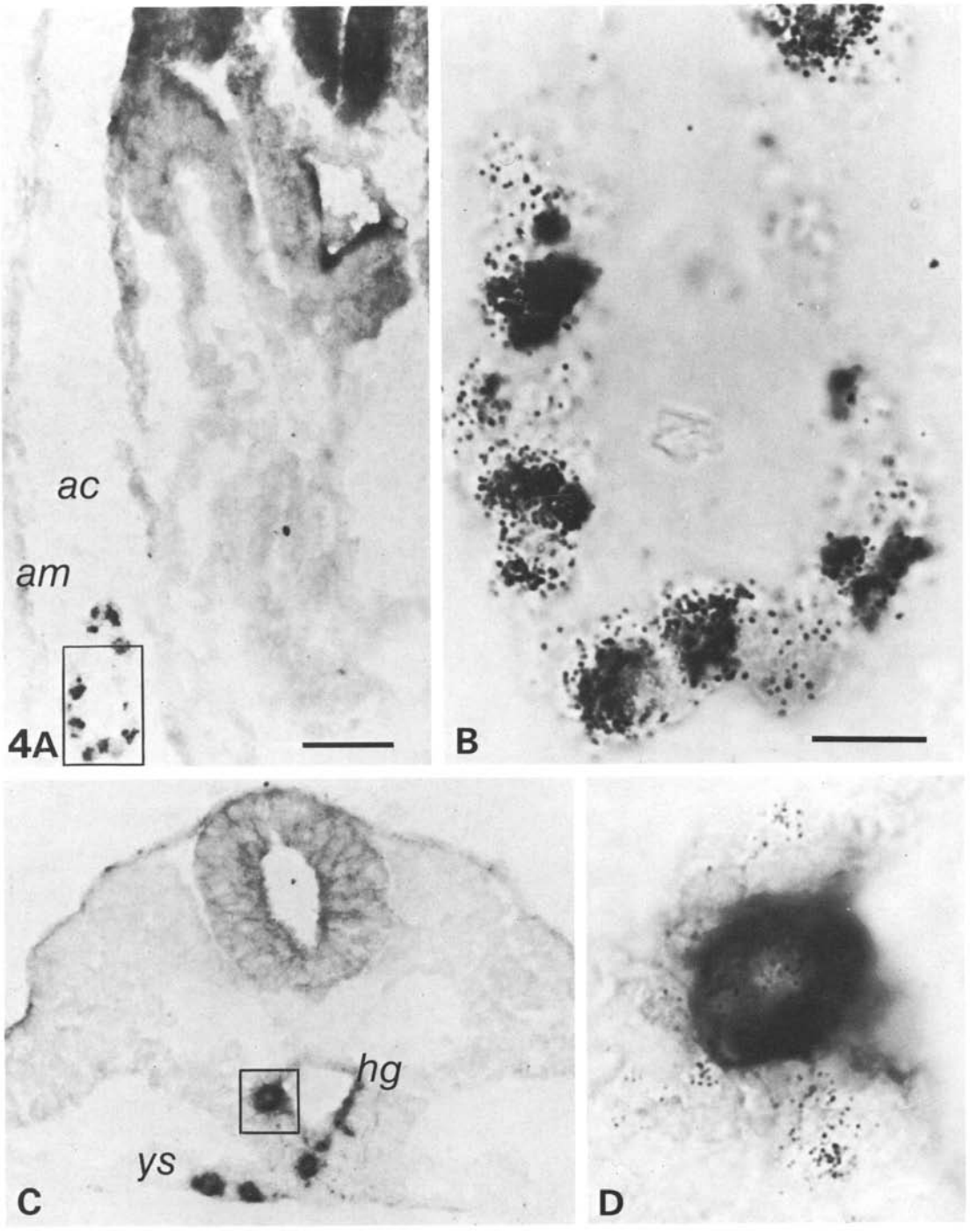
(D) High-magnification view of area outlined in (C). The PGC and adjacent surface ectoderm cells are labelled whereas underlying mesodermal cells are unlabelled. PGCs in the gut, in this section, were unlabelled. Magnification as in (B).

