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Commentary

Commentary on Tam and Zhou, 1996

For this commentary on classic papers published by *Developmental Biology* over the years, I chose to highlight this manuscript by Tam and Zhou (1996) on the plasticity of epiblast (EPI) cell contribution to the germ cell lineage in the mouse embryo. I teach this paper in my Developmental Biology class and students always love it.

It had been known for some time that single EPI cells transplanted from one preimplantation embryo to another could give rise to both primordial germ cells (PGCs) and somatic tissues (Gardner, 1968). Following implantation into the uterus however, heroic fate mapping experiments using dye injections indicated that only EPI cells neighboring the extraembryonic ectoderm (ExE) could give rise to PGCs, raising the possibility that these cells might be induced by an extraembryonic signal (Lawson and Hage, 1994). In this important paper, Tam and Zhou (1996) addressed the issue conclusively, by performing technically challenging transplantation experiments in gastrulating embryos. They found that distal EPI cells, far removed from the ExE, could form PGCs when transferred to a proximal location. Conversely, proximal EPI cells transplanted to a distal site generally did not form germ cells. These experiments indicated that EPI cells are plastic with respect to cell fate and depend on their localization in the embryo to form PGCs. This, in turn, strongly suggested that PGCs are formed at this stage through an induction process in response to a signal from the ExE. The paper was quite influential and had a marked impact on the field, as it set the stage for the search for such an inducer, subsequently shown to be BMP signaling (Lawson et al., 1999).

The paper by Tam and Zhou, and many before it, was based on transplantation experiments and the formation of chimeras to address the question of prospective potency. Classic papers such as this one are worth revisiting, as such approaches are the foundation of our knowledge about mammalian development.

References

Gardner, R.L., 1968. Mouse chimeras obtained by the injection of cells into the blastocyst. Nature 220, 596–597.

Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., Hogan, B.L., 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 13, 424–436.

Lawson, K.A., Hage, W.J., 1994. Clonal analysis of the origin of primordial germ cells in the mouse. Ciba Found. Symp. 182, 68–84 discussion 84-91.

Tam, P.P., Zhou, S.X., 1996. The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev. Biol. 178, 124–132.

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The Allocation of Epiblast Cells to Ectodermal and Germ-Line Lineages Is Influenced by the Position of the Cells in the Gastrulating Mouse Embryo

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The developmental potency of cells in the proximal and distal regions of the epiblast of pre- and early-primitive-streak-stage mouse embryos was assessed by their differentiation in the host embryo following orthotopic and heterotopic cell transplantation. Normally, cells in the distal epiblast differentiate predominantly into neuroectoderm and surface ectoderm. However, when they were transplanted to proximal regions of the epiblast, distal epiblast cells behaved like proximal epiblast cells: they colonised the extraembryonic mesoderm and other mesodermal tissues in the posterior region of the host embryo. In addition, about 3.7% of the transplanted distal epiblast cells differentiated into primordial germ cells. This proportion is comparable to the 3.9% of orthotopically transplanted proximal epiblast cells that became primordial germ cells. When proximal epiblast cells were transplanted heterotopically to distal sites, their descendants were generally absent from the extraembryonic mesoderm and the germ cell population of the host embryo. Like cells in the distal epiblast, they mostly colonised the neural plate and surface ectoderm. This plasticity of cell fate suggests that the epiblast cells are not irreversibly allocated to any specific lineages, including the germ line. The adoption of developmental fate that is typical of the cell population at the site of transplantation suggests that the specification of cell lineages is subject to certain site-specific influences in the epiblast. Allocation of cells to the ectodermal and germ cell lineages may be subject to local tissue interactions and the restriction of morphogenetic tissue movement of different epiblast cell populations during gastrulation.

INTRODUCTION

Fate-mapping studies of the gastrulating mouse embryo have revealed that the precursor populations for major tissue types are localised to specific regions in the epiblast, which contains approximately 660 cells (Lawson *et al.,* 1991; Lawson and Pedersen, 1992a,b; Quinlan *et al.,* 1995; Parameswaran and Tam, 1995). Fate maps of the epiblast have been constructed to depict the geographical organisation of the progenitor cells of various embryonic tissues. The maps also provide a description of the multitude of differentiation pathways and morphogenetic movements that cells will normally display during embryogenesis. For example, cells in the distal part of the epiblast of an early-primitive-streak-stage embryo will expand during germ layer morphogenesis to occupy most parts of the embryonic ectoderm by the late-primitive-streak-stage and eventually form the neuroectoderm and surface ectoderm of the early-somite-stage embryo (Quinlan *et al.,* 1995). By contrast, epiblast cells in the proximal region will ingress at the primitive streak and give rise to extraembryonic mesoderm while those cells in the lateral epiblast midway along the proximal–distal axis contribute mostly to embryonic mesoderm (Lawson *et al.,* 1991, Lawson and Pedersen, 1992b, Parameswaran and Tam, 1995).

Clonal analysis of cell differentiation in the gastrulating mouse embryo reveals that the precursors of primordial germ cells (PGCs) are localised in the proximal region of the epiblast close to the extraembryonic ectoderm (Lawson and Hage, 1994). PGCs are first identified as a cluster of alkaline phosphatase active cells within the extraembryonic mesoderm near the posterior part of the primitive streak (Chiquoine, 1954; Ginsburg *et al.,* 1990). Cell fate analysis further reveals that the germ-cell precursors are localised amongst precursors of extraembryonic mesoderm and are geographically separated from precursors of embry-

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onic tissues (Lawson *et al.,* 1991; Lawson and Pedersen, 1992a,b; Parameswaran and Tam, 1995). This strategic localisation of the germ cell precursors has provoked an interesting notion that the isolation is critical for the conservation of pluripotency of the germ-line cells by sparing them from the forces driving somatic cell differentiation and tissue patterning (Grant *et al.,* 1992; Dixon, 1994). If this sequestration is indeed critical to the existence of the germ-cell lineage, some localised environmental factors in the proximal epiblast may be involved with the maintenance of a preexisting germ line in the embryo.

