

# Analysis of cell lineage in two- and four-cell mouse embryos

Toshihiko Fujimori<sup>1,\*†</sup>, Yoko Kurotaki<sup>1</sup>, Jun-ichi Miyazaki<sup>2</sup> and Yo-ichi Nabeshima<sup>1</sup>

<sup>1</sup>Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe Cho, Sakyo-ku, Kyoto 606-8501, Japan

<sup>2</sup>Division of Stem Cell Regulation Research, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

\*PRESTO, Japan Science and Technology Corporation (JST)

†Author for correspondence (e-mail: fujimori@lmls.med.kyoto-u.ac.jp)

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## Summary

Compared with other animals, the embryos of mammals are considered to have a highly regulative mode of development. However, recent studies have provided a strong correlation between the first cleavage plane and the future axis of the blastocyst, but it is still unclear how the early axes of the preimplantation embryo reflect the future body axes that emerge after implantation. We have carried out lineage tracing during mouse embryogenesis using the Cre-loxP system, which allowed us to analyze cell fates over a long period of development. We used a transgenic mouse strain, CAG-CAT-Z as a reporter line. The descendants of the manipulated blastomere heritably express  $\beta$ -galactosidase. We examined the distribution of descendants of a single blastomere in the 8.5-day embryo after labeling at the two-cell and four-cell stages. The derivatives of one blastomere in the two-cell embryo randomly mix with cells originating from the second blastomere in all cell layers examined. Thus we find cells from different blastomeres intermingled and localized randomly along the body axis.

The results of labeling experiments performed in the four-cell stage embryo fall into three categories. In the first, the labeled cells were intermingled with non-labeled cells in a manner similar to that seen after labeling at the two-cell stage. In the second, labeled cells were distributed only in the extra-embryonic ectoderm layers. Finally in the third category, labeled cells were seen only in the embryo proper and the extra-embryonic mesoderm. Manipulated embryos analyzed at the blastocyst stage showed localized distribution of the descendants of a single blastomere. These results suggest that incoherent clonal growth and drastic cell mixing occurs in the early mouse embryo after the blastocyst stage. The first cell specification event, i.e., partitioning cell fate between the inner cell mass and trophectoderm, can occur between the two-cell and four-cell stage, yet the cell fate is not determined.

Key words: Mouse, Cell lineage, Cre recombinase

## Introduction

It is well known that the future embryonic body axes are specified prior to the first cleavage in a variety of animals. In the case of the mammalian embryo, however, the timing of the earliest specification events that control the future body axes are still unclear (Beddington and Robertson, 1999; Tam et al., 2001; Zernicka-Goetz, 2002). Two groups have reported that the earliest specification in the mouse embryo might occur before the first cleavage event (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001; Piotrowska et al., 2001). Studies showed that, the orientation of the first cleavage plane is strongly related to the axes of the blastocyst. The relationship between the axes of the blastocyst and the axes at the egg cylinder stage has also been investigated (Weber et al., 1999). It was concluded that descendants of cells located near the polar body at the blastocyst stage tend to contribute to the distal visceral endoderm in the egg cylinder. The relationship between this axis (near the polar body – away from the polar body) to the distal-proximal axis at egg cylinder stages was also examined. From these investigations, a possible relationship between the earliest axis manifestation and the final embryonic body axes at later stages was proposed. However, as yet, continuous observation from the fertilized egg

to the post-implantation period has not been performed. An important issue remains, namely how each cell changes its relative spatial position in an embryo. A second problem is how cell fates are specified during early stages of development. To ascertain this it is important to observe embryos without disturbing the original developmental program, because the mammalian embryo possesses a high potential to regulate its development after experimental manipulation. Our aim was to investigate the inherent developmental program in early stages of normal embryogenesis with minimal disturbance to the embryonic program.

A major reason for the paucity of experiments attempting to follow cell lineage in the early mouse embryo is one of technical difficulty. A number of approaches have been used to mark cells. Individual cells have been labeled with oil drops (Wilson et al., 1972), dyes (Lawson et al., 1986; Gardner, 2000; Tam and Beddington, 1992), reporter enzymes such as horseradish peroxidase (Balakier and Pedersen, 1982), or by mRNA injection (Zernicka-Goetz et al., 1997). However, it has been difficult to follow the lineage over a long period because these lineage tracers are easily diluted or alternatively may disturb normal development. We have traced cell lineages in the early mouse embryo using a Cre-loxP system (Araki et al., 1995). After the activation of Cre recombinase, cells express

the bacterially derived  $\beta$ -galactosidase enzyme. Activation of the reporter expression is irreversible and heritable via the changes of genomic organization. This system allows us to follow the fate of a cell and its progeny for a long period after activation of Cre recombinase.

We labeled individual cells during the pre-implantation period and analyzed the distribution of labeled descendants in the post-implantation embryo around day 8.5. By performing multiple experiments, we have established the generalized behavior of cells in the embryo after labeling between the two-cell and four-cell stages of development.

## Materials and methods

### Mouse strains and collection of embryos

CAG-CAT-Z (Araki et al., 1995) mice were maintained as a homozygous transgenic colony. To identify homozygous males, individual animals were crossed with wild-type females, embryos were collected and subjected to PCR genotyping. Males were confirmed as homozygous transgenic animals when all embryos were heterozygotes. For the lineage tracing experiments, wild-type BDF1 females were used. BDF1 females (Charles River Japan, Inc.) were super-ovulated by injection of 5 IU pregnant mares serum (PMS; Teikoku Zouki, Japan) followed by 5 IU human chorionic gonadotropin (hCG; Teikoku Zouki, Japan) as described previously (Hogan et al., 1994). Primed females were mated with homozygous CAG-CAT-Z transgenic males. To obtain one-cell stage embryos, swollen ampullae were flushed with M2 medium (Specialty Media) containing 0.5 mg/ml of hyaluronidase (Sigma, H3506). The embryos were washed several times in M2 medium to remove the cumulus cells. To obtain embryos at the two-cell or four-cell stages, embryos were flushed from the oviduct.

### Activation of Cre recombinase

To activate Cre recombinase in a specific blastomere, we used one of three methods. One was the injection of an expression vector pBS185 (Gibco-BRL), which drives expression of bacteriophage P1-encoded Cre recombinase under the control of human cytomegalovirus early promoter. The second method was injection of the expression vector CAG-Cre, in which Cre expression is controlled by a CAG promoter cassette. This plasmid was constructed using the Cre cDNA fragment from pBS185 subcloned into the pCAG-GS expression vector (Araki et al., 1995) to give pCAG-Cre. Third was the direct injection of Cre recombinase protein (Clontech). For all of these methods, injections were performed into blastomere nuclei using conventional injection techniques. Following injection, embryos were cultured in KSOM medium (Specialty Media) containing 0.1% BSA in an incubator at 37°C, 5% CO<sub>2</sub>. Healthy embryos were selected and transferred into the oviduct of pseudo-pregnant females.

