The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation

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SUMMARY

The cardiogenic potency of cells in the epiblast of the early primitive-streak stage (early PS) embryo was tested by heterotopic transplantation. The results of this study show that cells in the anterior and posterior epiblast of the early PS-stage embryos have similar cardiogenic potency, and that they differentiated to heart cells after they were transplanted directly to the heart field of the late PS embryo. That the epiblast cells can acquire a cardiac fate without any prior act of ingression through the primitive streak or movement within the mesoderm suggests that neither morphogenetic event is critical for the specification of the cardiogenic fate. The mesodermal cells that have recently ingressed through the primitive streak can express a broad cell fate that is characteristic of the pre-ingressed cells in the host when they were returned to the epiblast. However, mesoderm cells that have ingressed through the primitive streak did not contribute to the lateral plate mesoderm after transplantation back to the epiblast, implying that some restriction of lineage potency may have occurred during ingression. Early PS stage epiblast cells that were transplanted to the epiblast of the mid PS host embryos colonised the embryonic mesoderm but not the extraembryonic mesoderm. This departure from the normal cell fate indicates that the allocation of epiblast cells to the mesodermal lineages is dependent on the timing of their recruitment to the primitive streak and the morphogenetic options that are available to the ingressing cells at that instance.

Key words: mouse embryo, gastrulation, heart mesoderm, epiblast, cell position, cell movement

INTRODUCTION

The immediate outcome of gastrulation is the formation of the definitive germ layer and the organisation of a general body plan, which serves as a blueprint for fetal development. The establishment of the body plan is accomplished by the generation of diversified tissue lineages from a pluripotent progenitor cell population and the siting of the descendants of different lineages in their appropriate locations in the embryo. Of specific interest is an understanding of the mechanisms of lineage specification and the morphogenetic processes leading to body patterning.

Clonal analyses and fate-mapping studies have shown that cells in the epiblast of the early primitive-streak stage (early PS) embryo are not restricted in their lineage potency. Descendants of single epiblast cells are allocated to diverse lineages and the epiblast cells may acquire a different fate after transplantation to a new site (Lawson et al., 1991; Tam and Zhou, 1996). Despite this plasticity of cell potency, distinct regionalisation of cell fate has been found and fate maps depicting the location of the progenitors of major tissue lineages in the epiblast have been constructed. Essentially, in these fate maps, cells in different regions of the embryo are assigned a specific fate, in accordance with the probability that descendants of these cells may be allocated to tissues of a particular part of

the body. The allocation of cells to specific lineages is influenced by the morphogenetic behaviour of the cells (Lawson et al., 1991) and the inductive interaction experienced by the epiblast cells as they ingress at the primitive streak and in the newly formed germ layers (Sasaki and Hogan, 1993; Hogan, 1995; Yamaguchi et al., 1994; Ang and Rossant, 1993, 1994; Ang et al., 1994).

The heart is one of the first structures to form during organogenesis of the mouse embryo (DeRuiter et al., 1992). Previous fate-mapping studies have identified the lateral epiblast as the major source of embryonic mesoderm including the heart mesoderm, which is localised to the posterior regions of the lateral epiblast (Lawson et al., 1991; Lawson and Perdersen, 1992a,b; Parameswaran and Tam, 1995). Epiblast cells destined for the heart are co-localised with the cranial mesenchyme to the distal region of the newly formed mesodermal layer of the mid PS embryo. By the late PS stage, the prospective heart mesoderm is found in the anterior proximal regions of the mesodermal layer underneath the cephalic neural plate (Parameswaran and Tam, 1995). The localisation of the heart precursors in the mesoderm of 7.0-7.5-day embryos coincides with the distribution of the transcripts of the endothelium-specific *GATA4* and *Flt1* genes (Heikinheimo et al., 1994; Yamaguchi et al., 1993) and the *Nkx2.5* gene in the myocardium (Lints et al., 1993; reviewed by Lyons, 1996). There is therefore an

apparent shift of the precursors of the heart mesoderm from a distal location to an anterior-proximal position on the lateral aspect of the gastrulating embryo. However, this migratory pattern, which is re-constructed from the fate maps, has to be verified by tracking the movement of cells in the embryo.

Studies performed in the amphibian and chick embryos have shown that the developmental fate of the heart precursor may be specified early during gastrulation (Sater and Jacobson, 1989; Yutzey et al., 1995). The cardiogenic potency of the cells is initially labile and becomes progressively fixed through the cumulative effect of continuous inductive tissue interaction (Sater and Jacobson, 1990). The results of experiments that involve the ablation, recombination and transplantation of the germ layers of amphibian and avian embryos have shown that some inductive signals emanating from the endoderm may be required for the specification of cell fate, the maintenance of tissue competence and the induction of the cardiogenic mesoderm to terminal differentiation. There are, however, conflicting views as to how critical the role of the endoderm is to these cardiogenic processes in different vertebrate embryos (Gannon and Bader, 1995; reviewed by Jacobson and Sater, 1988).

In the present study, experiments were designed to address (1) whether the morphogenetic cell movement during gastrulation is critical for the allocation and the differentiation of epiblast cells to heart mesoderm; (2) if the recruitment of epiblast cells to the mesoderm leads to any concomitant restriction in cell potency; and (3) if the timing of ingression of the epiblast cells has any impact on their developmental fate. The basic experimental strategy involved the transplantation of groups of transgenically tagged cells to defined sites in the germ layers and testing of the potency of these cells to colonise the host tissues and to differentiate into cardiac cells.

MATERIALS AND METHODS

Embryo collection and culture

Mouse embryos were collected at 6.5 to 7.5 days post coitum from pregnant mice of the ARC/s strain and the transgenic H253 line. The transgenic mice express a *lacZ* transgene that is regulated by the promoter of the mouse gene encoding the 3′ hydroxyl-5′ methyl glutaryl (HMG) coenzyme A reductase. This reporter construct also contains the nuclear-localisation signal (nls) sequence derived from the SV40 T antigen, which results in the localisation of the β-galactosidase in the nucleus of transgenic cells. The transgenic cells are therefore stained blue by X-gal reagent, leaving the cytoplasm unstained, so that the expression of cytoplasmic lineage-specific marker such as the myofibrillar tropomyosin protein can be detected with little interference from the transgenic activity. This *HMG-nls-lacZ* transgene is expressed ubiquitously in all embryonic tissues during gastrulation and early organogenesis (Tam and Tan, 1992).

Embryos were explanted from the decidua and freed of the investing Reichert's membrane. Embryos were cultured briefly for 1-1.5 hours prior to micromanipulation and cultured afterwards in the same medium until they developed to the early somite stage. The culture period was 44-46 hours for 6.5-day embryos, 36-38 hours for 7.0-day embryos and 24-26 hours for 7.5-day embryos. The culture medium consisted of Dulbecco's modified Eagle medium supplemented with heat-inactivated human cord serum and rat serum (Sturm and Tam, 1993) and embryos were cultured either in wells on the 4-well chamber slide (NUNC) or in rotating bottles (BTC Engineering), depending on the stage of development.