(E) Section through the junction between trophoblast (*t*) and allantois (*al*) of embryo No. 2 which received a graft of PS cells. Two groups of PGCs are visible at the edge of the allantoic region. The majority of allantoic cells are labelled. Trophoblastic cells are stained for AP, although less heavily than the PGCs. Magnification as in (A).

(F) High magnification view of area outlined in E. All four PGCs have grains over their nuclei although the intensity of AP staining hampers their visualization in a single focal plane. The majority of adjacent allantoic cells are labelled. Magnification as in (B).

cases, showed a persistent association with labelled somatic components of the graft (Fig. 3C–F). Only a minority of such PGCs (9 out of 21) were found in close proximity to more than a single unlabelled PGC. It would appear that labelled

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PGCs in the allantois and surface ectoderm did not undergo significant migration following grafting.

Extent of labelling and numbers of PGCs in grafted and control embryos

Labelled PGCs comprised a small percentage (0·4 to 13·3 %) of the total PGC population of colonized embryos. An additional subpopulation of donor-derived PGCs may have gone undetected in grafted embryos: around 20 % of PGCs in nonoperated labelled control embryos were found to be unlabelled following the 36–40 h culture period. Nevertheless, it seems unlikely that the true incidence of donor-derived PGCs exceeded 20 % in any of the PGC-chimaeric embryos. This low incidence of PGC colonization in grafted embryos may be attributed to the following factors: (a) the procedure, adopted routinely in the experiments, of subdividing the primitive streak fragment of a single donor embryo into 5–8 clumps of cells for grafting into separate host embryos. Thus, each donor cell clump may have contained relatively few PGC precursors; (b) loss of PGCs due to trauma during grafting; (c) competition between donor- and host-derived PGCs following grafting.

The total number of PGCs varied between groups of embryos in two ways (Tables 1–4): among A/Strong embryos, those that received grafts contained significantly fewer PGCs than nonoperated controls (operated, $55{\cdot}7 \pm 28{\cdot}8$; nonoperated, $112{\cdot}0 \pm 65{\cdot}0$; $t = 3{\cdot}04$, d.f. $= 28$, $P < 0{\cdot}01$). A similar comparison among C3H embryos revealed no significant difference although in this case small numbers of embryos were studied. It seems possible, therefore, that the grafting procedure may be detrimental to survival and, or, proliferation of host-derived PGCs. There was also a significantly reduced number of PGCs in A/Strong embryos when compared with C3H embryos (operated and nonoperated embryos combined: A/Strong, $63{\cdot}2 \pm 39{\cdot}1$, C3H, $198{\cdot}1 \pm 43{\cdot}4$; $t = 9{\cdot}51$, d.f. $= 39$, $P < 0{\cdot}001$). It is unclear whether this strain difference in the extent of increase of PGC number can be attributed to a variation in the rate of PGC proliferation and, or, cell death *per se*, or whether there is a greater susceptibility of A/Strong PGCs to the culture conditions used in the experiments.

Colonization of somatic tissues following grafting (Tables 1, 2, 3)

PS grafts colonized the allantois in all cases (Fig. 3E) and in addition formed part of the amnion in three embryos and surface ectoderm and caudal mesoderm

Fig. 4. Photomicrographs of embryos cultured for 36–40 h from the allantoic bud stage. Sections stained to demonstrate AP activity.

(A) Transverse section through anterior region of embryo No. 18 which received a graft of END cells. A vesicle of labelled cells is visible in the amniotic cavity (*ac*). Cells of the definitive embryo and of the amnion (*am*) were unlabelled. Scale bar, 50 μm.

(B) High magnification view of area outlined in (A). Cells of the unincorporated vesicle are heavily labelled and AP negative. Scale bar, 10 μm.