Results of clonal analysis and assessment of cell fate by heterotopic transplantation have shown that epiblast cells display remarkable plasticity in lineage differentiation. Descendants of individual epiblast cells are not restricted to one particular cell lineage but are distributed to multiple lineages of different germ layer origins (Lawson *et al.,* 1991; Lawson and Pedersen, 1992a). Epiblast cells can also acquire a novel cell fate after heterotopic transplantation by giving rise to embryonic tissues that are different from those predicted by their normal fate (Beddington, 1982; Parameswaran and Tam, 1995). The adoption of different developmental fates by transplanted epiblast cells suggests that certain instructive signals or local tissue influences might have a significant impact on lineage differentiation during gastrulation. It is not known whether or not the plasticity of developmental fate is confined to the precursors of the somatic lineages. If similar plasticity is displayed by the germ cell precursors, then the specification of germ-line cells in the proximal epiblast is also subject to local tissue influence. To gain insight into this issue, it is imperative to resolve first if the conditions required for germ cell determination are found only locally in the proximal epiblast and second, if cells other than those in the proximal epiblast may respond to the specification signals. Since only cells in the proximal epiblast have been tested by clonal analysis for the formation of germ cells, it is not clear whether this germ-line potency is unique to the proximal epiblast cells. In the present study, the developmental potency of the epiblast cells was tested by confronting the cells with different tissue environments. This was accomplished by transplanting cells to heterotopic sites in the epiblast of gastrulating embryos. We have compared the differentiation of two epiblast populations with vastly divergent fates: the distal epiblast that gives rise to ectodermal tissues versus the proximal epilast that differentiates into extraembryonic mesoderm and primordial germ cells. The histogenetic potency of the epiblast cells was assessed by examining the pattern of tissue colonisation displayed by descendants of the transplanted cells in the host embryo. Specific attention was given to the formation of PGCs in the heterotopic transplantation experiments.

MATERIALS AND METHODS

Embryo Dissection, Micromanipulation, and Culture

Pre- and early-primitive-streak-stage embryos were explanted from pregnant transgenic *HMG–lacZ* mice (Tan *et al.,* 1993; Tam *et al.,* 1994) and ARC/s mice at 6.5 days pc in PB1 medium + 20% (v/v) foetal calf serum. The transgenic donor embryos were dissected into three fragments by cutting across the egg cylinder as indicated in Fig. 1. The epiblast and the endoderm layers of the fragments were separated by dissecting using fine glass needles. Clumps of 5–20 cells isolated from the distal and the proximal epiblast were transplanted to early-primitive-streak-stage ARC/s mouse embryos using micromanipulators (Leica). Four types of transplantations were performed (Fig. 1A): (a) proximal epiblast cells to the lateral and posterior regions of the proximal epiblast (P → P), (b) distal epiblast cells to the distal part of the epiblast (D → D), (c) proximal epiblast cells to distal sites (P → D), and (d) distal epiblast to proximal sites (D → P). The host embryos were checked immediately after transplantation to ascertain the proper positioning of the graft. Some embryos were cultured without any cell transplantation. They served as control for *in vitro* development and alkaline phosphatase detection of PGCs.

Embryos were cultured in NUNC four-chamber slides at 37°C under 5% CO_2 in air for 45–48 hr. The culture medium is made up of Dulbecco's modified Eagle's medium, 25% (v/v) heat-inactivated human cord serum, and 50% (v/v) rat serum (Sturm and Tam, 1993).

Detection of lacZ-Expressing Cells by Enzyme Histochemistry of β-Galactosidase

Cultured embryos were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) for 3–5 min. The yolk sac and amnion were punctured at several places using fine alloy needles to enhance the penetration of reagents. Fixed embryos were washed twice in PBS and once in washing buffer containing NP-40 detergents, then stained for 6–16 hr in X-gal staining solution (0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase, 2 m*M* $MgCl_2$, 5 m*M* EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Nonidet P-40, 5 m*M* $K_3Fe(CN)_6$, and 5 m*M* $K_4Fe(CN)_6 \cdot 6H_2O$; Cepko *et al.,* 1993). Whole embryos were assessed for the pattern of distribution of the graft-derived cells and then processed for wax histology. Serial 8-μm sections were examined after counterstaining with 0.1% (w/v) nuclear fast red.

Combined Detection of lacZ-Expression and Primordial Germ Cell Markers

Whole mount staining. Embryos were fixed as for X-gal staining. Specimens were washed twice in fresh PBS, washed once in Tris buffer (0.1 *M* Tris–Cl, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5), and incubated with AP-Red substrate (Boehringer-Mannheim, No. 1465 341) for 30 min at room temperature. After colour development, the embryos were washed twice in PBS and once in X-gal washing buffer. Embryos were stained overnight at 37°C in X-gal solution and examined for the distribution of the X-gal-stained and alkaline phosphatase-active cells.