Cell transplantation

Germ layer tissues used for transplantation were isolated from 6.5- and 7.0-day embryos derived from the H253 transgenic line. The 6.5-day embryos were dissected with electrolytically polished alloy metal needles into anterior and posterior halves, with reference to the newly formed primitive streak as the landmark for the posterior side of the cylindrical embryo. Small fragments of tissues were isolated from the distal portion of the posterior epiblast, which has previously been shown by fate mapping to contain the mesoderm precursor for the heart and the cranial mesenchyme (Lawson et al., 1991; Parameswaran and Tam, 1995), and the proximal part of the anterior epiblast, which is fated for amnion, surface ectoderm and neuroectoderm (Lawson et al., 1991; Quinlan et al., 1995; Fig. 1). Dissection of tissue fragments and the removal of the primitive endoderm were done by cutting with pairs of
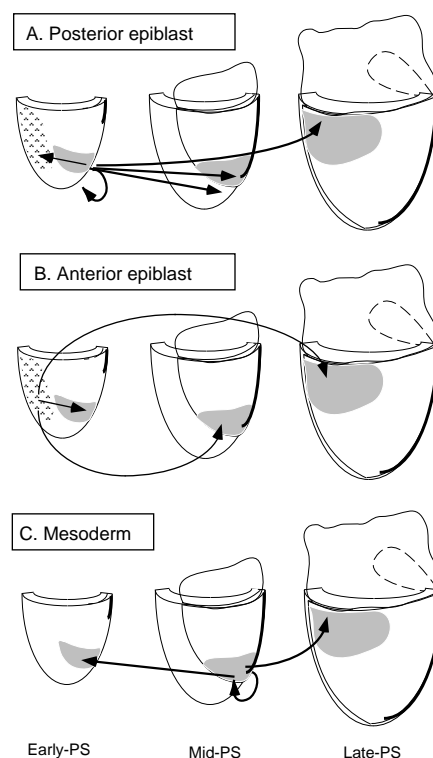

Fig. 1. The experimental strategy for testing the developmental potency of 6.5-day (early primitive-streak stage, early PS) epiblast cells and 7.0-day (mid-primitive-streak stage, mid PS) mesodermal cells by cell transplantation. (A) Cells isolated from the posterior epiblast of the early PS embryo, containing the precursor populations of the heart and other mesodermal cells (stippled area), were transplanted orthotopically. They were also transplanted heterotopically to the anterior epiblast of the early PS embryo, to the posterior epiblast and the most distal part of the nascent mesodermal layer of the mid PS embryo and to the anterior-proximal region of the mesoderm of the 7.5-day (late primitive-streak stage, late PS) embryos. (B) Cells isolated from the anterior epiblast of the early PS embryo, which are fated to form the surface ectoderm, the neural plate and the amnion (∧ hatched), were transplanted heterotopically to the posterior epiblast of the early PS embryo, to the most distal part of the nascent mesodermal layer of the mid PS embryo and to the anterior-proximal region of the mesoderm of the late PS embryo. (C) Cells in the most distal part of the nascent mesodermal layer of the mid PS embryo were transplanted heterotopically to the posterior epiblast of the early PS embryo and to the anterior-proximal region of the mesoderm of the late PS embryo.

finely drawn glass needles. For the 7.0-day embryo, the newly formed mesoderm was dissected free of the ectoderm and the endoderm by teasing the germ layers with fine glass needles without any prior enzymatic digestion. The distal portion of the mesodermal layer lying next to the distal end of the primitive streak (Fig. 1), which contains the recently ingressed mesodermal cells destined for the heart and the cranial mesenchyme (Parameswaran and Tam, 1995), was isolated. The epiblast and mesodermal fragments were then dissected into clumps of 5-15 cells for transplantation to host embryos.

The transplantation of cell clumps was done using a Leitz micromanipulation system in conjunction with a Leitz Fluovert fixed-stage inverted microscope. The holding of the embryo and the microinjection of cell clumps were achieved with oil-filled micropipettes controlled by a micrometer syringe (Wellcome Agla syringe assembly) and a pressure pump (de Fonbrune, Alcatel). The dissection of tissues and the micromanipulation of embryos were done at room temperature in PB1 medium supplemented with bovine serum albumin and 10% fetal calf serum.

Cells from the anterior epiblast and the posterior epiblast of the 6.5-day (early primitive-streak stage, early PS) and the mesoderm of the 7.0-day (mid primitive-streak stage, mid PS) embryos were transplanted to sites where the precursors of the heart mesoderm were found at different stages of gastrulation (Fig. 1). The posterior epiblast was also transplanted heterotopically to the anterior epiblast and to the posterior epiblast of the 7.0-day embryo. The strategy of transplantation was designed to test the developmental potency of the donor tissues for differentiation into mesodermal derivatives, and specifically to the heart mesoderm.

Tracing the movement of the mesodermal cells in mid- to late PS embryo

The mesodermal cells in the distal region of the newly formed mesoderm of nine 7.0-day embryos were labelled by a carbocyanine dye (DiO) in order to follow their movement during the next 12-14 hours of development in vitro. The dye solution was prepared by dissolving 50 µg of 5,5′-Ph$_2$-DiOC$_{18}$(3) (#D-7770, Molecular Probes) in 1 ml absolute ethanol, which was then diluted 1:10 in 0.2 M sucrose to a working concentration of 5 µg/ml. A small volume (approx. 10 pl) of the diluted dye solution was force-injected into the mesoderm of the 7.0-day embryo. The labelled embryos were cultured and harvested after either 5-6 hours or 12-14 hours of culture.

Analysis of results
Localisation of graft-derived cells

Cultured embryos were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) for 3-5 minutes. Fixed embryos were washed twice in PBS and in washing buffer (PBS containing 2 mM MgCl2, 5 mM EGTA, 0.01% sodium deoxycholate, 0.02% Nonidet P-40) and then stained overnight at 37°C in X-gal staining solution (0.1% 5-bromo-4-chloro-3-indoly-β-D-galactopyranosidase, 5 mM K$_3$Fe(CN)$_6$ and 5 mM K$_4$Fe(CN)$_6$.6H$_2$O in washing buffer solution). Whole embryos were assessed for the localisation of the *lacZ*-expressing cells in the embryonic tissues. They were then processed for wax histology and serial 8-µm sections were counterstained with 0.1% nuclear fast red (Tam and Zhou, 1996). The localisation and the number of the X-gal-stained cells in different tissue types were scored.

Immunostaining of sarcomeric tropomyosin in myocardial cells

In order to assess if the graft-derived cells in the heart were displaying the appropriate myocardial phenotype, the expression of a myocardial myofibrillar protein was examined by immunostaining using a monoclonal antibody against the sarcomeric tropomyosin (clone CH1, Sigma #T9283). This antibody has been used sucessfully