(C) Transverse section through posterior region of nonoperated labelled control embryo No. 45. PGCs are visible in the hind gut (*hg*) and yolk sac (*ys*) endoderm. Magnification as (A).

(D) High magnification view of area outlined in (C). Grains are visible over the nuclei of the PGC, gut endoderm cells and adjacent mesoderm cells. Magnification as (B).

of the definitive embryo in two cases. In a single embryo some graft-derived cells were visible as an unincorporated vesicle of heavily labelled cells attached to the cranial neural folds.

END grafts produced a very different result. In only one case, donor-derived cells were found to be integrated into the host embryo, forming part of the yolk-sac endoderm. In the majority of embryos, grafts were present as vesicles of heavily labelled cells attached either to extraembryonic structures (six cases) or to parts of the definitive embryo (two cases), or else unattached within the yolk sac cavity (one case) or in the amniotic cavity (three cases, Fig. 4A,B).

Five out of nine LEM grafts failed to colonize the host embryo. Of the other four grafts, the pattern of somatic colonization was similar to that of PS grafts.

Intermingling of host and donor cells was noted in caudal embryonic mesoderm and allantois of chimaeric embryos. In contrast, donor cells in the amnion and surface ectoderm showed a more coherent distribution. This difference has been observed previously by Beddington (1982) and suggests that there may be a greater extent of cell mixing during the development of mesenchymal than epithelial tissues in the early embryo.

The lack of clear definition of individual somatic cells using the AP histochemical method precluded accurate quantitation of somatic colonization in grafted embryos. However, cells belonging to incorporated grafts were found to have a similar density of labelling as labelled control embryos whereas cells in unincorporated vesicles exhibited considerably heavier labelling (e.g. compare Figs 3D and 4B). This finding suggests that there was a greater increase in cell numbers in grafts that became incorporated into host embryos than in grafts that remained as unincorporated vesicles.

There was no correlation between the apparent extent of somatic colonization (as judged by the number of serial sections occupied by the graft) and the presence of PGC chimaerism. It seems likely that colonization of the PGC lineage reflects the presence of cells competent for formation of germ cells and not merely the state of well being of the graft.

Effect of grafting, labelling and culture on embryonic growth and development (Table 5)

In culture experiments, unlabelled and grafted embryos did not differ in parameters of growth or developmental progression (t-tests, $P > 0{\cdot}05$) indicating that the grafting procedure is not detrimental to subsequent development *in vitro*. Labelling with [^{3}H]thymidine had no deleterious effects on growth or increase in somite number but the incidence of malformations was somewhat higher than in unlabelled embryos. 38 % of cultured embryos initiated, but failed to complete, axial rotation during the culture period. The incidence of this abnormality did not differ among the three groups of cultured embryos and is probably a nonspecific complication of cultures begun at primitive streak, rather than the later head-fold, stage of development (Buckley, Steele & New, 1978).

Embryos that developed entirely *in vivo* were significantly larger ($t = 5{\cdot}0$, d.f. = 39, $P < 0{\cdot}001$), contained more protein ($t = 7{\cdot}5$, d.f. = 51, $P < 0{\cdot}001$) and had more somites ($t = 3{\cdot}0$, d.f. = 52, $P < 0{\cdot}005$) than cultured embryos. *In vivo* control and cultured embryos are not, however, strictly comparable. Embryos were preselected for culture: only those at the early allantoic bud stage were chosen for experiments and littermates at more advanced stages of development were excluded. *In vivo* control embryos, by contrast, were obtained unselected from entire litters. It is difficult to assess, therefore, how far the observed difference in growth parameters between *in vivo* and cultured embryos is due to relative growth retardation *in vitro*.