Alkaline phosphatase histochemistry and immunostaining for β-galactosidase. Embryos were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in PBS for 5–10 min, washed twice in PBS, dehydrated in ethanol, and embedded in water-miscible polyester wax (melting point at 37°C, BDH). Serial 7-μm sections were dewaxed and hydrated in a descending ethanol series. Alkaline phosphatase activity was detected by incubation with Vector-Red enzyme substrate (Vector Lab, No. SK-5100) at 37°C for 30–45 min. The sections were washed in running distilled water. For immunostaining, PBS-washed sections were treated with 10% goat normal serum and incubated with rabbit

IgG anti-β-galactosidase antibody (5 μg/ml, Cappel No. 55976). The sections were washed with PBS + 0.2% BSA and then incubated with goat biotinylated anti-rabbit IgG (Vector No. BA-1000, 1:2000 dilution). After washing with PBS + 0.2% BSA, the sections were incubated with streptavidin-β-galactosidase conjugate (Boehringer-Mannheim, No. 1112481, 1:200 dilution), washed with PBS and X-gal washing buffer, and stained (for 30–45 min at 37°C) with X-gal solution. The sections were mounted in Glycergel (DAKO, No. C563) for microscopic examination.

Histochemical staining for alkaline phosphatase and β-galactosidase activity. Paraformaldehyde (4%)-fixed embryos were bathed in Tris buffer (pH 9.5) followed by incubation in NBT/BCIP substrate solution (GIBCO BRL No. 8280SA) for 15–20 min at room temperature. Embryos were washed in five changes of X-gal washing buffer and stained overnight at 37°C with X-gal solution. Stained embryos were processed for wax histology. Serial sections were dewaxed and mounted directly in Canada balsam.

The number of *lacZ*-expressing cells was counted on serial sections of the embryo. Every labelled cell was counted and the total score was corrected by applying Abercrombie's formula (1946) to compute the final cell number. At the sites where host PGCs were found (see Results and Figs. 1C–1E), cells that showed X-gal staining or positive immunostaining (blue coloration in both cases) and strong alkaline phosphatase activity [red coloration after Vector-Red staining (Figs. 1G–1I) or purple coloration after NBT/BCIP incubation (Figs. 1J and 1K)] were scored. This score represents the number of graft-derived cells that have differentiated into PGCs.

RESULTS

Epiblast Cells Display the Developmental Fate Characteristic of Cells at the Site of Transplantation

LacZ-expressing epiblast cells were transplanted to early-primitive-streak-stage host embryos. The distribution of cells derived from the transplanted epiblast in the host tissues was analysed when the embryo has developed to the 1- to 6-somite-stage after 44–45 hr of culture [mean somite number = 3.3, standard error of the mean (SEM) = ±0.2, 120 embryos from 6 experiments]. Table 1 summarises the results obtained from experimental embryos that have been assayed for both β-galactosidase and alkaline phosphatase activity. Additional observations on embryos that were stained only for β-galactosidase activity are discussed separately in the text.

Orthotopic transplantation. Proximal epiblast that was transplanted orthotopically to a proximal site (P → P) colonised predominantly the extraembryonic mesoderm of the yolk sac (55% of all graft-derived cells), allantois (32%), and amnion (8%). Some contribution (about 5% of the graft-derived population) was found in the chorionic mesoderm, hindgut (endoderm and mesoderm), ectoderm in the posterior neural tube, surface ectoderm and caudal mesoderm (Table 1). In another 27 P → P embryos that were examined only for lacZ expression (data not shown), a similar pattern of tissue colonisation by graft-derived cells was found mostly in the extraembryonic mesoderm.

Distal epiblast cells, when transplanted orthotopically to a distal site (D → D), colonised the neural tube (91% of graft-derived cells), with a minor contribution to the surface ectoderm (4%) and the cranial mesoderm (3%) (Table 1).

Heterotopic transplantation. Proximal epiblast cells transplanted to distal sites (P → D) behaved like distal epiblast cells and contributed predominantly to the neuroectoderm (86% of graft-derived cells). A minor contribution was found in the surface ectoderm (10%) and the cranial mesoderm (4%) (Table 1). Of another 27 P → D embryos,

FIG. 1. (A) The schematic illustration of the four types of transplantation performed on early-primitive-streak-stage mouse embryos. The epiblast of the *HMG-lacZ* donor embryo was dissected into proximal and distal fragments by cutting along the dotted lines. Small clusters of cells were isolated from the proximal (P) and distal (D) fragments of the epiblast (hatched areas). Groups of 5–20 cells were transplanted orthotopically to sites that are equivalent to their origin (orthotopic transplantations: P → P, D → D) or they were transplanted to sites that are different from where they were explanted (heterotopic transplantations: P → D, D → P). The primitive streak (PS) is a landmark of the posterior side of the egg cylinder. (B) *lacZ*-expressing cells in chimaeric embryos after cell transplantation and culture *in vitro*. Clusters of X-gal stained cells are present in the allantois (al), the lateral plate mesoderm (arrowhead), and the endoderm (arrow) in the posterior region of a D → P embryo. Bar, 500 μm. (C–E) Primordial germ cells (PGCs) localised in (C) the primitive streak, (D) the mesenchyme of the allantoic bud, and (E) the base of the allantois and the hindgut endoderm (en) of embryos of D → P embryos. The PGCs display the distinctive cytoplasmic spot of alkaline phosphatase activity and a higher enzyme activity on the cell surface than that of other cell types in their vicinity. These PGCs do not express any *lacZ* transgene activity and are therefore derived from the host tissues. Bar, 25 μm for C–E. (F) Colocalisation of *lacZ*-expressing cells (blue cells, black arrows) and alkaline phosphatase positive primordial germ cells (red cells, white arrows) in the allantois and the tissues in the posterior region of a D → P embryo. Bar, 500 μm. (G–I) *lacZ*-expressing PGCs (arrows) derived from distal epiblast cells that have been transplanted to proximal epiblast. They are identified by the alkaline phosphatase cytoplasmic spot and *lacZ* activity revealed by the blue immunostaining reaction with anti-β-galactosidase antibody. Graft-derived PGCs are found in (G) the primitive streak, (H) the allantois, and (I) the endoderm in the posterior region of D → P embryos. Bar, 30 μm for G–I. (J and K) *lacZ*-expressing cells (with dark blue core due to nuclear localisation of the X-gal stained protein, arrows) that show strong alkaline phosphatase on the cell surface (purple cell outline) of (J) a P → P embryo and (K) a D → P embryo. In both embryos, the enzyme-positive cells are localised in the posterior endoderm and display the spherical shape and large soma size that are typical of primordial germ cells. In (K), a nearby PGC of the host embryo has no *lacZ* activity (arrowhead) and shows only alkaline phosphatase activity on the cell surface. Bar, 10 μm for J and K. (L and M) *lacZ*-expressing cells (with blue X-gal stained nucleus) in the neuroepithelium of (L) a D → D embryo and (M) a P → D embryo. The graft-derived cells (arrows) are properly integrated into the pseudostratified epithelium of the neural plate and have acquired the histological characteristic of the host neuroepithelial cells. The graft-derived cells do not show the cytoplasmic or cell surface staining that is characteristic of PGCs. The apical surface of the neuroepithelium shows moderate alkaline phosphatase activity. Bar, 20 μm (L) and 10 μm (M).