Fig. 2. The displacement of DiO-labelled cells in the mesoderm during gastrulation. Cells in the most distal region of the newly formed mesodermal layer and in the vicinity of the distal end of the primitive streak of 7.0-day (mid PS) embryo were labelled by injecting DiI. Embryos were examined after 5-6 hours (A-C) and 15 hours (D) of in vitro culture. The figures show the left lateral view of the primitive-streak-stage embryo. The confocal fluorescent image is the summation of optical sections taken in step at 5 µm through the mesodermal layer and the bright-field picture of the egg cylinder was taken in the sagittal plane. (A) In this embryo, most labelled cells stay near the site of labelling but some (small arrows). are displaced anteriorly (in the direction of the hollow arrow). with the expanding mesodermal layer.
(B) Displacement of the labelled mesoderm away from the primitive streak side of the embryo and towards the proximal-lateral region of the embryo.
(C) Anterior and proximal displacement of the labelled mesodermal cells after 6 hours of in vitro development. (D) Localisation of the labelled mesoderm in the anterior and proximal parts of the fully formed mesodermal layer in a late PS stage embryo after 14 hours of culture. The field covers approximately one-third of the anterior-lateral aspect of the embryo. The hollow arrow points anteriorly. ps, primitive streak side; ant, anterior side of the embryo. Bar, 50 µm.
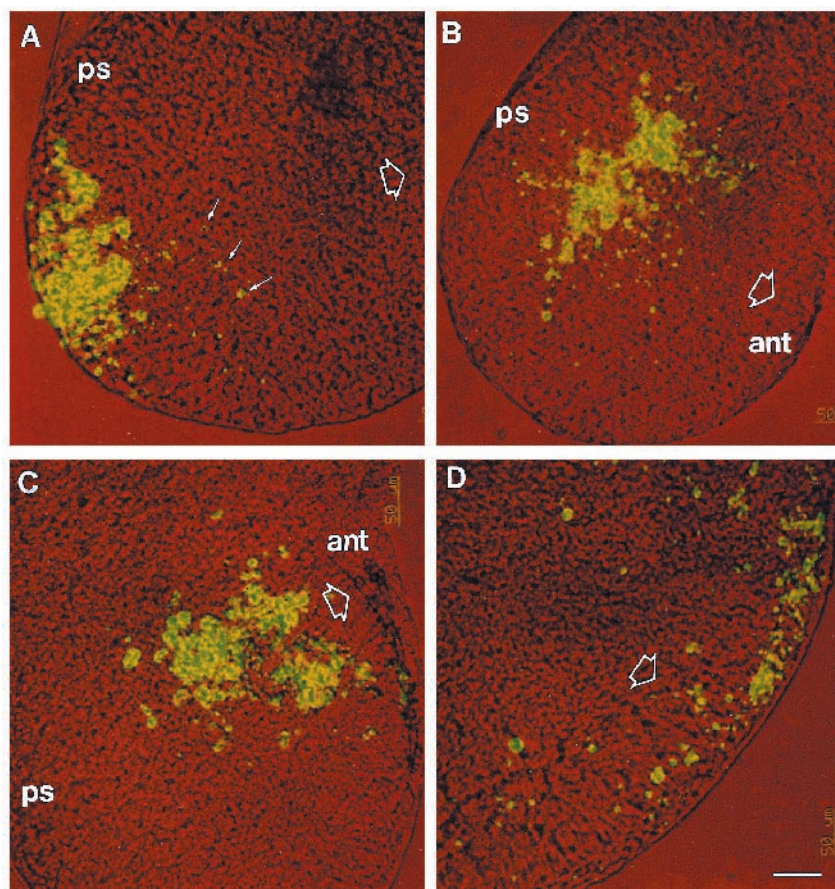
to detect the myofibrillar protein in the myocardial and skeletal cells in the zebrafish embryo (Stainier et al., 1995). In the mouse, only the myocardial population in the early somite-stage embryo was stained with this antibody. Other mesodermal tissues, including the dermomyotome of the somite, did not give a positive staining at this stage (R. P. Weinberger and M. Parameswaran, unpublished results).

Cultured embryos were fixed in 4% paraformaldehyde in PBS for 5 minutes and then stained with X-gal solution, as described in the previous section. Stained embryos were washed in PBS and water and then assessed for the distribution of the *lacZ*-expressing cells in the host tissues. The specimens were dehydrated through the ethanol series and embedded in water-miscible low-melting-point (37°C) polyester wax. Serial 8-μm sections were treated with Target Unmasking Fluid (Boehringer Mannheim), blocked with normal goat serum and then incubated for 1 hour in a 1:150 dilution of anti-sarcomeric tropomyosin monoclonal antibody (clone CH1, Sigma #T9283) at room temperature. After the post-incubation washes, the sections were incubated for 1 hour in a humid environment with a 1:200 goat anti-mouse IgG conjugated to alkaline phosphatase (Jackson Laboratory #115-055-146). Following the incubation of the secondary antibody, the treatment with the same primary and secondary antibodies was repeated to achieve further amplification of the immunoreaction. The alkaline phosphatase activity was revealed by staining with reagents of a Vector Red colour detection kit (Vector Laboratories). The sections were then mounted directly in water-miscible glycergel (DAKO) or in Canada balsam after dehydration and clearing. The stained sections were examined using bright-field and phase-contrast microscopy.

Confocal microscopy of DiO-labelled embryos

At specific times after labelling with DiO, embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C. The embryos were mounted flat under extra-thin cover slips (thickness grade 1.5, Lomb) in the fixative or PBS and sealed with varnish. The specimens were examined under a Leica confocal laser scanning Diaplan microscope. The green fluorescence of the DiO label was detected at the excitation wavelength of 488 nm and emission wavelength of about 560 nm using a FITC fluorescence setting. Optical sections were taken at a defined depth in the core of the specimen, which corresponds to the position of the mesoderm. Composite images were generated by stacking optical sections superimposed on a bright-field view of the specimen.

RESULTS

Heart mesoderm moves proximally and anteriorly during gastrulation

Results of the dye-labelling experiment have shown that recently ingressed mesodermal cells located near the distal end of the primitive streak of the mid PS embryo were displaced towards the anterior and proximal regions of the mesoderm during in vitro development. There were some variations in the extent of population movement in the first 5-6 hours of in vitro development. In one embryo, the majority of labelled cells congregated near the site of labelling but some cells dispersed anteriorly in the mesoderm (Fig. 2A). In four other embryos, the labelled cell population has moved en masse away from the primitive streak and the cells were found in the mesoderm at the lateral region of the egg cylinder (Fig. 2B) or in the anterior-proximal quadrant of the embryo (Fig. 2C). In four embryos examined after 12-14 hours of culture, most of the labelled cells were now found in the anterior-proximal region of the mesoderm (Fig. 2D),

where the cardiogenic mesoderm is localised. The results of this time-course study of the localisation of the labelled mesoderm suggests that cells are displaced from the distal posterior region of the mid PS embryo to the proximal-anterior region of the mesoderm of the late PS embryo via the lateral aspect of the embryo.

Potency of posterior epiblast cells is not restricted during early gastrulation

For the 6.5-day posterior epiblast cells, transplantation experiments were performed (Fig. 1A) to test if the act of cellular ingression through the primitive streak and the post-ingression movement in the mesodermal layer are critical for the determination of cell fate.

Posterior epiblast cells contributed to embryonic mesodermal tissues (heart, cranial mesenchyme, somites and lateral plate mesoderm), gut endoderm and extraembryonic mesoderm when transplanted orthotopically to 6.5-day embryos (Fig. 3D). When the posterior epiblast cells were transplanted to the distal mesoderm of the 7.0-day embryo, the graft-derived cells colonised the same types of host tissues as those transplanted orthotopically to the posterior epiblast (compare Fig. 3B and D). The posterior epiblast clearly behaved differently from the mesodermal cells of the 7.0-day host. Distal mesoderm transplanted orthotopically colonised predominantly the heart and the yolk sac mesoderm (compare Figs 3G and 3B). This finding may suggest that without the experience of ingression through the primitive streak, the epiblast cells can retain their original tissue potency even when they are relocated to a mesodermal site. They colonise a wider range of host tissues than the cells that have completed ingression.

With respect to the contribution to heart mesoderm, the transplantation experiments have demonstrated that posterior epiblast cells can colonise the heart irrespective of whether they have undergone ingression or movement in the mesoderm, provided they are placed at sites normally populated by the heart precursor cells. Posterior epiblast cells grafted directly to the 7.0-day (mid PS) mesoderm were able to colonise the heart and other mesodermal tissues (Fig. 3B). When these epiblast cells were transplanted to the site occupied by the heart mesoderm (the heart field) in the 7.5-day (late PS) embryo, descendants of the transplanted cells were almost exclusively found in the heart by the early somite stage (Fig. 3A).