### Embryo culture

For direct observation at the blastocyst stage, embryos were cultured at 37°C in a drop of KSOM covered with light mineral oil. Alginate gel was used to maintain the position of embryos in culture (Gardner, 2001). Embryos were put into a drop of KSOM (MR-106-D, Specialty Media) containing 0.7% alginate acid sodium salt (A-2158, Sigma) and covered with paraffin oil. The alginate was induced to gel by application of a small amount of solution containing 1.5% CaCl<sub>2</sub> and 0.9% NaCl. After 1 minute, residual KSOM containing alginate was removed. The alginate drop was rinsed more than five times with alginate-free KSOM, and the alginate gel was covered with KSOM under paraffin oil. Embryos were cultured in a 37°C, 5% CO<sub>2</sub> incubator. Photographs of embryos in culture were taken with an inverted microscope (IX70, Olympus) equipped with a 35 mm film camera.

### X-gal staining and histology

Manipulated embryos were collected from uteri and fixed with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS at 4°C for 1 hour. Fixed embryos were washed with PBS containing 0.1% Triton X-100 at 4°C overnight, and  $\beta$ -gal was visualized with X-gal staining buffer (1  $\mu$ M MgCl<sub>2</sub>, 3 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 3 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 0.1% Triton X-100 and 0.05% 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) in PBS). For the X-gal staining of embryos embedded in alginate gel, buffer without Triton X-100 was used. Embryos were incubated in the staining buffer at 37°C overnight and post-fixed in PBS containing 4% paraformaldehyde and 50 mM EDTA. Whole-mount images were captured with a M-420 Leica microscope equipped with CCD camera (Progress 1012, Zeiss Vision). Embryos were dehydrated through 70% ethanol, 90% ethanol, 100% ethanol and chloroform, and then embedded in paraffin wax. 7.5  $\mu$ m serial sections were stained with Haematoxylin and Eosin. These sections were observed under the microscope (DMRB, Leica), and photographic images were taken with a CCD camera (Progress 1012, Zeiss Vision). Images were processed with Adobe Photoshop.

## Results

### The CAG-CAT-Z transgenic mouse is an appropriate model for tracing cell lineage in early development

We used the transgenic CAG-CAT-Z (Araki et al., 1995) mouse line for our experiments. These mice were designed so that  $\beta$ -galactosidase becomes active in a cell-autonomous fashion following activation by Cre recombinase. Control experiments were performed to ensure that the cell marking system worked appropriately. Mice homozygous for the CAG-CAT-Z transgene were shown to develop normally and give rise to viable fertile animals. We crossed homozygous transgenic males to super-ovulated wild-type BDF1 females to obtain heterozygous embryos. Following induced activation of Cre recombinase in a specific blastomere, embryos were transferred into the oviducts of pseudo-pregnant females. Manipulated embryos were recovered from uteri at approximately day 8.5 of development and subjected to X-gal staining. We tested several methods for activating Cre recombinase in the embryo (Table 1) and compared the efficiency of labeling. No positive staining was obtained in embryos directly injected with Cre recombinase protein. We tested two Cre recombinase expression vectors. The first was pBS185, which drives expression of Cre under control of the CMV promoter. This induced recombination but the X-gal-positive embryos was less than 10% of those injected. Next we tested pCAG-Cre, which utilizes the regulatory sequence of CMV and chick  $\beta$ -actin and a rabbit  $\beta$ -globin poly(A). Injection of this plasmid into the nuclei of specific blastomeres gave the most consistent results: 30% and 11.2% X-gal-positive embryos following injection at the 1-cell stage, and at the two-cell stage respectively. Accordingly we decided to use this method to mark individual cells.

Initially we examined the efficiency of reporter enzyme activation in embryos. Fig. 1 shows the representative embryos recovered following injection of pCAG-Cre into fertilized eggs. The manipulated embryos developed normally regardless of expression of the  $\beta$ -galactosidase reporter. From whole-mount observation, both embryonic and extra-embryonic tissues were stained, with the exception of the extra-embryonic membranes (Fig. 1A,E). Next, we performed a detailed

**Table 1. The efficiency of Cre activation in CAG-CAT-Z transgenic embryos**

Injected DNA/protein		Number transferred	Number implanted	Number of blue embryos
Injection at the one-cell stage				
CMV Cre (pBS185)	5 ng/μl	169	69	3 (4.3%)
pCAG-Cre	5 ng/μl	28	20	6 (30%)
Cre recombinase	10 ng/μl	123	60	0 (0%)
Injection at the two-cell stage				
CMV Cre (pBS185)	5 ng/μl	295	180	3 (1.7%)
pCAG-Cre	5 ng/μl	180	125	14 (11.2%)
Cre recombinase	10 ng/μl	47	32	0 (0%)

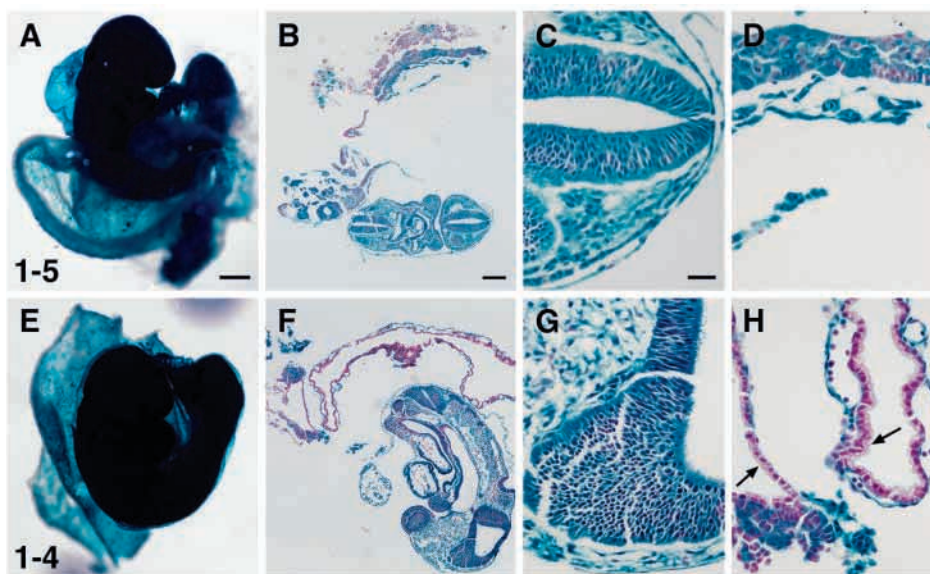
Three types of methods for Cre activation were tested in the one-cell and the two-cell stage embryos. pBS185 or pCAG-Cre plasmids were injected into the nucleus of the embryo. After culturing for specified periods, healthy embryos were transferred into the uteri of pseudo-pregnant females. These embryos were recovered 8 days later and subjected to X-gal staining. The numbers and percentage of the X-gal-positive embryos are shown. pCAG-Cre gave the best results, 30% and 11.2% X-gal-positive embryos following injection at the one-cell stage, and at the two-cell stage respectively. We could not obtain X-gal-positive embryos after injections with Cre recombinase protein.

examination of serial sections (Fig. 1B-D,F-H). Almost all cells in every germ layer were uniformly positive for β-gal activity. Variation in strength of staining between layers reflects the promoter activity in these cell types. Two cell populations persistently failed to stain; they were the primitive endoderm derivatives including the parietal endoderm and visceral endoderm. Few X-gal-positive cells were detected in parietal endoderm and no stained cells were observed in visceral yolk sac endoderm (Fig. 1H, arrows). Although all the cells were stained, variation of staining intensity was apparent in the trophoblast giant cells (data not shown). Most of the giant cells were clearly stained, however, punctuate staining within the cytoplasm was seen in some cells. Similar variation in staining was also observed in chorionic ectoderm. We also stained embryos obtained from wild-type females crossed with males carrying the reporter cassette in which the floxed CAT

gene had been excised (Fig. 2). The same staining pattern, namely lack of activity in the extra-embryonic endoderm, was observed (Fig. 2C, arrow and arrowhead), suggesting a lack of CAG promoter activity in this cell lineage. Accordingly, we were unable to trace the lineage of extra-embryonic endoderm derivatives in this system.