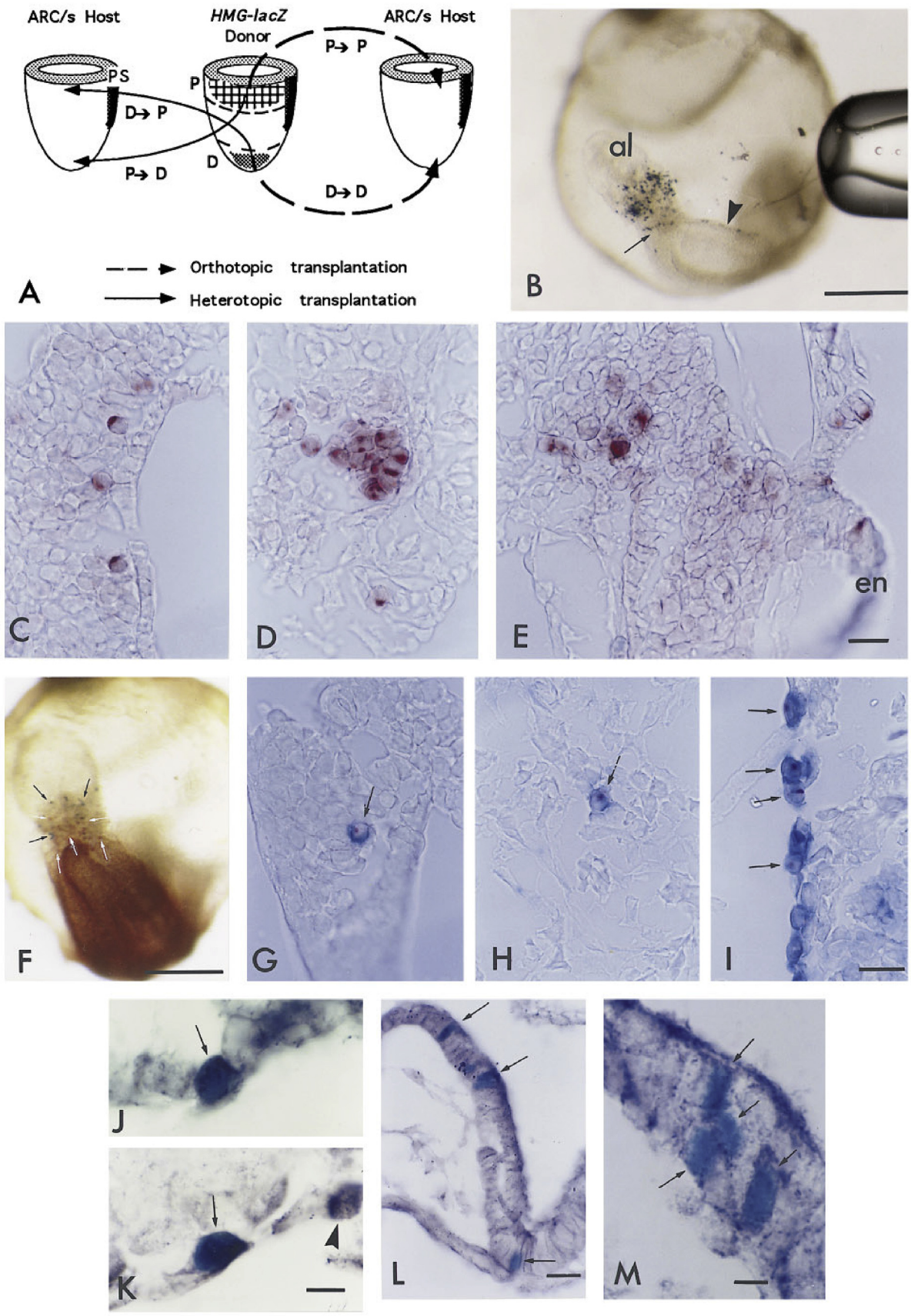
ARC/s Host
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TABLE 1
Distribution and Mean Number of *lacZ*-Expressing Cells Derived from Grafts of Distal and Proximal Epiblast in the Chimaeric Mouse Embryos