There was also no restriction in the contribution by the epiblast cells to different cardiac cell lineages, although there was a distinct bias for the myocardium. In 17 chimaeric embryos (a subset of the embryos shown in Fig. 3B,D), in which the posterior epiblast cells colonised the heart, a total of 386 *lacZ*-expressing cells were found in the tissues of the heart. The majority (82.9%, 320 cells) were found in the myocardium of the ventricle. Of the remaining cells, 5.9% (23 cells) colonised the myocardium of the sinus venosus and atrium, 3.9% (10 cells) were in the endocardium and 3.1% (12 cells) in the endothelium of the outflow tract. This finding is consistent with the concept that precursors of the myocardium and the endocardium are localised to similar regions of the epiblast. About 4.2% (16 cells) were found in the mesocardium and the pericardium. The contribution to epicardium is not known because the specimens were analysed prior to the formation of this cardiac tissue (Viragh et al., 1993; Van den Eijnde et al., 1995).

The developmental fate of epiblast cells is influenced by the timing of ingression and their position in the epiblast

When the 6.5-day posterior epiblast cells were transplanted to the posterior epiblast of the 7.0-day embryo, the graft-derived cells were restricted largely to the embryonic mesoderm and gut endoderm, but were not found in the extraembryonic mesoderm (Fig. 3C). This finding indicates that the timing of ingression of the epiblast has a significant impact on the allocation of cells to tissue lineages.

When the posterior epiblast cells were transplanted to the anterior epiblast (Fig. 3E), they did not contribute to any embryonic mesoderm, but instead descendants were predominantly distributed to the ectodermal tissues (surface ectoderm, neural plate and amnion). The allocation of cells to the mesodermal lineages is therefore influenced by the strategic localisation of the epiblast cells.

Cells in the mesoderm display restricted tissue potency when they re-engage in cell ingression through the primitive streak

The lineage potency of recently ingressed mesoderm cells in the 7.0-day embryo was tested by transplantation back to the posterior epiblast of 6.5-day embryo. These experiments were to find out if a previous ingression event may result in any lineage restriction. Cells in the distal mesoderm of the 7.0-day embryo usually contribute to the heart and extraembryonic mesoderm (Fig. 3G). These mesodermal cells, after they were relocated to the posterior epiblast of younger (6.5-day) embryos, were capable of undergoing another round of cellular ingression at the primitive streak and displayed an expanded cell fate. In addition to the expected contribution to the heart and extraembryonic mesoderm (Fig. 3G), the transplanted cells also colonised the cranial mesoderm and the somites (Fig. 3H). These findings suggest that the post-ingressed cells still retain nearly the full lineage potency of the ancestral epiblast cells (compare Figs 3H and 3D). There was, however, a notable lack of contribution of the mesodermal cells to the lateral plate mesoderm, indicating that some cell potency may be lost during ingression.

Transplantation of 7.0-day distal mesodermal cells to the 7.5-day anterior mesoderm enhanced their colonisation of the heart tissues (Fig. 3F). This may suggest that the post-ingression cell movement within the mesoderm is not essential for the initiation of heart differentiation. This result, when con-

sidered together with the finding that posterior epiblast cells transplanted directly to the 7.5-day mesoderm can participate in heart differentiation, suggests that the acquisition of the heart cell fate does not depend on the process of cellular ingression and migration of the heart precursor during gastrulation.

Commitment to heart differentiation after the cells reach the heart field in the late PS embryo

The cardiogenic potency of the anterior and posterior epiblast cells was compared by assessing their differentiation following transplantation to sites where host heart precursors are localised during gastrulation (Fig. 1). These two epiblast populations display different developmental fates: the anterior epiblast cells give rise to ectodermal tissues and the posterior epiblast cells contain the precursors of mesodemal tissues, including the heart. The developmental potencies of these two populations were assessed by their ability to colonise the heart
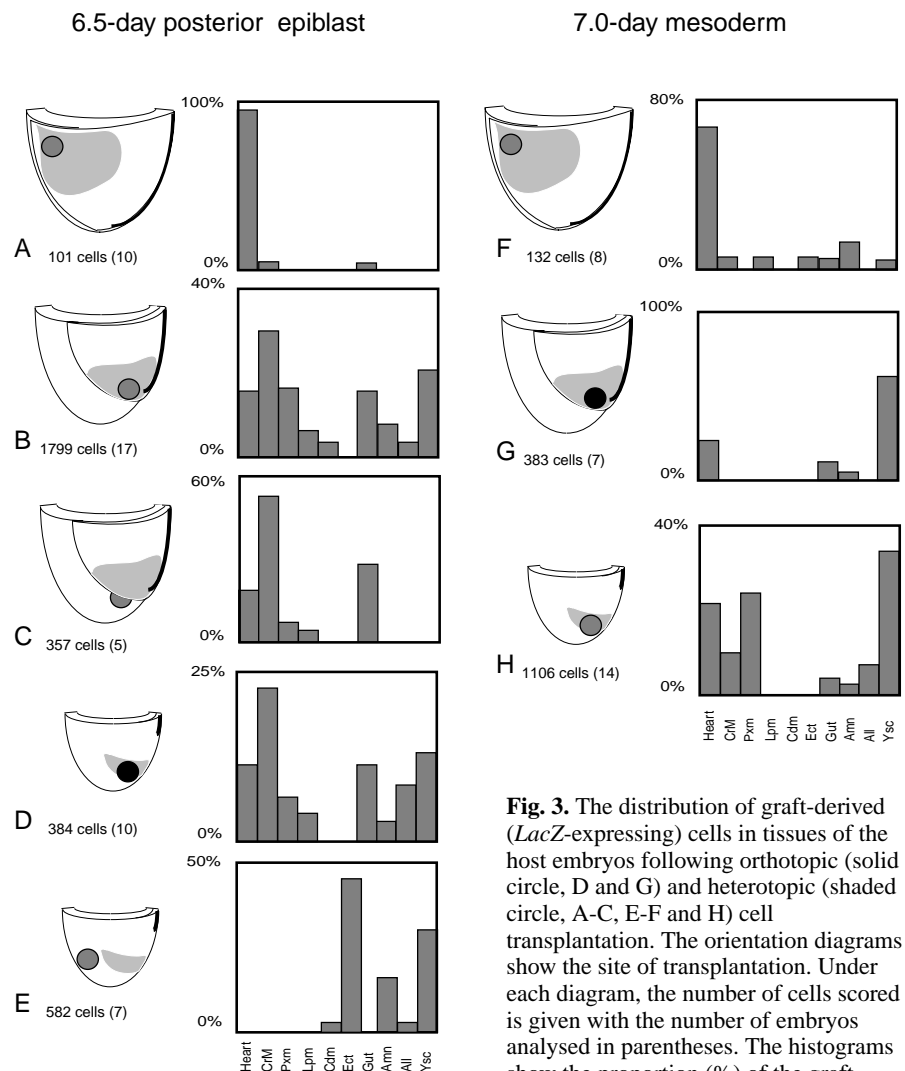

6.5-day posterior epiblast

7.0-day mesoderm

A   101 cells (10)

B   1799 cells (17)

C   357 cells (5)

D   384 cells (10)

E   582 cells (7)

F   132 cells (8)

G   383 cells (7)

H   1106 cells (14)