### Injection at the two-cell stage resulted in random distribution of labeled cells

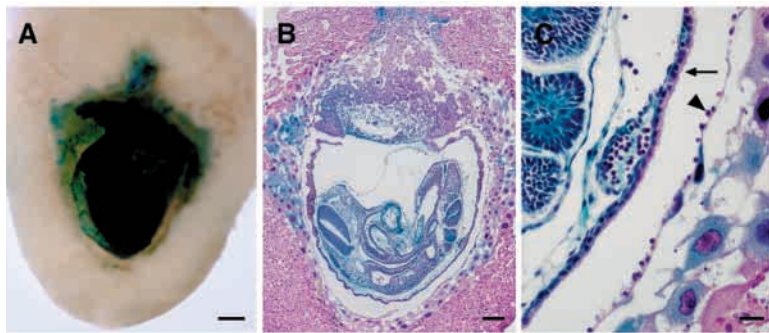
Two-cell stage embryos were collected from oviducts and the expression vector pCAG-Cre was injected into the nucleus of one blastomere. The embryos were then transferred into oviducts of pseudo-pregnant females. Eight days after transfer they were isolated from the uteri and subjected to X-gal staining. Representative embryos are shown in Fig. 3. While overtly similar to the embryos injected at the one-cell stage, staining was more uneven, with a mixture of X-gal-positive (blue) cells and white cells within a single embryo. These embryos were serially sectioned and the distribution of stained cells was examined in detail. We analyzed 37 embryos that were positive for X-gal staining and morphologically normal. One embryo showed a restricted distribution of the X-gal-positive cells only in extra-embryonic ectoderm, while in a second embryo there were no positive cells in the extra-embryonic ectoderm. In other embryos, the X-gal-positive and X-gal-negative cells were distributed uniformly throughout all lineages except for the primitive endoderm derivatives. There was no clear predominance of positive cells along these body axes. To obtain more quantitative data, we counted the number of X-gal-positive and -negative cells in nine independent embryos after Haematoxylin staining. Positive cells were identified by their blue cytoplasmic X-gal staining. The sections that were counted are shown in low magnification in Fig. 3B,G,J. Because we could not see clear preferential distribution, we selected a representative section of each embryo where we could observe the most tissues. The number of cells in several different cell layers are shown in the tables and histogram of Fig. 3E,H,K. The contribution of X-gal-positive cells in each layer varied between embryos. An exceptional case, was embryo 2-2 in which the amnion was



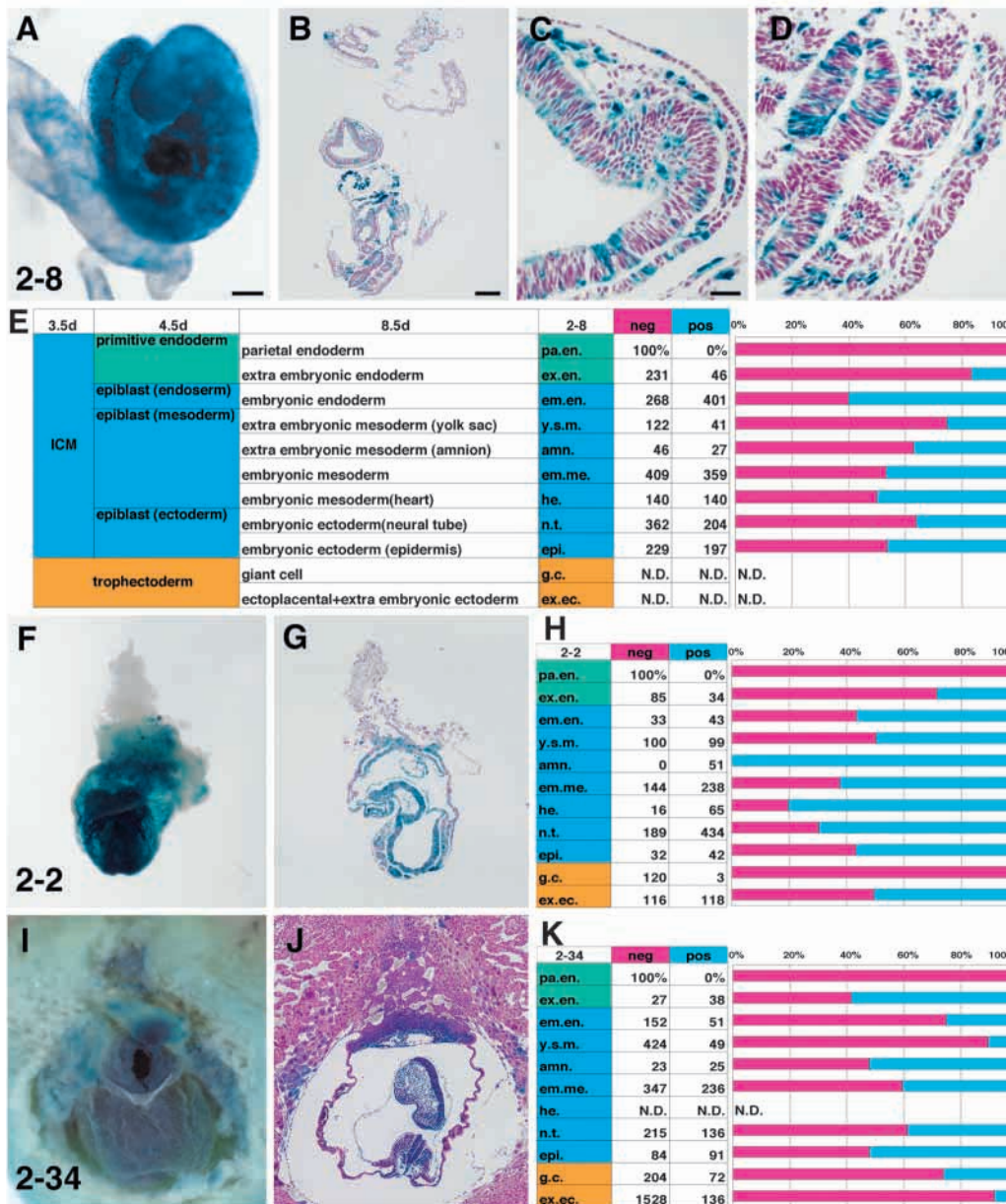
**Fig. 1.** Expression of β-galactosidase in embryos injected at the one-cell stage. Two representative embryos, 1-5 (A-D) and 1-4 (E-H) are shown. (A,E) Whole mounts and (B-D,F-H) Hematoxylin and Eosin-stained sections. Almost all parts of the embryos were positive for X-gal staining (A,B,E-F). (C,D,G,H) Higher magnifications of B and F respectively. (H) Derivatives of primitive endoderm (arrows) did not show X-gal staining. Scale bars: 300 μm (A,E), 150 μm (B,F), 30 μm (C,G).

completely X-gal-positive (Fig. 3G,H). In other embryos, the overall number of X-gal-positive cells was in the range of 20-80%. Positive cells made clusters. The contribution of X-gal-

positive cells to the trophoblast giant cells was lower than in other tissues, which may reflect the coherent growth of this lineage.