DISCUSSION

Microsurgical grafting and embryo culture techniques in the study of PGC development

Two microsurgical methods have been described in recent years for producing chimaerism in early postimplantation embryos (Beddington, 1981, 1982; Copp, 1983). Using one of these techniques, we have found that cells from the caudal end of the primitive streak, but not from the adjacent embryonic endoderm, give rise to migrating PGCs when grafted orthotopically into host embryos at the allantoic

Table 5. *Parameters of growth and developmental progression in embryos cultured for 44 h or developed entirely* in vivo

	Embryonic growth		Developmental progression				
				Number of embryos with:			
Embryo type	CR length*	Protein content*	Somite number*	Heart beat	Yolk sac circulation	Axial rotation	Malformations
1. Cultured embryos							
Unlabelled	4·0 ± 0·4 (9)†	48·7 ± 13·7 (13)	17·4 ± 2·1 (15)	15 (15)	15 (15)	9 (15)	Pericardial oedema (1)
Labelled	4·8 ± 0·3 (6)	58·1 ± 19·7 (9)	20·3 ± 0·8 (7)	9 (9)	9 (9)	6 (9)	Caudal agenesis (2), epidermal blebs (2)
Grafted	4·2 ± 0·2 (10)	50·6 ± 10·6 (15)	18·2 ± 1·3 (16)	16 (16)	16 (16)	10 (16)	Forebrain hypoplasia (1)
2. *In vivo* control embryos							
	5·0 ± 0·4 (16)	91·8 ± 24·3 (16)	19·9 ± 1·5 (16)	16 (16)	14 (16)	16 (16)	None

* Mean ± standard deviation.

† Number of embryos analysed.

CR length: crown–rump length in arbitrary units, determined only for embryos that completed axial rotation.

Protein content in μg.

Somite number could not be determined for embryos with caudal agenesis.

bud stage of development. The method described in this paper for producing specific chimaerism of the PGC lineage in postimplantation embryos, when combined with the technique of whole-embryo culture (New *et al.* 1973), provides an experimental system in which a variety of questions may be asked concerning PGC development. For instance, it is unclear whether sex differences between germ line and soma may affect the migration of PGCs. PGC migration appears to proceed normally in mouse chimaeras produced by aggregating XX and XY morulae (Mystkowska & Tarkowski, 1970; McLaren, Chandley & Kofman-Alfaro, 1972). However, germ line and soma were both chimaeric in these embryos, raising the possibility that interactions between somatic and germ cells of the same genotypic sex may have promoted migration of PGCs to the genital ridges. In the present study, approximately half of the PGCs that arose from PS grafts exhibited migration towards the genital ridges of host embryos alongside host-derived PGCs. Experiments are underway to determine whether operated embryos in which PGC migration occurred may represent 'sex compatible' graft–host combinations (i.e. XX< >XX and XY< >XY) whereas grafted embryos that contained nonmigratory donor-derived PGCs may be of the 'sex incompatible' type (i.e. XX< >XY).

Technical considerations in the grafting experiments

The usefulness of [^{3}H]thymidine as a marker of cell lineage is limited by problems of cytotoxicity and dilution with successive cell cycles. In the present experiments, although labelling medium with low specific activity was utilized to minimize damage to embryonic cells, around 80% of PGCs, and a similar percentage of somatic cells, were found to be labelled above background after 36–40 h of culture. It is known that autoradiograms employing thick histological sections rarely yield labelling indices of 100%, even for populations of cells that are expected to have undergone full incorporation of tritiated precursor (Modak, Lever, Therwath & Uppuluri, 1973). A variable number of nuclei lie outside the 0·6 to 1·5 μm autoradiographic range and, although radioactive, give only background grain counts. The percentage of apparently unlabelled nuclei in autoradiograms is proportional to section thickness and related inversely to nuclear diameter (Modak *et al.* 1973). In the present study, the use of 6 μm sections, together with a PGC nuclear diameter of approximately 10 μm (see Clark & Eddy, 1975), leads to an expectation of around 70% of nuclei falling within the tritium autoradiographic range (Modak *et al.* 1973). The observed labelling index of 80% probably represents, therefore, incorporation of [^{3}H]thymidine by essentially all PGC precursors during the labelling period.