Heart, CrM, Pxm, Lpm, Cdm, Ect, Gut, Amn, All, Ysc

Fig. 3. The distribution of graft-derived (*LacZ*-expressing) cells in tissues of the host embryos following orthotopic (solid circle, D and G) and heterotopic (shaded circle, A-C, E-F and H) cell transplantation. The orientation diagrams show the site of transplantation. Under each diagram, the number of cells scored is given with the number of embryos analysed in parentheses. The histograms show the proportion (%) of the graft-derived population that colonised the different tissue types: Heart; CrM, cranial mesenchyme; Pxm, paraxial mesoderm; Lpm, lateral plate mesoderm; Cdm, caudal mesenchyme, Ect, neural and surface ectoderm; Gut, gut endoderm; Amn, amnion, All, allantois, Ysc, yolk sac mesoderm. The solid line on the posterior side of the embryo represents the length of the primitive streak.
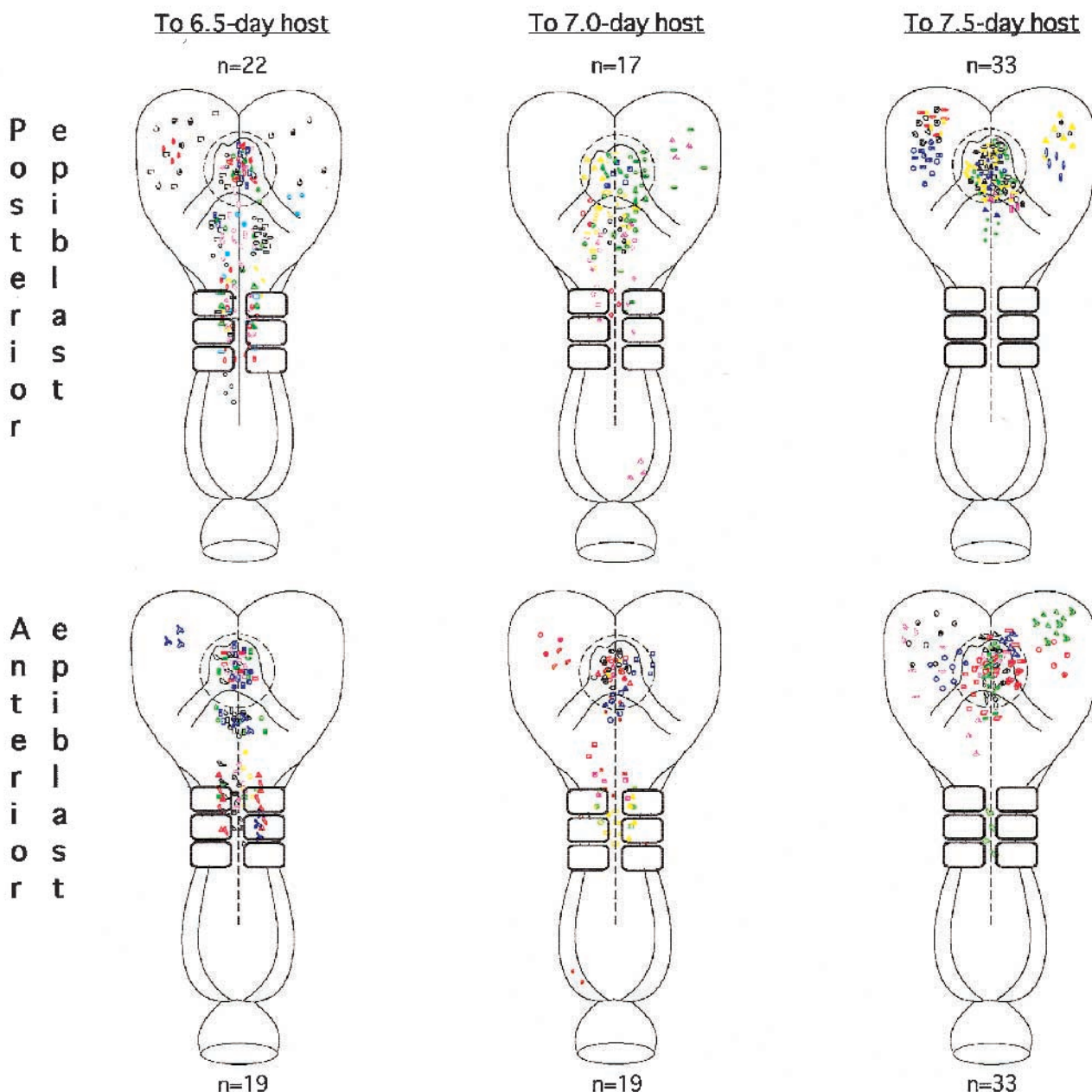
Fig. 4. The spatial distribution of the graft-derived cells in the host embryo after the transplantation of cells from the posterior epiblast (heart precursor) and the anterior epiblast to the 6.5-day host (posterior epiblast), 7.0-day host (distal mesoderm) and 7.5-day host (anterior-proximal mesoderm) and cultured to the early somite stage in vitro. The graft-derived cells are found almost exclusively in the mesoderm (the heart, the cranial mesenchyme and the somites). Differently coloured symbols represent results from different host embryos. *n*, number of embryos analysed for each type of transplantation.

(Fig. 4) and to undergo myocardial cell differentiation, as revealed by the expression of the tropomyosin (Fig. 5A,B).

The results of these transplantation experiments show that the anterior and posterior epiblast cells display a similar pattern of distribution in the host embryos (Fig. 4). In the 6.5-day host, the graft-derived cells were found in the heart, the cranial mesenchyme and the somite (Fig. 4: 6.5-day host). A minor contribution to the neural ectoderm were found in only

six host embryos, three in each of the anterior and posterior epiblast groups. Of the 41 host embryos that received either anterior or posterior epiblast grafts, 19 showed heart colonisation and 22 showed colonisation of other mesodermal tissues (Table 1). In both cases, distribution of the graft-derived cells outside the heart was more often bilateral, suggesting that the cells transplanted to the epiblast have participated in the gastrulation movement of the host cells. Anterior

and posterior epiblast cells transplanted to the distal mesoderm (Fig. 4: 7.0-day host) colonised the mesoderm exclusively. Of the 36 embryos examined, 19 had graft-derived cells in the heart. In these embryos, the graft-derived cells in the cranial and paraxial mesoderm were found only on one side of the embryos, suggesting that the epiblast cells had stayed within the mesodermal layer of the side to which they were transplanted. By contrast, in the other 17 embryos that showed no heart colonisation, more of them (8/17) showed colonisation of the mesoderm on both sides of the axis. This bilateral distribution of cells may suggest that some epiblast cells might have been transplanted into or very close to the primitive streak so that they were recruited to the non-heart mesoderm of both sides of the axis. In the 7.5-day host