**Fig. 2.** Distribution of X-gal-positive cells in an embryo derived from a mating with a F<sub>1</sub> male lacking the floxed CAT gene. (A) Whole-mount embryo in the deciduum. All embryonic cells expressed β-gal except for the layers of primitive endoderm origin. (B,C) Sections stained with Hematoxylin and Eosin. The staining pattern was similar to that of embryos after injection at the one-cell stage. Almost all cells were stained except for the parietal endoderm (arrowhead) and visceral yolk sac endoderm (arrow). Scale bars, 300 μm (A), 150 μm (B), 30 μm (C).

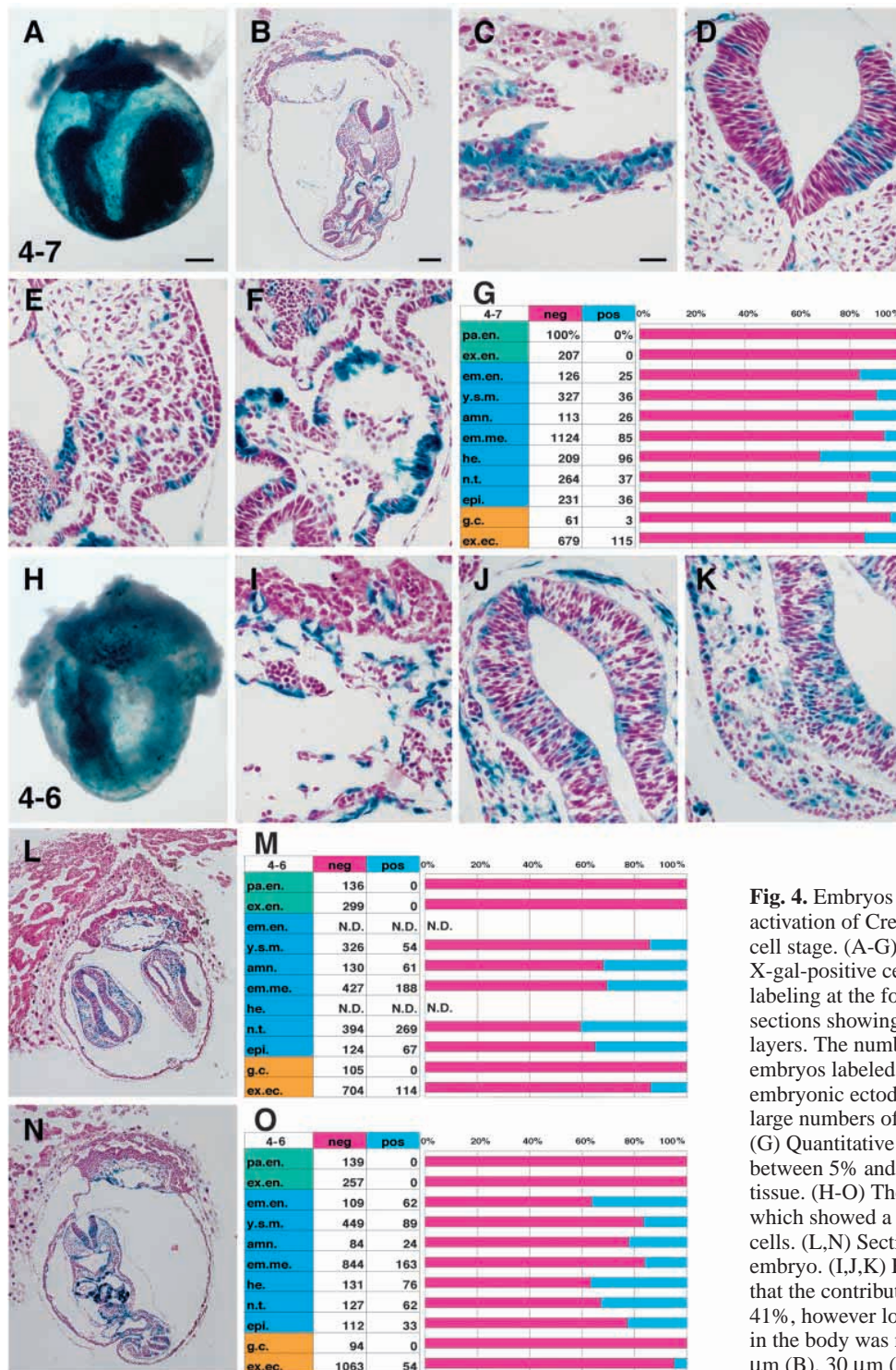


**Fig. 3.** Embryos injected at the two-cell stage showed random distribution of labeled cells. Embryos showed spotted distribution of X-gal-positive cells, and this pattern was seen evenly throughout the body axis (A,B) without regard to any embryonic axes. C and D are higher magnifications of B. In all cell layers, X-gal-positive and -negative cells were randomly intermingled. (E) Quantitative analysis. Classification of cell layers, and the result from one embryo, 2-8, are shown. Left panels indicate the origin of the each layer at 3.5 days and 4.5 days based on classic studies, and the name and abbreviation of the each tissue is also shown. The actual numbers of positive and negative cells are given and also represented by the histograms. F,G,H and I,J,K show the results of embryos 2-2 and 2-34, respectively. In the amnion of embryo 2-2, all the cells were positive for X-gal, however, this was an exceptional case because positive cells were intermingled with negative cells in other embryos. Scale bars: 300 μm (A) 150 μm (B), 30 μm (C).

**Injection at the four-cell stage resulted in three types of distribution of labeled cells**

Next we labeled single blastomeres of four-cell stage embryos by nuclear injection of the expression vector. Embryos were recovered from the uteri 8 days after transplantation and subjected to X-gal staining. Whole-mount images of the embryo are shown in Figs 4 and 5. A total of 54 positive embryos were recovered that developed normally. Three classes of staining patterns were observed.