A. General tissue distribution

	lacZ-expressing cells in tissues[a]							Mean number of *lacZ*-expressing cells per embryo[b]
Alm	Ysc	Am	Ch	Hgn	N-ect	S-ect	Mes	
Proximal to Proximal (P → P)								
46.3 ± 6.2 (26)	35.3 ± 7.0 (21)	11.6 ± 4.2 (18)	3.6 ± 0.8 (5)	4.6 ± 2.1 (5)	7.5 (2)	np	4.0 ± 0.6 (3)	77.2 ± 10.3 (30)
Distal to Proximal (D → P)								
54.1 ± 10.5 (49)	28.8 ± 3.7 (39)	13.0 ± 3.0 (24)	29.3 ± 13.1 (7)	8.2 ± 2.0 (14)	5.8 ± 2.2 (8)	np	12.5 ± 4.4 (11)	75.2 ± 10.1 (63)
Distal to Distal (D → D)								
np	np	np	np	np	62.3 ± 14.6 (12)	17.5 ± 9.5 (4)	7.3 ± 3.7 (4)	70.5 ± 17.0 (12)
Proximal to Distal (P → D)								
27 (1)	np	np	np	np	53.4 ± 13.7 (18)	5.8 ± 1.9 (4)	15 (1)	57.2 ± 14.6 (19)

B. The primordial germ cell population

Graft type	Number of embryos analysed	Number of presumptive PGCs in tissues[c]			Percentage of total graft-derived populations
		Base of allantois	Yolk sack endoderm	Hindgut	
P → P	30	5.5 ± 1.7 (15)	1.7 ± 0.3 (3)	1.3 ± 0.3 (3)	3.97% (92 of 2316 cells)
D → P	63	4.0 ± 0.6 (34)	2.5 ± 1.2 (4)	3.1 ± 0.5 (16)	3.67% (196 of 5329 cells)
D → D	12	np	np	np	0% (0 of 846 cells)
P → D	19	5 (1)	np	np	0.46% (5 of 1086 cells)

Note. Abbreviations used include: Alm, allantoic bud and the mesenchyme adjoining the posterior part of the primitive streak; Ysc, yolk sac mesoderm and blood islands of vitelline vessels; Am, ectodermal and mesodermal layers of the ammion; Ch, the mesodermal component of the chorion; Hgn, endoderm of the prospective hindgut portal; N-ect, neuroepithelium of the brain and spinal cord; S-ect, surface ectoderm; Mes, paraxial and lateral mesoderm, and mesenchyme adjacent to the primitive streak; np, transgenic cells were not present in the tissue.

[a] The number in parentheses under each tissue type represents the number of specimens that showed colonisation of that particular tissue by the transplanted cells. The cell number does not include those showing alkaline phosphatase activity (Part B).

[b] The mean number ± SEM of transgenic cells per embryo (including all count of somatic cells and PGCs in both parts of the table) are not different between types of transplantations (Student's *t* test, $P > 0.05$). The numbers in parentheses indicate the number of embryos analysed for each group.

[c] The numbers in parentheses indicate the number of embryos showing presence of *lacZ*-expressing cells and alkaline phosphatase active cells that may be PGCs at (i) the base of the allantois: mesenchyme at the junction of the allantois and the primitive streak, (ii) the yolk sac endoderm: the visceral endoderm of the yolk sack adjacent to the base of the allantois, and (iii) the hindgut: endoderm and adjacent mesoderm of the prospective hindgut.

analysed only for *lacZ* expression (data not shown), 26 showed the preponderant presence of *lacZ*-expressing transplanted cells in the neuroectoderm and cranial paraxial mesoderm. A minor contribution was found in the lateral mesoderm (four embryos) and the yolk sac mesoderm and amnion (one embryo). Proximal epiblast cells therefore have

adopted a predominantly ectodermal fate after heterotopic transplantation.

When distal epiblast cells were transplanted to proximal sites (D → P), their descendants were found mostly in extraembryonic tissues (Fig. 1B): 58% of graft-derived cells in allantois, 26% in yolk sac mesoderm, 8% in amnion and chorion (Table 1). A minor contribution (about 6%) was observed in the endodermal and mesodermal tissues in the caudal part of the embryo (Table 1). Despite their prospective neuroectodermal fate, few of the distal epiblast cells (<2%) in D → P embryos became neuroepithelial cells in the posterior neuropore and trunk neural tube. In another 20 D → P embryos analysed only for *lacZ* expression (data not shown), most of the graft-derived cells colonised the extraembryonic tissues. However, an additional contribution was also found in lateral mesoderm (3 cases) and caudal surface ectoderm (16 cases).

Results of these transplantation experiments clearly show that cells taken from either the proximal or the distal regions of the epiblast will colonise the same types of tissues that are derived from host cells at the site of heterotopic transplantation and not those that are characteristic of their original sites.

Distal Epiblast Cells May Form PGCs after Transplantation to the Proximal Region of the Epiblast

After *in vitro* culture, control and experimental (P → P and D → P) 6.5-day early-primitive-streak-stage embryos were examined for alkaline phosphatase (AP) staining. In both types of embryos, some cells in the primitive streak (Fig. 1C), the allantoic bud (Figs. 1D and 1E), the endoderm (Fig. 1E) and mesenchyme of the hindgut, and the adjacent yolk sac endoderm were found to express strong enzyme activity that is typical of primordial germ cells (Ginsburg *et al.,* 1990).

Transplantation of epiblast cells to proximal sites. When proximal epiblast cells were transplanted orthotopically, they colonised the mesoderm of the yolk sac, the chorion, the amnion, the allantois, the hindgut, and the caudal mesenchyme (Table 1). Some AP-active transgenic cells were found amongst PGCs of the host embryo. Of the 2316 transgenic cells scored in all P → P embryos, about 3.9% also expressed strong enzyme activity. These cells were found in the mesenchyme at the base of the allantois posterior to the primitive streak and in the endoderm (Fig. 1J) and mesenchyme in the posterior region of the embryo (Table 1).