Fig. 5. (A) Specific expression of the sarcomeric tropomyosin in the myocardium (my) of the early somite-stage embryo that has been cultured for 48 hours since the early primitive-streak stage. The endocardium (en) and the pericardium (pc) show no staining with antibody. The histology of the unstained tissues is revealed by phase contrast microscopy. (B) A magnified view of the early myocardial plate (my; DeRuiter et al., 1992) of the heart primordium in a pre-somite-stage embryo. The myocardial cells are stained strongly by the CH-1 antibody. This staining pattern is similar to that of the myosin antibody (Baldwin et al., 1991). A graft-derived cell is found in the thin endocardial layer (en). The large nucleus, which is characteristic of the early endocardial cells (Cohen-Gould and Mikawa, 1996), is stained with an intense blue color by the X-gal reaction but the cytoplasm shows no immunochemical reaction with the anti-tropomyosin antibody. (C-F) The colonisation of the myocardium by cells isolated from the posterior epiblast after transplantation to three different sites: the posterior epiblast of a 6.5-day embryo (C), the distal mesoderm of a 7.0-day embryo (D_1 and D_2) and the anterior-proximal region of the mesoderm of 7.5-day embryos (E,F). The graft-derived cells are identified by intense X-gal-stained (blue) nuclei. These graft-derived cells are integrated into the myocardium and express the specific myofibrillar tropomyosin protein in the cytoplasm. In C, *lacZ*-expressing cells (arrowhead) in the myocardium show staining of tropomyosin in the cytoplasm and the cell membrane, which is marked by the more intense red color. This staining pattern outlines the cell membrane and is similar to that displayed by CH-1 staining of the myocardial cells of the zebrafish (Stainier et al., 1995). Other examples of nuclear localisation of the β-galactosidase activity and cytoplasmic tropomyosin staining (intense red coloration) are shown at a higher magnification in D_2 and F. (G-J) The colonisation of heart tissues by cells isolated from the anterior epiblast after transplantation to the same three sites as (C-F): (G) The posterior epiblast of a 6.5-day embryo, (H_1 and H_2) the distal mesoderm of a 7.0-day embryo, and (I,J) the anterior-proximal region of the mesoderm of 7.5-day embryos. The morphology of the myocardial cells derived from the anterior epiblast is the same as those from the transplanted posterior epiblast. Examples of graft-derived cells (with blue nuclei) in the myocardium that express the tropomyosin protein in the cytoplasm (arrowheads) are shown at higher magnification in H_2 and J. my, myocardium; en, endocardium; pc, pericardium. Bars, 50 μm (A,E and I), 20 μm (C,G,D_1,H_1 and F) and 10 μm (B,D_2,H_2 and J).
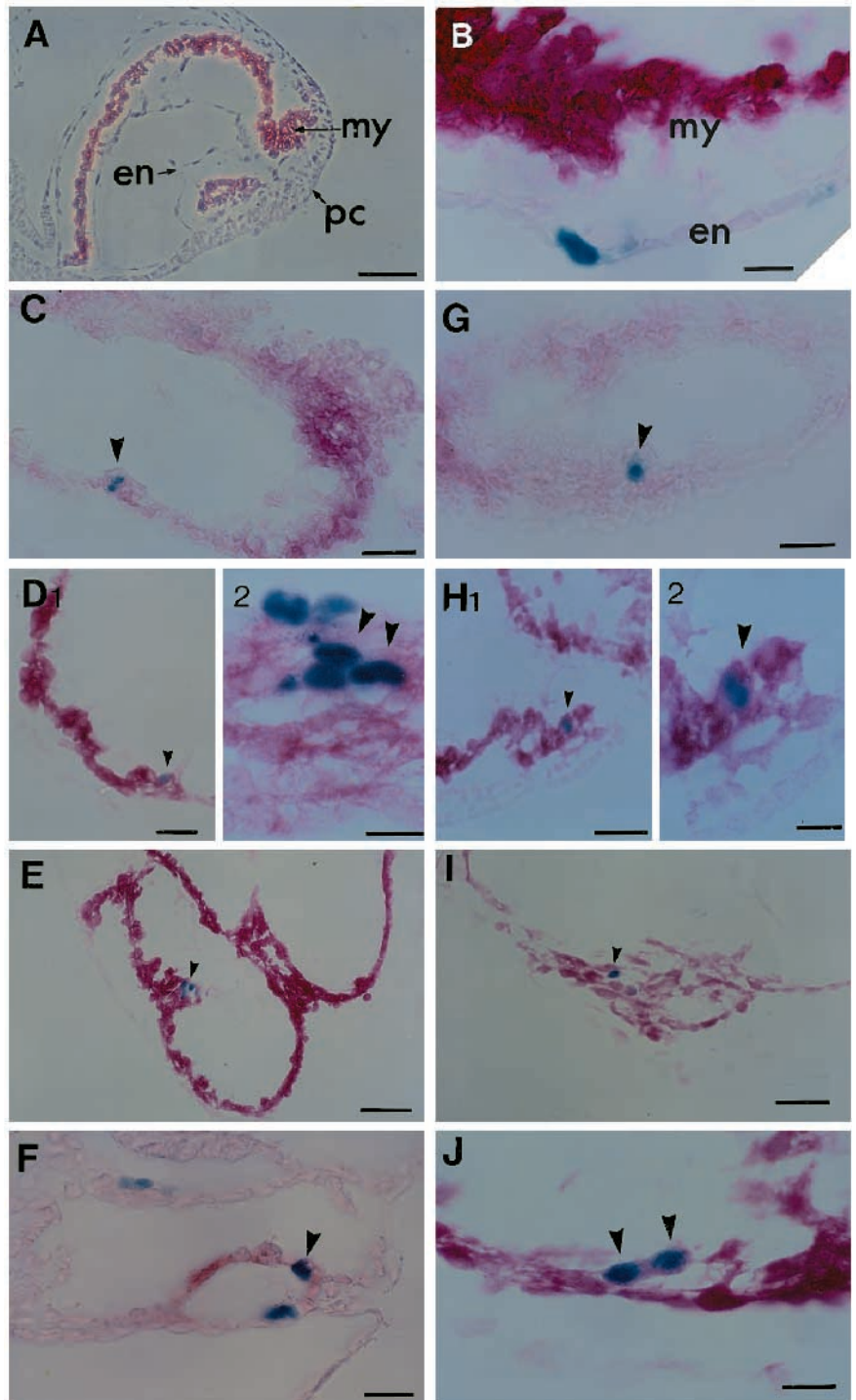
Table 1. The distribution of *lacZ*-expressing cells derived from the transplanted anterior and posterior epiblast cells in the ARC/s host embryos

		Number of embryos showing colonisation of:					
		Heart and other tissues			Other tissues only		
Donor tissue	Site of transplantation (Total number of embryos)	Total	Unilateral distribution	Bilateral distribution	Total	Unilateral distribution	Bilateral distribution
Posterior epiblast	Posterior epiblast (22)	10	2	8	12	2	10
	Distal mesoderm (17)	9	8	1	8	3	5
	Anterior-proximal (33) mesoderm	19	19	0	14	13	1
Anterior epiblast	Posterior epiblast (19)	9	1	8	10	0	10
	Distal mesoderm (19)	10	10	0	9	6	3
	Anterior-proximal (33) mesoderm	20	20	0	13	12	1

For the assessment of unilateral versus bilateral distribution of the graft-derived cells in the host embryos, only the *lacZ*-expressing cells localised outside the heart were studied. The overall pattern of distribution of graft-derived cells for each transplantation type is summarised in Fig. 4.

embryos, both the anterior and posterior epiblast cells contributed exclusively to the mesodermal tissues on one side of the embryo (Table 1). The graft-derived cells were found mostly in the heart and the cranial mesenchyme but rarely in the somites. A comparison of the distribution of transgenic cells in the host embryos reveals that epiblast cells transplanted to the epiblast of host embryos at early gastrulation were able to colonise more caudal embryonic tissues than those transplanted to the mesodermal tissue, which has already been allocated to a more rostral region of host embryos at late gastrulation (Fig. 4).

Immunostaining for the sarcomeric tropomyosin protein revealed that posterior epiblast cells grafted to gastrulating embryos were able to integrate into the heart tissues and differentiate into early myocardial cells (Fig. 5C-F). The posterior epiblast cells displayed cardiogenic potency even when they were transplanted directly to the mesoderm of the late PS embryo, suggesting that commitment to cardiac differentiation is independent of cellular ingression and tissue movement associated with gastrulation. These posterior epiblast cells may have acquired the heart fate either before gastrulation (i.e. it is pre-determined) or only after they have reached the heart-forming region in the mesoderm of the late PS embryos. Further support for the latter comes from the result of the transplantation of anterior epiblast cells, which normally do not display a cardiogenic fate. Like the posterior epiblast cells, graft-derived cells were properly integrated into the host heart tissues and expressed tropomyosin when they were a part of the myocardium (Fig. 5G-J). The acquisition of the myocardial phenotype by the anterior epiblast cells after they were transplanted directly to the 7.5-day mesoderm provides compelling evidence that the most critical process for specifying the myocardial cell fate occurs after the cells have reached the heart field in the 7.5-day embryo. The morphogenetic process of gastrulation therefore plays no essential role in cardiac specification, but only serves to move the precursor cells to the heart field during embryonic development.