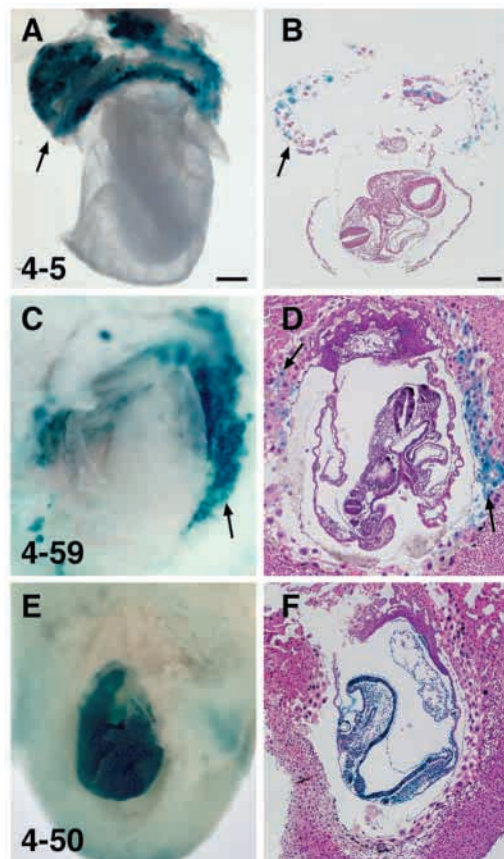
In the first type, the staining patterns were similar to those of the embryos injected at the two-cell stage (Fig. 4). 34 embryos were placed in this category. The X-gal-positive cells were distributed both in embryonic tissue and extra-embryonic ectoderm. In these cases, X-gal-positive cells were distributed randomly and there was no clear localization of these cells along the body axes. We quantified the results by counting the cell numbers (Fig. 4G,M,O). In all embryos in this category, the numbers of X-gal-positive cells in all germ layers were less



**Fig. 4.** Embryos with one type of X-gal distribution after activation of Cre recombinase in a blastomere at the four-cell stage. (A-G) The results of embryo 4-7. In this embryo, X-gal-positive cells were distributed randomly following labeling at the four-cell stage. (A) Whole mount, (B-F) sections showing that the stained cells were in all cell layers. The numbers of positive cells were less than in those embryos labeled at the two-cell stage. (C) In the extra-embryonic ectoderm of this embryo, clusters of relatively large numbers of X-gal-positive cells were observed. (G) Quantitative analysis. X-gal-positive cells accounted for between 5% and 31% of the total cells depending on the tissue. (H-O) The results of another example, embryo 4-6, which showed a similar pattern of distribution of labeled cells. (L,N) Sections at different positions of the same embryo. (I,J,K) High magnifications of L. Histograms show that the contribution of labeled cells is between 5% and 41%, however localization to any specific tissue or position in the body was not observed. Scale bars: 300  $\mu$ m (A), 150  $\mu$ m (B), 30  $\mu$ m (C).

than that seen after labeling of a blastomere at the two-cell stage. The ratio of positive to negative cells was variable between the germ layers. However, no embryo had more than 50% blue cells, and no specific germ layer showed preferential accumulation of X-gal-positive cells. We could observe relatively large clusters of positive cells in the extra-embryonic ectoderm (Fig. 4C), which may suggest coherent clonal growth in this lineage. In other tissues, such as the neural ectoderm (Fig. 4D,J), embryonic ectoderm (Fig. 4E,K) and embryonic mesoderm (Fig. 4D-F,K), the number of labeled cells in a cluster of X-gal-positive cells were relatively small and similar to that seen after injection at the two-cell stage. We also compared the distribution of labeled cells in different sections of the same embryo (Fig. 4L-O). We could not detect any clear preference of localization, which may also suggest random cell mixing in this embryonic population.

The second and third classes of distribution patterns (Fig. 5) were different from the pattern seen after labeling at the two-



**Fig. 5.** Two other types of labeled cell distribution after injection at the four-cell stage. (A-D) In the second type of distribution, only extra-embryonic ectoderm contained X-gal-positive cells. (A,C) Whole mounts; (B,D) sections of the embryos in A and C, respectively. In both embryos, a portion of trophoblast giant cells (arrows) and a part of the ectoplacental cone is for X-gal-positive. Note that embryo 4-59 (C,D, arrows) has labeled cells localized to the trophoblast giant cell layer. (E,F) The last type of cell distribution. In this embryo, there were no X-gal-positive cells in the extra-embryonic ectoderm region. In other regions, X-gal-positive and -negative cells were intermingled. Scale bars: 300  $\mu$ m (A), 150  $\mu$ m (B).

cell stage. In the second type, X-gal-positive cells were seen only in the extra-embryonic region (Fig. 5A-D). The trophoblast giant cells and some cells in the ectoplacental cone were stained blue. No positive cells were found in the embryo proper or in the extra-embryonic mesoderm layers in these embryos, while both X-gal-positive and -negative cells were found in the extra-embryonic ectoderm. As shown in Fig. 5D, more labeled giant cells were seen in one part than the other. In the final class, cells of the extra-embryonic ectoderm were completely unlabeled, although positive cells were found in other tissues (Fig. 5E,F). The numbers of embryos obtained from the experiments are summarized in Tables 2 and 3.

### Distribution of labeled cells in the blastocyst

Piotrowska et al. (Piotrowska et al., 2001) previously reported that the descendants of one blastomere in the two-cell stage embryo have a localized distribution in the blastocyst. Our results from analyzing cell distribution at later stages, however, showed that the descendants of a single blastomere of two-cell stage embryos intermingled completely in the embryo. To understand the timing of this cell mixing, we next examined the distribution of labeled cells in the blastocyst following labeling of a blastomere of the 2 or the four-cell stage embryo. This allowed us to address when the change in relative position of cells occurs; i.e. prior to or following the blastocyst stage. A blastomere of two-cell stage embryos was injected with the Cre expression vector and embryos were cultured in vitro until the blastocyst stage. The results are shown in Fig. 6A-F and in

**Table 2. Summary of the labeling experiments**

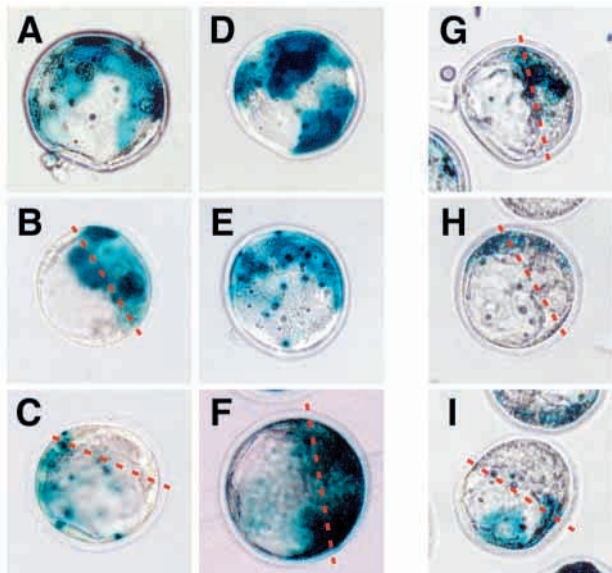
	Number transferred	Number implanted	Number of blue embryos
One cell	64	38	13 (34%)
Two cell	533	401	52 (13%)
Four cell	411	319	64 (20%)

Embryos injected with pCAG-Cre were used for the analysis. Following the injection, healthy embryos were selected and transferred. Number transferred, number of transferred embryos; Number implanted, the number of decidua found in the uterus of the foster mother; Number of blue embryos, the number of X-gal-positive embryo; the percentage is the number of blue embryos out of total implanted embryos.