It is possible that donor-derived PGCs may have gone undetected in host embryos receiving END grafts due to the failure of PGC precursor cells to incorporate [^{3}H]thymidine during the labelling period. If this were the case, unlabelled PGCs would be expected among labelled somatic cells derived from such grafts. AP-positive cells were never seen within unincorporated END vesicles making this hypothesis appear unlikely. A related question is whether labelled

AP-positive cells represent host-derived PGCs that became labelled following grafting. LEM cells could be expected to release potential radioisotopic precursors in a manner similar to PS cells but did not yield labelled PGCs. 'Secondary labelling' was, therefore, probably not an important factor in the present experiments.

The use of elevated AP activity as a histochemical marker for PGCs has been criticized on the grounds that it is not specific for the germ line (e.g. Heath, 1978). In the present study, apart from putative PGCs, trophoblast, neurectoderm and somitic cells were AP positive but could be distinguished from PGCs by their well-defined positions in the embryo and the lesser intensity of their AP staining. Independent evidence that AP histochemistry identifies PGCs in the present study comes from the finding of migratory behaviour, which characterizes pregonadal PGCs, in a proportion of the AP-positive donor-derived cells.

It was noted during preparation of cell clumps for grafting that isolated END cell layers, both before (Fig. 1H) and after (Fig. 1I) subdivision into cell clumps, showed a marked tendency to roll up into spheroids with apical cell surfaces outermost. It seems probable that the observed failure of END grafts to incorporate into host embryos may be attributed to this rapid formation of cellular vesicles. The consequent sequestration of the basal cell surfaces which, during undisturbed development are responsible for the interaction with underlying PS cells, may have precluded formation of cellular contacts necessary for integration of the grafted cells. It is possible that PGC formation is prevented by failure of incorporation of grafted cells into the host embryo. However, Snow (1981) has demonstrated the appearance of AP-positive PGCs in isolated fragments of day-7 egg cylinders undergoing allantoic differentiation *in vitro*. In preliminary experiments (A.J.C., unpublished), we have introduced PS fragments into the exocoelom of day-8 embryos and find that PGC differentiation is not precluded by lack of graft incorporation: although the clumps of PS cells became attached to extraembryonic membranes, as was the case with many END grafts, several clumps contained PGCs as judged by AP staining.

Emergence of the PGC lineage during early mammalian development

The results of the present study, taken together with the blastocyst injection experiments of Gardner & Rossant (1976) and Gardner *et al.* (1985) indicate strongly that mouse PGCs separate from the multipotential embryonic ectodermal lineage, *via* caudal end of the primitive streak, during early postimplantation development. Although an origin of PGCs from the embryonic endoderm cannot be definitely ruled out, the positive identification of PGC precursors in embryonic ectoderm at both blastocyst and primitive streak stages of development, together with the failure of endoderm grafts to form PGCs in the present study, make this possibility appear unlikely.

Snow (1981) has suggested that cells in the posterior region of the embryonic ectoderm become 'allocated' to the formation of PGCs by the early-primitive-streak stage of development. This conclusion was based on experiments in which

fragments of embryonic egg cylinder were grown in isolation *in vitro*: PGCs developed only in certain fragments obtained from the posterior end of the embryo. Moreover, embryos from which these fragments were extirpated failed to develop PGCs. It is not clear whether this apparent localization of cellular developmental potential for PGC formation indicates that cells have undergone commitment to PGC differentiation, as suggested by Snow (1981). An alternative explanation is that ectoderm cells at this embryonic stage remain developmentally labile and that their subsequent pathway of differentiation is dictated by local cellular interactions. For instance, in Snow's experiment, all isolated embryonic fragments contained embryonic endoderm in addition to embryonic ectoderm cells. Posterior endoderm may have influenced adjacent ectoderm cells to promote PGC differentiation in posterior fragments, whereas anterior endoderm may lack this inductive property.