In the D → P embryo where distal epiblast cells were transplanted to proximal sites, some *lacZ*-expressing cells were found in the same tissues as the AP-positive cells (Fig. 1F). An average of 92.4 (SEM = 7.3, results of cell count in 16 D → P embryos from 6 different experiments) AP-active cells were found at the base of allantois, in the yolk sac endoderm, and in the hindgut of the embryo. Between 6 and 9 cells coexpressing AP and *lacZ* activity were found in each D →P embryo, which constitute about 7–10% of the total AP-positive PGC population. About 3.7% of the 5329 graft-derived cells in the D → P embryos displayed AP activity characteristic of PGCs (Figs. 1G–1I and 1K, Table 1). Distal epiblast cells that have been transplanted to proximal sites therefore display a germ-line potency similar to that of the proximal epiblast cells.

Transplantation of epiblast cells to distal sites. When distal epiblast cells were transplanted orthotopically (D → D embryos), their descendants colonised the neuroectoderm (Fig. 1L) and the surface ectoderm primarily in cranial and the trunk regions of the embryos. None of the 846 descendants were found at the PGC sites (Table 1). When proximal epiblast cells were transplanted to the distal site (P → D embryos), over 90% of the graft-derived cells were incorporated into the neuroepithelium of the host embryo. Similar to the host neuroepithelial cells, the graft-derived cells also expressed a moderate level of AP activity (Fig. 1M). However, the *lacZ*-expressing cells were histologically indistinguishable from the neighbouring neuroepithelial cells (Fig. 1M) and did not express the cytoplasmic pattern of AP activity typical of the PGCs. Only 0.46% of graft-derived cells, which were all found in one P → D embryo, met the criteria for PGCs. In this embryo, *lacZ*-expressing AP-positive cells were found in the mesenchyme at the base of the allantois, and other non-AP-expressing graft-derived cells were confined to the allantois.

DISCUSSION

Cells in the Epiblast of the Gastrulating Embryo May Have Similar Developmental Potency

Previous fate-mapping studies have shown that epiblast cells in different regions of the embryos display different developmental fates. In the early-primitive-streak-stage embryo, the distal epiblast is destined for the formation of neuroectoderm and surface ectoderm while the proximal epiblast has a predominantly mesodermal fate. We have shown in this study that, following heterotopic transplantation, the epiblast cells of pre- and early-primitive-streak-stage embryos acquire the developmental fate of the cells in the environment into which they have been translocated. Distal epiblast cells differentiate like cells in the proximal region of the epiblast and give rise to extraembryonic mesoderm, gut endoderm, and embryonic mesoderm. Conversely, proximal epiblast cells colonise the neuroectoderm and surface ectoderm when they are placed in the distal epiblast. In both cases, the heterotopically transplanted cells are able to integrate properly into the host tissues and acquire the appropriate cellular phenotype. Previous clonal analysis of epiblast cells has shown that epiblast cells at early gastrulation show no restriction of lineage potency and clonal descendants of single epiblast cells can participate in the differentiation of multiple types of tissue (Lawson *et al.,* 1991). The present heterotopic transplantation study further demonstrates that epiblast cells of the mouse

embryo at early gastrulation, which normally display vastly different fates, have similar developmental potencies. Therefore, although a regionalisation of cell fate could be demonstrated by fate-mapping, lineage restriction or irreversible determination of cell fate seems not to have occurred in the epiblast population. Considerable plasticity of developmental fate can still be found in the cells of the embryonic ectoderm at late gastrulation (Beddington, 1982). Ectodermal cells that are destined for the formation of extraembryonic mesoderm (Beddington, 1981; Tam and Beddington, 1987) can still contribute to neuroectodermal cells after hetertopic transplantation. However, a bias for neural differentiation is already evident in the prospective brain cells localised in the anterior region of the ectodermal layer (Beddington, 1982), suggesting that some populations of ectodermal cells may be less amenable to the manipulation that alters cell fate.

We have also shown in this study that the potency to form germ cells is not limited to cells in the proximal epiblast. Cells in the epiblast outside the proximal region can differentiate into cells that show the characteristics of PGCs of the host embryo, such as the colocalisation to a similar site and the expression of germ cell markers. This potency is revealed following the translocation of the distal epiblast cells to the proximal region of the epiblast. The introduction of 5–20 distal epiblast cells to the proximal epiblast may have facilitated the incorporation of the transplanted cells into the community of host PGC precursors, resulting in their transformation and recruitment to the germ line. The transplantation of a similar number of proximal epiblast cells to sites away from the host PGC precursors seems to have diminished the chance of PGC formation. The inability to realise the prospective germ-line potency in heterotopic sites may be due to the absence of the site-specific signals or the provision of the appropriate community effects to promote cell differentiation (Cossu *et al.,* 1995; Gurdon *et al.,* 1993). Alternatively, the lack of contribution to the germ line could result from the physical constraint imposed by the heterotopic site that prevents the germ cell precursors from homing to the normal sites for PGCs.

The finding that cells in different parts of the epiblast have similar developmental potencies raises the question of how the regionalisation of cell fate may arise. The pattern of gene activity known to date has not provided a simple correlation to the fate map of lineage precursors in the gastrulating embryo (Boncinelli and Mallamaci, 1995; Quinlan and Tam, 1995). It has been proposed that the allocation of epiblast cells to tissue lineages occurs as the cells ingress through the primitive streak (Sasaki and Hogan, 1993; Yamaguchi *et al.,* 1994; Ang and Rossant, 1994; Tam and Trainor, 1994). Lineage specification therefore depends critically on the timing and the position of ingression taken by different epiblast cells. Both parameters may be governed by the range of morphogenetic options that are available to specific groups of epiblast cells during gastrulation. Analyses of the displacement of clonal descendants of epiblast cells have revealed a reproducible pattern of cell movement during germ layer formation (Lawson *et al.,* 1991; Quinlan *et al.,* 1995). The regionalisation of cell fate might reflect the order of the assignment of pluripotent epiblast cells to various lineages as a function of their position in the epiblast and the region-specific morphogenetic activity. It is conceivable that the transplantation of cells to a different region of the epiblast may impart a new time table and a different pattern of cell movement, which lead to the acquisition of the developmental fate of the cells in the new environment.