**Table 3. Summary of distribution patterns of the X-gal-positive cells**

ICM derivatives	+	+	-	
TE derivatives	+	-	+	Total
One cell	13	0	0	13
Two cell	35	1	1	37
Four cell	34	4	16	54

Distribution patterns were categorized into three types. Numbers of embryos categorized in each type are shown. ICM derivatives indicate the cell layers derived from inner cell mass such as embryo proper and extra-embryonic mesoderm. TE derivatives indicate extra-embryonic ectoderm such as trophoblast giant cells and the cells of ectoplacental cone. The first type showed existence of labeled cells in both groups of cell layers, the second type showed distribution of labeled cells only in ICM origin, and the third type exclusively in TE derivatives. Out of the blue embryos in Table 2, those embryos recovered with extra-embryonic ectoderm that were morphologically normal were analyzed. Because we did not dissect out embryos with extra-embryonic ectoderm in early experiments, the numbers counted were less than the numbers shown in Table 2.



**Fig. 6.** Distribution of labeled cells at the blastocyst stage. Embryos were cultured *in vitro* following injection of Cre recombinase into a blastomere at the two-cell (A-F) or at the 4-cell (G-I) stage. Labeled cells were seen as clusters in these embryos. The boundary between the embryonic and abembryonic parts is indicated by the dashed line.

Table 4. We obtained similar results to those described by Piotrowska et al. (Piotrowska et al., 2001) who used an alternative labeling method. In 73% of the blastocysts, the labeled cells stayed in clusters and did not intermingle completely. In Fig. 6 the boundary between the embryonic and the abembryonic parts is indicated where visible. This boundary was clear in 84 out of 147 embryos. Labeled cells were found in the embryonic part in 35 of those embryos and in the abembryonic part in 26 embryos. These results indicate that the descendants of a two-cell blastomere could be located either in the embryonic or the abembryonic part of the blastocyst in 73% of embryos, supporting the idea that the first cleavage plane is nearly orthogonal to the embryonic-abembryonic axis of the blastocyst. Similar results were obtained after labeling a single blastomere at the four-cell stage (Fig. 6G-I and Table 4), although the number of X-gal-positive cells was lower than that seen following labeling at the two-cell stage. To further examine the relationship between the first cleavage plane and embryonic-abembryonic axis of the blastocyst, we cultured embryos in alginate gel to maintain the position. Two-cell stage embryos with zona pellucida were

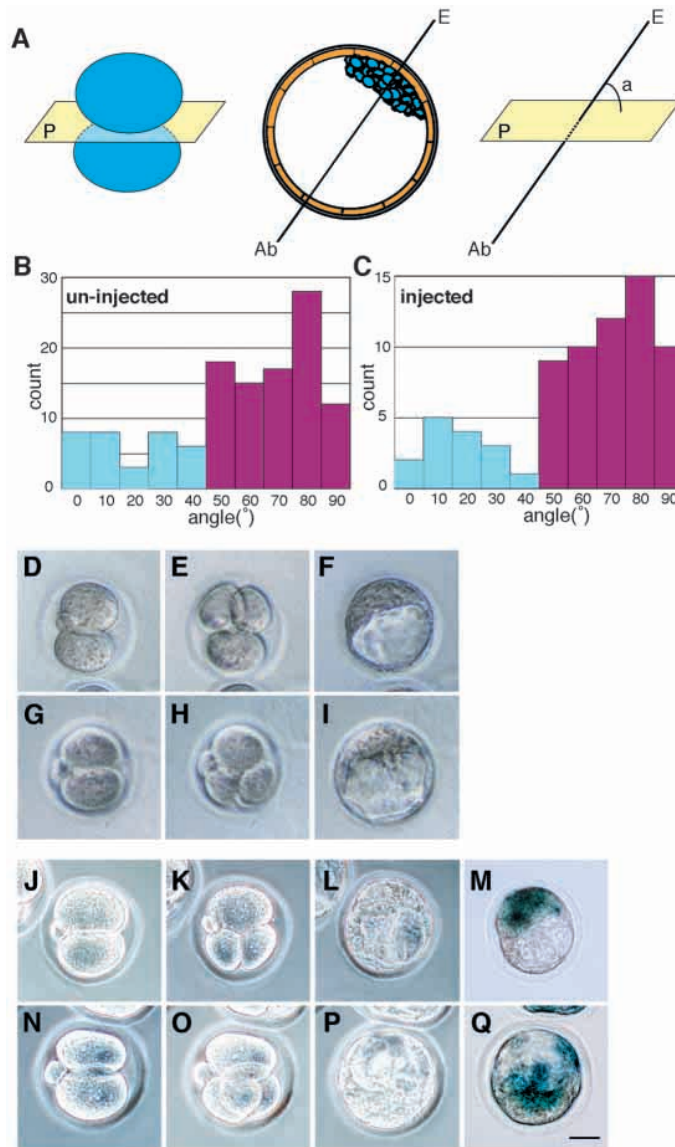
**Table 4. Distribution of X-gal-positive cells in blastocysts after labeling single blastomeres of two-cell or four-cell stage embryos**

Injection stage	Injected	Blue	Normal	Localized
Two cell	278	178	147	107 (73%)
Four cell	172	23	23	22 (96%)

Embryos were cultured *in vitro* after the injection with pCAG-Cre and the blastocysts were stained. Most X-gal-positive embryos (No. blue) were morphologically normal (normal). 73% (injection at the two-cell stage) and 96% (injection at the four-cell stage) of embryos showed localized distribution of labeled cells.

embedded in alginate gel, and cultured in a CO<sub>2</sub> incubator. Several embryos were cultured in a drop of alginate gel covered with alginate-free KSOM. In these drops, the relative positions of embryos were maintained, and this relative position was used to identify the orientation of each embryo. At the two-cell stage, polar bodies were located on or very close to the plane of the first cleavage. Although embryos had zona pelluciditas, the positions of polar bodies did not changed while they were visible, until the eight-cell stage (data not shown). Because it was not always easy to reliably distinguish the second polar body from the first polar body in the living embryo without damage as Gardner described (Gardner, 2002), the plane of the first cleavage was used as a landmark in the two-cell stage embryo. Photographs of embryos at the two-cell, three-cell and blastocyst stages were taken and compared. We measured the angle between the plane of the first cleavage and the embryonic-abembryonic axis of the blastocyst (Fig. 7A). Those embryos in which two blastomeres were positioned side by side in a drop of gel were used to measure the angle of cleavage. Embryos in which the first cleavage plane was not orthogonal to the bottom of the culture plate were not counted. We also analyzed the division order of blastomeres at second cleavage in cases where the angle between first cleavage plane and the embryonic-abembryonic axis was more than 50°. First, we examined intact embryos without injection. The angle was analyzed in 123 embryos (Fig. 7B). Although we observed a wide variety of distribution, more embryos in which the angle was greater than 50° (dark red columns in Fig. 7B) were mapped. Out of 123 embryo analyzed, 90 embryos (73%) were mapped in this category.