DISCUSSION

Precursors of different cardiac cell types are co-localised in the epiblast

The results of the present study have provided additional

information on the origin and morphogenetic movement of heart precursor cells during gastrulation. Previous fate-mapping studies have shown that cells in the lateral epiblast of the early PS embryo display a predominantly mesodermal fate (Lawson et al., 1991; Lawson and Pedersen, 1992a,b; Parameswaran and Tam, 1995). Broadly, the lateral epiblast can be divided into two mesodermal domains: proximal for extraembryonic mesoderm and distal for precursors of the heart and cranial mesoderm, which are localised to a region posterior and proximal to the neural tissue precursor. A more detailed analysis of the lineage composition of the heart precursor in the epiblast has shown that descendants of this population contribute predominantly to the myocardium and, to a lesser extent, to the endocardium and the pericardium. These findings suggest that the precursors for these three cardiac tissues are localised to the same regions of the epiblast. The transplantation of groups of 5-15 cells in our experiment precludes the possibility of revealing whether these cardiac lineages share a common progenitor. In the zebrafish blastula, the myocardial and endocardial precursors are both found in the heart field at the yolk margin of the blastoderm, but the endocardial precursors are mostly sequestered to the ventral-marginal regions (Stainier et al., 1993; Lee et al., 1994). By clonal analysis, about 52% of the ventral-marginal cells tested give rise to both endocardial and myocardial cells. However, at the midblastula stage, only 14% of the blastomeres contribute to both cardiac cell types (Lee et al., 1994). These findings suggest that in the zebrafish, the endocardium and the myocardium may be derived from common progenitors and that these two lineages may segregate later during development. Mutation of the *cloche* gene in the zebrafish results in the selective deletion of the endocardial cells without affecting the myocardial lineage (Stainier et al., 1995). This seems to suggest that these two cell lineages may be derived from independent progenitors, but it is also possible that the mutation only impacts upon cell differentiation after the segregation of the lineages. The issue of whether there is a common progenitor for myocardial and endocardial lineages is also unresolved in the avian embryo. At early gastrulation, precursors of the pericardial, myocardial and endocardial lineages are co-localised in the rostral half of the primitive streak (Garcia-Martinez and Schoenwolf, 1993). Later in the lateral anterior mesoderm (the heart-forming region), some cells are found to co-express markers of the

myocardial (N-cadherin) and endocardial (QH-1) lineages (Linask and Lash, 1993). Furthermore, a clonal cell line (QCE-6) derived from this mesodermal population displays both myogenic and endothelial potency (Eisenberg and Bader, 1995). However, by retroviral tracing of cell clones from the similar population of mesoderm in the chick, distinct progenitors of myocardial and endocardial cells are found (Cohen-Gould and Mikawa, 1996). This may suggest that if the two lineages do have a common progenitor, they may have been segregated before the cardiogenic mesoderm has reached the heart-forming region. Immunostaining with an endothelial marker further shows that by the somite stage, the endocardial population is distinctly separated from the myocardium (Coffin and Poole, 1988).

Restriction of lineage potency after cell ingression

Cell populations in different regions of the primitive streak display a diverse cell fate (Tam and Beddington, 1987; Smith et al., 1995), although considerable plasticity of cell fate is still evident (Inagaki et al., 1993). It is not known at present what constitutes the signal(s) for lineage specification or restriction. The analysis of gene activity in the primitive streak has revealed some regionalisation of transcription activity that is consistent with the allocation of the ingressing cells to various mesodermal lineages (Hogan, 1995; Sasaki and Hogan, 1993; Tam and Trainor, 1994). This concept has been applied to the interpretation of the inappropriate allocation of cells to different mesodermal derivatives in mutant mouse embryos that have lost the FGFR1 and HNF3β function (Ang et al., 1994; Yamaguchi et al., 1994).

The experiments involving the reciprocal transplantation of the posterior epiblast and newly ingressed mesoderm cells reveal that the ingression of cells through the primitive streak has led to a restriction of lineage potency. Cells taken directly from the posterior epiblast to the mesoderm, and therefore not subject to any influence within the primitive streak, retain full epiblast potency and colonise a range of host tissues concordant with the expected cell fate and not that of the mesoderm at the host site. When the ingressed cells in the distal mesoderm of the mid PS embryo are transplanted to the epiblast of early PS embryo, the transplanted cells can ingress for the second time through the primitive streak and contribute to a wider variety of mesodermal tissues than would be expected from their original fate. The transplanted cells, however, behave differently from the epiblast cells and do not colonise the lateral plate mesoderm. This finding, when considered in conjunction with the retention of potency of epiblast cells that have not undergone ingression, indicates that the process of ingression may result in some restriction of lineage potency. The previous ingression through the primitive streak, however, does not seem to reduce the ability of the mesodermal cells to re-enact the morphogenetic movement of gastrulation.

Studies in the avian embryo have shown that the commitment of embryonic cells to their myocardial fate occurs at gastrulation. Both the pre-ingressed and post-ingressed cardiac myocyte progenitors can differentiate into myocardial cells in vitro. The post-ingressed cells, however, display a lesser dependence on cell-cell contact and are more resistant to the inhibitory effect of bromodeoxyuridine (BrDU) and phorbol ester (TPA) on the expression of myofibrillar proteins (Gonzalez-Sanchez and Bader, 1990; Montgomery et al.,

1994). The acquisition of increasing resistance to BrDU inhibition also seems to occur during the post-ingression migration of the myocyte progenitor to the heart-forming region. These findings therefore seem to suggest that the commitment to myocardial differentiation takes place during the morphogenetic movement of the mesodermal cells.

An early association of the heart mesoderm and the foregut endoderm during germ layer morphogenesis

At the onset of gastrulation (6.5 days), the primitive streak first forms at the most proximal site on the posterior mid-line of the epiblast (Hashimoto and Nakatsuji, 1989) and is adjacent to the precursors of the extraembryonic mesoderm. The primitive streak extends distally during gastrulation (Tam et al., 1993) and reaches the domain occupied by the heart and cranial mesoderm at about 7.0 days (mid-primitive-streak stage). The allocation of epiblast cells to specific lineages seems to be influenced by the timing and order of cellular recruitment to the new germ layers, and this is largely determined by the position of the cells in the epiblast (Lawson et al., 1991; Tam and Zhou, 1996). The strategic location of the mesodermal precursors in the epiblast thus predicts that the recruitment of epiblast cells to the nascent germ layer first involves the extraembryonic mesoderm, followed by the heart and cranial mesoderm about 12 hours after the onset of gastrulation (G. A. Quinlan and P. P. L. Tam, unpublished). In the chick gastrula, the precursors of the extraembryonic mesoderm and the heart mesoderm are recruited to the primitive streak at similar stages of gastrulation (HH stages 3-4; Garcia-Martinez and Schoenwolf, 1993).

Results of the fate-mapping (Parameswaran and Tam, 1995) and cell-tracking experiments (this study) show that the mesodermal cells destined for the heart are displaced in a distal to anterior-proximal direction along the lateral aspect of the mid- to late PS embryos (Fig. 6). This movement of the heart mesoderm in the mouse gastrula is comparable to that seen in *Xenopus* (Keller and Tibbetts, 1989) and the chick (Garcia-Martinez and Schoenwolf, 1992; Schoenwolf et al., 1992; Psychoyos and Stern, 1996). It is interesting to note the a generally similar direction of cellular displacement also occurs in the endoderm of the embryos at gastrulation (Lawson et al., 1986; Lawson and Pedersen, 1987). The definitive endoderm for the foregut is recruited at early to mid-PS stages and progressively spreads to the anterior-lateral position in the developing embryo (Tam and Beddington, 1992). The spatial overlap of the heart mesoderm and foregut endoderm therefore strongly suggests that a neighbour relationship may be established early at the inception of these two tissues. In *Xenopus*, the heart mesoderm is found next to the pharyngeal endoderm of the late blastula (Keller, 1976), and during gastrulation these two tissues move together anteriorly along the lateral sides of the neural plate (Wilens, 1955).