The Establishment of the Germ Line

Analyses of developmental potency of blastomeres and inner cell mass cells of preimplantation embryos have shown that clonal descendants of these early embryonic cells may contribute, but never exclusively, to the germ line (Gardner and Beddington, 1988). Even at early gastrulation, single proximal epiblast cells can still contribute to both the somatic and germ-line lineages (Lawson and Hage, 1994), implying that lineage restriction has not yet occurred in the epiblast population that is derived from the inner cell mass. These findings suggest that during mouse embryogenesis, the segregation of the germ line from other somatic lineages has not happened before early gastrulation (Gardner and Rossant, 1979; Gardner *et al.,* 1985; McMahon *et al.,* 1983). That the somatic cells and germ cells share common progenitors suggests that a predetermined germ line does not exist in the early mouse embryo.

PGCs are first identified as a cluster of alkaline phosphatase active cells within the extraembryonic mesoderm near the posterior part of the primitive streak (Chiquoine, 1954; Ginsburg *et al.,* 1990; Lawson and Hage, 1994; MacGregor *et al.,* 1995). Our finding that distal epiblast cells could form PGCs after transplantation to proximal epiblast provides support for the concept that specification of germ-line cells takes place amongst the precursors of extraembryonic mesoderm. It has been postulated that the specification of the germ line in the mouse embryo is subject to the influence of somatic tissues on the germ cell precursors (McLaren, 1981). The allocation of epiblast cells to the germ line may depend on the provision of localised signals either found within the proximal epiblast or emanating from tissues nearby. Before the emergence of a distinct PGC population, some markers for PGCs such as SSEA-1, F9, EMA-1, *Oct4,* and alkaline phosphatase (Chiquoine, 1954; Eddy and Hahnel, 1983; Enders and May, 1994; Ginsburg *et al.,* 1990; Scholer *et al.,* 1990) are expressed widely in the epiblast of the mouse embryo at early gastrulation. These germ cell markers are later absent in the epiblast or ectodermal cells that are not involved in the formation of the germ-cell lineage. The down-regulation of germ cell marker may suggest that the ability to become germ cells may be lost progressively by the epiblast cells except for those in the proximal epiblast, where the local environment is more conducive for conserving the germ-line potency or totipotency (Yeom

et al., 1996). Distal epiblast cells can therefore differentiate to give PGCs if their germ-line potency is retained by heterotopic transplantation to a region of the epiblast containing permissive signals for germ cell specification. Proximal epiblast cells that are transplanted to distal sites may be deprived of the environmental factors or cues that maintain the germ-line potency and therefore fail to contribute to the PGC population. Whether these epiblast cells might form PGCs in other ectopic sites cannot be resolved in the present study. The presence of moderate levels of AP activity in tissues such as the neuroectoderm and the somitic mesoderm of the early-somite-stage embryo precludes a definitive identification of the ectopic germ cells based on the expression of this enzyme marker.

Insight into the nature and mode of action of local interactive signals might be obtained by examining PGC formation in embryos that display aberrant gastrulation as a result of spontaneous and targeted gene mutation. The formation of mesoderm and PGCs has been examined in *eed* mutants. Although gastrulation and mesoderm patterning in homozygous *eed* embryos are disrupted, normal numbers of PGCs are apparently formed (Faust *et al.,* 1995). Mutations such as W^e (*extreme dominant spotting*) and *Sl* (*Steel*), which result in loss of c-kit tyrosine kinase and its receptor, also do not affect the initial establishment of the PGC population (Buehr *et al.,* 1993; McCoshen and McCallion, 1975). PGCs are also present in parthenogenetic embryos (Kaufman and Schnebelen, 1986), suggesting that the presence of only the maternal alleles or the absence of paternal alleles of the genome does not affect PGC specification. There are many other spontaneous and targeted mutations that may interfere with gastrulation (Copp, 1995; Brandon *et al.,* 1995). A systematic investigation of the formation of PGCs in these mutants might be rewarding in identifying the factors that are critical to the specification of the germ line.

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REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* 94, 239–247.

Ang, S-L., and Rossant, J. (1994). *HNF-3β* is essential for node and notochord formation in mouse development. *Cell* 78, 561–574.

Beddington, R. S. P. (1981). An autoradiographic analysis of the potency of embryonic ectoderm in the 8th day postimplantation mouse embryo. *J. Embryol. Exp. Morphol.* 64, 87–104.

Beddington, R. S. P. (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic egg cylinder. *J. Embryol. Exp. Morphol.* 69, 265–285.

Boncinelli, E., and Mallamaci, A. (1995). Homeobox genes in vertebrate gastrulation. *Curr. Opin. Genet. Dev.* 5, 619–627.

Brandon, E. P., Idzerda, R. L., and Knight, G. S. (1995). Targeting the mouse genome: A compendium of knockouts (Part 1). *Curr. Biol.* 5, 625–634.

Buehr, M., McLaren, A., Bartley, A., and Darling, S. (1993). Proliferation and migration of primordial germ cells in We/We mouse embryos. *Dev. Dyn.* 19, 182–189.

Cepko, C. L., Ryder, E. F., Austin, C. P., Walsh, C., and Fekete, D. M. (1993). Lineage analysis using retroviral vectors. *Methods Enzymol.* 225, 933–960.

Chiquoine, A. D. (1954). The identification, origin and migration of the primordial germ cells in the mouse embryo. *Anat. Rec.* 118, 135–146.

Copp, A. J. (1995). Death before birth: Clues from gene knockouts and mutation. *Trend Genet.* 11, 87–93.