This result was consistent with our results of the labeling experiments at the two-cell stage, and the results reported by two other groups (Gardner, 2001; Piotrowska et al., 2001). We then analyzed the order of cell division of two-cell blastomeres. It is known that mammalian two-cell blastomeres divide asynchronously. Piotrowska et al., (Piotrowska et al., 2001) suggested that the cell that is first to divide preferentially contributes to the embryonic part of the blastocyst. We examined this possibility using an alternative method. Embryos embedded in alginate gel were observed and photographed every 30 minutes until most of the embryos had developed to the three-cell stage, and then they were allowed to develop to the blastocyst stage in a 37°C CO<sub>2</sub> incubator. Out of 90 embryos in which the angle between the first cleavage plane and the embryonic-abembryonic axis of the blastocyst was greater than 50°, we could capture images of the three-cell stage in 67 embryos. The first blastomere to divide was seen in the position corresponding to the future embryonic part of the blastocyst in 31 embryos (46%), as is shown in Fig. 7D-F. However, in 36 embryos (54%) the first two-cell blastomere to divide was located in the future abembryonic part. Thus in our experiments, intact embryos did not show a clear correlation between the division order of the two-cell blastomeres and the axis of the blastocyst. We then analyzed this relationship in embryos injected with Cre expression plasmid. These injected embryos were fixed and subjected to X-gal staining following the same procedures used for uninjected embryos. A summary of the angles between the first cleavage plane and the embryonic-abembryonic axis of the blastocysts is shown in Fig. 7C. Variation in the angle was similar to that of un-injected embryos. The distribution was also similar to that of uninjected



**Fig. 7.** Analysis of alginate-embedded embryos. Normal embryos without injection or embryos labeled with Cre recombinase were cultured in alginate gel to maintain their position. (A) The angle (a) between the first cleavage plane (P) and the embryonic-abembryonic axis (E-Ab) of the blastocyst was measured by comparing photographs. Only the embryos in which the first cleavage plane was orthogonal to the bottom of culture plate were used for the analysis. (B) The angle was found to be  $80^\circ$  in 28 out of 123 of the normal uninjected embryos. The angle was greater than  $50^\circ$  in 90 embryos (73%) (columns are colored in dark red). (C) In the case of DNA-injected embryos, similar distribution in angles were observed after analyzing 71 embryos. (D-I) Examples of uninjected embryos used for analysis. In the embryo in D-F, the angle was  $70^\circ$ , and the first blastomere to divide contributed mainly to the embryonic part of the embryo. In another normal embryo shown in G-I the angle was  $80^\circ$ , and the early-dividing blastomere contributed to the abembryonic part of the embryo. (J-Q) Two examples of labeled embryos. (J-M) The injected blastomere divided later than the other blastomere and contributed to the embryonic part in the embryo as can be seen from the X-gal staining in this region (M). In the embryo shown in N-Q, the injected blastomere also divided late. The labeled cells were seen in the trophectoderm of the abembryonic part and around the boundary zone. Scale bar:  $30\ \mu\text{m}$ .

embryos with the peak of distribution at around  $80^\circ$ . Out of 71 embryos analyzed, 56 embryos (79%) were in the category in which the angle was more than  $50^\circ$  (Fig. 7C, dark red columns). This result suggests that injection of expression plasmid did not have a clear influence on the specification of the embryonic-abembryonic axis in the blastocyst. We also analyzed division order of two-cell blastomeres in 18 embryos. The injected blastomere divided first in four embryos (22%). Out of these 4 embryos, X-gal-positive cells were found in the embryonic half in two and in the abembryonic half in the other two. The injected blastomere divided later in 14 embryos (78%). Nine embryos had X-gal-positive cells in the embryonic part, and in five embryos they were mainly in the abembryonic part. These results suggest that injection of DNA may slightly affect the timing of cell division. However, the labeled cells could contribute to both embryonic and abembryonic parts of the blastocyst.

## Discussion

We carried out cell lineage tracing experiments in the early mouse embryo. We took advantage of a recombination-based method, using expression of Cre following permanent activation of reporter gene expression to label a specific cell. This method generates a non-diffusible, cell-autonomous and heritable marking of cells, and thus is suitable for lineage tracing during extended periods of embryonic development. Only low levels and transient expression of Cre recombinase is required to mediate recombination of the target DNA. We first tested the efficiency of the cell labeling method with Cre recombinase in heterozygous CAG-CAT-Z (Araki et al., 1995) transgenic mouse embryos. Injection of the Cre expression plasmid (pCAG-Cre) gave the most consistent results for cell labeling. The manipulated embryos recovered from the uteri were morphologically normal, indicating that the manipulation did not perturb normal development. After injection at the one-cell stage, the majority of cells became positive for X-gal staining with the exception of cells derived from the primitive endoderm. The lack of X-gal staining in these cells was not due to inefficient recombination in their cell lineage because no negative cells were found at the blastocyst stage after labeling at the one-cell stage (data not shown), and embryos derived from matings with males that carry a transgene without the CAT gene insertion showed a similar staining pattern. Lack of staining suggests that the CAG expression cassette inserted in this transgenic mouse was silent or very weakly expressed in these cells. However, combining the CAG-CAT-Z transgene and pCAG-Cre provided a very suitable system for analyzing other cell lineages in developing embryos. An important concern is the effect of the manipulation on the developmental potential of the cell. For example, the manipulation may disturb the normal fate of the marked cell or affect the potential to contribute to a certain type of tissue. This possibility, however, was ruled out by the results of marking a single blastomere in two-cell and four-cell stage embryos. Analysis of the cleavage pattern of two-cell blastomeres following the injection showed that the manipulation had only a slight effect on the timing of cell division. However, the relationship between the first cleavage plane and the embryonic-abembryonic axis of the blastocyst was not affected. The manipulated blastomere could contribute to both embryonic



and abembryonic parts in the blastocyst, suggesting that the manipulation did not affect the specification of cell fates. We also found the labeled cells could contribute to the cell types examined in 8.5-day embryos. Accordingly in this paper we exploited this transgenic mouse to analyze cell lineage.