The grafting experiments in the present study provide a direct test of the potential of ectoderm cells for formation of PGCs in the primitive-streak-stage embryo. LEM cells, isolated from a region outside the posterior primitive streak, yielded somatic derivatives but not PGCs after heterotopic grafting to the posterior primitive streak site. In contrast, PS cells grafted orthotopically to the same site yielded PGC chimaerism in the majority of cases. It would appear, therefore, that anterolateral ectoderm and mesoderm cells of the early allantoic bud stage embryo, under the experimental conditions utilized in this study, are unable to form PGCs.

A further question relates to the state of commitment of posterior primitive streak cells with regard to formation of PGCs *versus* somatic derivatives. PS cell clumps were produced by subdividing each posterior primitive streak region into five to eight pieces. It would be expected that a hypothetical localized subpopulation of cells competent to form PGCs, existing among a majority of cells committed to somatic differentiation, would be included in relatively few of the grafts. The finding of PGC formation by 75 % of PS grafts is not consistent with such a hypothesis. Alternatively, subpopulations of cells, already committed to somatic or PGC differentiation, may be codistributed within the posterior primitive streak. Support for this idea comes from the observation of Ożdżenski (1967) that AP-positive cells, at their first appearance in the posterior primitive streak, are intermingled with AP-negative cells. On this hypothesis, most PS grafts in the present study would have contained both PGC and somatic precursors and would be expected to yield both PGC and somatic chimaerism in high frequency, in agreement with the findings. A third possibility, that cells in the posterior primitive streak region are uncommitted as regards PGC or somatic differentiation, is also consistent with the findings of the grafting experiments. In this case, PS grafts would have contained cells competent to form both PGCs and somatic cells; the precise mode of differentiation taken by individual donor cells was presumably dictated by cellular interactions operating within the host embryo following grafting. A final answer to the question of the state of commitment of posterior primitive streak cells requires an approach in which the differentiative

ability of single cells is assayed in both normal and abnormal developmental environments.

Pattern of somatic colonization

PS cells contributed to the allantois of all chimaeric embryos and in addition formed amnion and definitive embryonic structures in a few cases. This pattern of colonization is similar to that observed by Beddington (1982) following orthotopic injection of cells into the posterior end of the primitive streak. In the latter experiment, however, colonization was more often of embryonic rather than extraembryonic structures. This difference may be attributed to the varying microsurgical approaches used for introduction of donor cells in the two studies: Beddington (1981, 1982) injected cell clumps directly through the wall of the egg cylinder whereas grafts in the present study were positioned at the base of the allantois by an approach through the exocoelom. The more caudal positioning of grafts in the present study probably accounts for the increased tendency to extraembryonic, particularly allantoic, colonization that was observed. Support for this idea comes from the finding of Snow (1981) that the most posterior embryonic fragments from primitive-streak-stage embryos form allantois when cultured *in vitro*.

Heterotopic grafts, of LEM cells, to the posterior embryonic site produced colonization of host embryos with lower frequency than orthotopic grafts, as was also noted by Beddington (1982). LEM cells that colonized host embryos contributed to a similar spectrum of cell types as PS cells. This result provides support for the suggestion of Beddington (1982) that ectoderm cells are unlikely to be committed to particular developmental fates, with regard to their somatic derivatives, according to their position in the primitive-streak-stage embryo.

The failure of most END grafts to integrate into host embryos precluded an accurate assessment of the somatic developmental potential of END cells. The morphological resemblance of unincorporated END vesicles to yolk sac endoderm vesicles, described by Sherman (1975), is consistent with the previous findings that embryonic endoderm isolated from the early egg cylinder is committed to formation of yolk sac endoderm (Rossant, Gardner & Alexandre, 1978).

We thank Dr Rosa Beddington for commenting on the manuscript. This work was supported by grants from the Mental Health Foundation, The Association for Spina Bifida and Hydrocephalus, Action Research for the Crippled Child and the Royal Society.

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(*Accepted 13 February 1986*)