Cossu, G., Kelly, R., DiDonna, S., Vivarelli, E., and Buckingham, M. (1995). Myoblast differentiation during mammalian somitogenesis is dependent upon a community effect. *Proc. Natl. Acad. Sci. USA* 92, 2254–2258.

Dixon, K. E. (1994). Evolutionary aspects of primordial germ cell formation. *In* "Germline Development," Ciba Foundation Symposium 182, pp. 92–120. Wiley, Chichester.

Eddy, E. M., and Hahnel, A. C. (1983). Establishment of the germ cell line in mammals. *In* "Current Problems in Germ Cell Differentiation" (A. McLaren and C. C. Wylie, Eds.), British Society for Developmental Biology Symposium No. 7, pp. 41–70. Cambridge Univ. Press, Cambridge, UK.

Enders, G. C., and May, J. J., II (1994). Developmentally regulated expression of mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. *Dev. Biol.* 163, 331–340.

Faust, C., Schumacher, A., Holdener B., and Magnuson, T. (1995). The *eed* mutation disrupt anterior mesoderm production in mice. *Development* 121, 273–285.

Gardner, R. L., and Beddington, R. S. P. (1988). Multi-lineage 'stem' cells in the mammalian embryos. *J. Cell Sci.* 10(Suppl.) 11–27.

Gardner, R. L., and Rossant, J. (1979). Investigation of the fate of 4.5 day post coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. Exp. Morphol.* 52, 141–152.

Gardner, R. L., Lyons, M. F., Evans, E. P., and Burtenshaw, M. D. (1985). Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. *J. Embryol. Exp. Morphol.* 88, 349–363.

Ginsburg, M., Snow, M. H. L., and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* 110, 521–528.

Grant, M., Zuccotti, M., and Monk, M. (1992). Methylation of CpG sites of two X-linked genes coincides with X-inactivation in the female mouse embryo but not in the germ line. *Nature Genet.* 2, 161–166.

Gurdon, J. B., Tiller, E., Robert, J., and Kato, K. (1993). A community effect in muscle development. *Curr. Biol.* 3, 1–11.

Kaufman, M. H., and Schnebelen, M. T. (1986). The histochemical identification of primordial germ cells in diploid parthenogenetic mouse embryos. *J. Exp. Zool.* 238, 103–111.

Lawson, K. A., and Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. *In* "Germline Development," Ciba Foundation Symposium 182, pp. 68–92. Wiley, Chichester.

Lawson, K. A., and Pedersen, R. A. (1992a). Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. *In* ''Postimplantation Development in the Mouse,'' Ciba Foundation Symposium 165, pp. 3–26. Wiley, Chichester.

Lawson, K. A., and Pedersen, R. A. (1992b). Early mesoderm formation in the mouse embryo. *In* ''Formation and Differentiation of Early Embryonic Mesoderm'' (R. Bellairs, E. J. Sanders, and J. W. Lash, Eds.), pp. 33–46, Plenum, New York.

Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* 113, 891–911.

MacGregor, G. R., Zambrowicz, B. P., and Soriano, P. (1995). Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* 121, 1487–1496.

McCoshen, J. A., and McCallion, D. J. (1975). A study of the primordial germ cells during their migratory phase in *Steel* mutant mice. *Experientia* 81, 589–590

McLaren, A. (1981). ''Germ Cells and Soma.'' Yale Univ. Press, New Haven, CT.

McMahon, A., Foster, M., and Monk, M. (1983). X-chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo *J. Embryol. Exp. Morphol.* 74, 207–220.

Parameswaran, M., and Tam, P. P. L. (1995). Regionalisation of cell fate and morphogenetic movement of the mesoderm during mouse gastrulation. *Dev. Genet.* 17, 16–28.

Quinlan, G. A., and Tam, P. P. L. (1995). Organisation of the body plan: Cell fate and gene activity during gastrulation of the mouse embryo. *In* ''Towards a Molecular Analysis of Mammalian Development'' (P. Lonai, Ed.), pp. 1–26. Harwood Press, Chur.

Quinlan, G. A., Williams, E. A., Tan, S.-S., and Tam, P. P. L. (1995). Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: Implication for body plan organisation during early embryogenesis. *Development* 121, 87–98.

Sasaki, H., and Hogan, B. L. M. (1993). Differential expression of multiple fork head-related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* 118, 47–59.

Scholer, H. R., Dressler, G. R., Balling, R., Rohdewohld, H., and Gruss, P. (1990). Oct-4: A germline-specific transcription factor mapping to the mouse *t*-complex. *EMBO J.* 9, 2185–2195.

Sturm, K. A., and Tam, P. P. L. (1993). Isolation and culture of whole postimplantation embryos and germ layer derivatives. *Methods Enzymol.* 225, 164–190.

Tam, P. P. L., and Beddington, R. S. P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* 99, 109–126.

Tam, P. P. L., and Trainor, P. A. (1994). Specification and segmentation of the paraxial mesoderm. *Anat. Embryol.* 189, 275–305.

Tam, P. P. L., Williams, E. A., and Tan, S.-S. (1994). The expression of an X-linked *HMG–lacZ* transgene in mouse embryos: Implication of chromosomal imprinting and lineage-specific X-chromosome activity. *Dev. Genet.* 15, 491–503.

Tan, S.-S., Williams, E. A., and Tam, P. P. L. (1993). X-chromosome inactivation occurs at different times in different tissues of the postimplantation mouse embryos. *Nature Genet.* 3, 170–174.

Yamaguchi, T. P., Harpal, K., Henkemeyer, M., and Rossant, J. (1994). *fgfr-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* 8, 3032–3044.

Yeom, Y. I., Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Hubner, K., and Scholer, H. R. (1996). Germline regulatory element of *Oct-4* specific for the totipotent cycle of embryonal cells. *Development* 122, 881–894.

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