After marking a blastomere at the two-cell stage, we observed random distribution of the labeled cells in 8.5-day embryos. These results suggested that rearrangement of the relative position of descendant cells from the two blastomeres occurred in the embryo between the two-cell stage and 8.5-day, the stage at which we performed the analysis. Although there are experiments showing indirectly incoherent clonal outgrowth of cells in the epiblast (Gardner and Cockroft, 1998; Weber et al., 1999), our report is the first observation to clearly demonstrate this phenomenon by tracing the cell lineages in normal mouse embryos over a long developmental period. This feature of cell mixing during early embryogenesis is specific to the mouse embryo. For example, in *Xenopus* embryos, a relatively clear segregation of cells along the left-right body axis is observed after labeling of a blastomere at the two-cell stage (Jacobson and Hirose, 1978). The first cleavage plane thus reflects the midline of the resulting tadpole. Our results suggest the program of embryonic development in mouse has inherent plasticity and that mixing of epiblast cells along the body axis happens during early stages. Piotrowska et al., (Piotrowska et al., 2001) showed that descendants of a two-cell stage blastomere analyzed in the blastocyst tend to be localized and the cells originating from one blastomere form a distinct boundary with those cells derived from the other blastomere. Similarly, we showed, with our labeling method, that drastic cell mixing does not occur until the blastocyst stage (Fig. 6 and Table 4), which is consistent with previous reports. Our data suggests that extensive intermingling of cells occurs subsequently to the blastocyst stage. The coherency of clonal cell growth may vary between cell layers (Gardner and Cockroft, 1998; Weber et al., 1999). Gardner and Cockroft and Weber et al. suggested that there is coherent clonal outgrowth of visceral endoderm and incoherent growth of ICM derivatives. Unfortunately, we could not examine the coherency of growth in cells originating from the primitive endoderm because our reporter system failed to mark primitive endoderm derivatives. The previous work of Gardner and a colleague (Gardner and Cockroft, 1998) showed cell mixing in the epiblast of chimaeric embryos after transplantation of cells into the blastocyst. They also showed two daughter cells located in non-adjacent positions following cell division in a non-chimeric 7-day epiblast, suggesting possible incoherent clonal growth in this cell layer. We could clearly show incoherent clonal growth of cells during development of the non-chimeric embryo, showing that cell mixing occurs in the post-implantation embryo. In contrast, we found larger patches of labeled cells in the trophoblast giant cell population (Fig. 3J and Fig. 5), indicating relatively coherent clonal growth of cells in this tissue.

Recently, the relation between the sperm entry point, the first cleavage plane, and the embryonic-abembryonic axis of the blastocyst has been discussed (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001; Davies and Gardner, 2002). Piotrowska et al. (Piotrowska et al., 2001) proposed a model in which the first cleavage plane is determined by the position of the polar body and the sperm entry point. However, other groups claimed

that the first cleavage plane is not related to the sperm entry point (Davies and Gardner, 2002). It is also suggested that the first cleavage plane is nearly orthogonal to the embryonic-abembryonic axis of the blastocyst (Gardner, 2001; Piotrowska et al., 2001). Kelly et al. (Kelly et al., 1978) suggested that the descendants of the first cell to divide to produce a three-cell stage embryo divide ahead of those cells derived from its slower partner. They also showed that the first pair of cells to reach the eight-cell stage contribute more descendants to the ICM than the last cell to produce the eight-cell stage. Piotrowska et al. (Piotrowska et al., 2001) suggested that a two-cell blastomere that divides early tends to contribute to embryonic parts of the blastocyst. However, their data revealed that neither of the two blastomeres contributes exclusively to the cells of the ICM. We also analyzed the relationship between the first cleavage plane, the division order of two-cell blastomeres and the orientation of embryo-abembryonic axis of the blastocyst. The labeling of the two-cell stage blastomere resulted in labeled cells in either the embryonic or in abembryonic part of blastocyst in 73% of embryos. This suggests a strong relationship between the first cleavage plane and the axis of the blastocyst. We observed a similar relationship by analyzing the embryos embedded in the alginate gel. It seemed that the variation of the angle of the first cleavage plane and the embryonic axis was greater in our results than those reported before. This may be because of the difference in mouse strains and methods. Gardner showed that the angle differs somewhat between mouse strains (Gardner, 2001). Our results support the idea that the first cleavage plane is close to orthogonal to the embryonic-abembryonic axis of the blastocyst. However, our results of the relationship between the cell division order and the allocation of the ICM in the blastocyst were different from that previously reported (Piotrowska et al., 2001). We observed that the first cell to divide contributed to the embryonic part in 46% of uninjected normal embryos in which the angle between the first cleavage plane and the axis of the blastocyst was greater than 50°. The early dividing blastomere was located to the abembryonic part of the blastocyst in the remaining 54% of embryos (Fig. 7G-I). We could only examine the topological relationship in this study although the position of cells in embryos were basically maintained in zona pellucida, and we did not trace actual cell lineages from three-cell to blastocyst stages because of technical limitations. As we noticed that the DNA injection affected division order of two-cell stage blastomeres, alternative methods are needed to further examine this relationship. In the manipulated embryos, labeled blastomeres contributed to both the embryonic part and the abembryonic part. The variation in angle between the first cleavage plane and the axis of the blastocyst was similar to that of normal embryos. These suggest that the DNA injection has little effect on the specification of cell fate, and division order does not necessarily determine the fates of blastomeres. Our results after the labeling at the two-cell stage showed that the descendants of either blastomere contribute to embryonic tissues at every axial level of the resulting embryonic body in 8.5-day embryos. These suggest that incoherent clonal growth and drastic cell mixing occurs later than the blastocyst stage, and that the actual body axis may be specified during later development.

The earliest segregation of cell fate is believed to occur between trophectoderm and inner cell mass during formation

of the blastocyst. Our data corroborate this. We classified the pattern of labeled cell distribution. Labeling at the four-cell stage resulted in three types of distribution patterns of X-gal-positive cells in 8.5-day embryos. The first pattern type was similar to the results of labeling at the two-cell stage, namely that the X-gal-positive cells were distributed randomly in all germ layers. As expected, the number of positive cells was less than that seen in the embryos labeled at the two-cell stage, and there were no specific biases in contribution. In the second type, only trophoblast giant cells and part of the ectoplacental cone were positive. This suggests that one blastomere of the four-cell stage embryo contributed exclusively to extra-embryonic tissue. However we note that in these embryos, X-gal-negative cells were also present in these extra-embryonic layers suggesting that descendants of other blastomere also contributed to this lineage. The reciprocal patterns of staining were also seen where X-gal-positive cells were absent from extra-embryonic ectoderm, while contributing to the embryo proper. These results also ruled out the possibility that the manipulation affected the potential of the cell to differentiate into certain cell lineages. From these data, we can propose two models for the segregation of cell fate. The first possible explanation is that the specification of cells starts at the four-cell stage and one blastomere can contribute only to the extra-embryonic tissue, another one only contributes to other cell types, and the remaining two retain the potential to contribute to both tissues. However, because we could not test the variation between embryos, the possibility remains that this segregation of future cell fates is not a common event observed in all the embryos. We should also pay attention to the loss of cells during development. The limitation of this type of experiment is that we can analyze only surviving cells and not the pattern of cell death that is routinely seen during development. An alternative explanation for the segregation of fate is that it depends on the cleavage pattern and the position of blastomeres in an embryo as proposed by Wilson et al. (Wilson et al., 1972). In their model, the cortical cytoplasm contributed to the trophectoderm and the deep cytoplasm mostly contributed to the ICM. Considering this, it might be possible that if the cleavage pattern and the allocation of blastomeres are relatively fixed, developmental programs of each blastomere are also regulated via these factors. To resolve this issue, we need to examine embryos in further detail and obtain statistical data. In any case, our data do not show that the cell fate is determined in this stage. As Kelly showed clearly (Kelly, 1977), all of the isolated four-cell stage blastomeres still possess the potential to contribute to all cell types of the body. In conclusion, our experiments show that the first segregation of cell fates is between ICM and trophectoderm, and that it may happen between the two-cell and four-cell stages.

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