Induction of myocardial differentiation takes place in the heart field

The present transplantation studies have shown that the descendants of cells taken from two different regions of the epiblast can contribute the heart of the host embryo. The graft-derived cells also express the appropriate tropomyosin isoform when they colonise the myocardium of the host. The only prerequisite for heart differentiation is the placement of the epiblast

Fig. 6. Epiblast cells destined for the heart ingress through the primitive streak at early to mid PS stages. Results of the cell-labelling study support the concept that the mesoderm layer expands in an anterior and proximal direction during gastrulation. The heart precursor cells in the mesoderm are displaced, in concert with the expansion of the mesoderm layer, anteriorly and proximally to the cardiogenic region in the late PS embryo (straight arrow, Parameswaran and Tam, 1995). The difference in the heart-forming fate of cells in the mesoderm during gastrulation reflects the actual movement of heart precursors, instead of being a consequence of the concomitant loss and acquisition of cell fate by different mesodermal populations. The movement of the heart mesoderm matches spatially and temporally with that of the definitive endoderm that contributes to the foregut endoderm (Lawson et al., 1986; Lawson and Pedersen, 1987; Tam and Beddington, 1992). This suggests that a neighbour relationship has been established between the cardiogenic mesoderm and the foregut endoderm as they are formed. This early association may facilitate the inductive interaction that is critical for heart morphogenesis. The crossed arrows indicate the orientation of the embryonic axes and the dashed lines show the approximate border between the embryonic and the extraembryonic mesoderm.

MESODERM

ENDODERM

Early-PS   Mid-PS   Late-PS
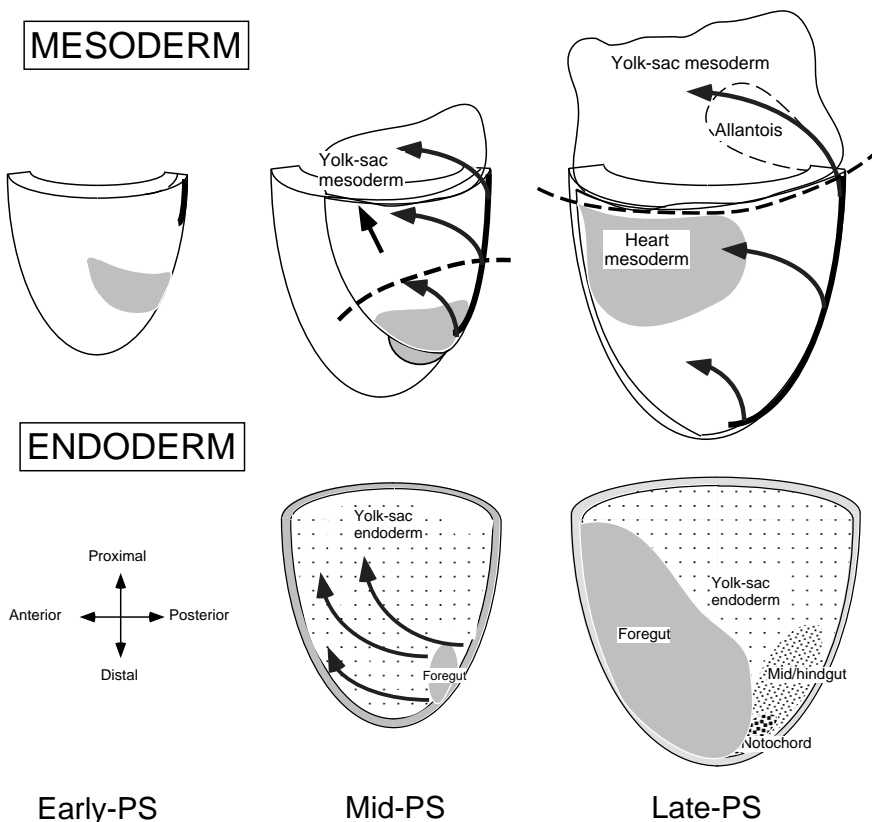
cells at those sites in the germ layers where the heart precursor cells of the host embryos are normally found. Most interestingly, epiblast cells can acquire a cardiogenic fate after direct transplantation to the heart field of the late PS embryo. This observation provides the most compelling evidence that the necessary information for specifying heart differentiation is given to the cells only after they have reached their destination, and not while they are travelling to the heart field. This information may be either the inductive signals derived from the endoderm (Sugi and Lough, 1995) or the cellular interaction with the host heart mesoderm community (Gonzalez-Sanchez and Bader, 1990). Our results do not preclude the possibility that some steps of specification of the heart mesoderm may have taken place during the initial phases of gastrulation. In the avian embryo, non-heart precursor cells (stage 4 posterior primitive streak cells, which normally form haematopoietic cells) are induced to differentiate into cardiac myocytes following translocation to the heart field in the anterior lateral region of the stage 4-5 embryo (Schultheiss et al., 1995). The inductive signal comes from the anterior endoderm and is absent from the central mesoendoderm, probably as a result of the suppressive activity emanating from the node or its derivative (Yuan et al., 1995).

The specific requirement of endoderm interaction in cardiac differentiation has been disputed (Jacobson and Sater, 1988). Cardiac differentiation, as assayed by the expression of heart-specific transcripts in co-explants of anterior ectoderm and mesoderm of stage 6-9 chick embryos, suggests that the initial steps of myocardial differentiation may occur in the absence of the endoderm. The endoderm of early gastrula (stage 3-4) is also not required for the patterning of tissues in the embryonic heart

(Inagaki et al., 1993), but may be necessary for establishing the diversity of myocardial cell types (Yutzey et al., 1995). Furthermore, the proper assembly of sacromeric proteins and the initiation of contractile activity require the support of the anterior endoderm, at least until stage 6 (Sugi and Lough, 1994; Antin et al., 1994; Gannon and Bader, 1995). These findings raise the possibility that the specification of heart mesoderm is initiated early during gastrulation by the inductive activity of the endoderm (Sugi and Lough, 1994). At a later stage, the inductive activity of the anterior endoderm is dispensable, since the assembly of sarcomeres and the differentiation of the atrial myocytes (Yutzey et al., 1994) can occur in the absence of the endoderm (Gannon and Bader, 1995; Yutzey et al., 1995). In *Xenopus*, the heart mesoderm seems to be specified at early gastrulation and its differentiation does not require any interaction with the superficial pharyngeal endoderm (Sater and Jacobson, 1989). The anterior endoderm is needed at the proneurula (stage 20) stage only to maintain the cardiogenic competence of the lateral mesoderm (Sater and Jacobson, 1990). Heart differentiation was, however, assayed by the appearance of foci of contractile cells and not by the expression of heart-specific molecules. Recently, the deep endoderm at the dorsal marginal zone of the early *Xenopus* stage 10-10.5 gastrula has been shown to be involved in the induction of heart mesoderm (Nascone and Mercola, 1995). The nature of the endoderm-derived inducing molecules is presently not known, but members of the TGFβ, BMP and FGF families of growth factors are strong candidates (Kokan-Moore et al., 1991; Sugi and Lough, 1995; Lough et al., 1996). Overexpression of the *cerberus* gene, which is normally transcribed in the foregut and anterior midgut endoderm of the *Xenopuc* embryos, has resulted

in the duplication of the body axis, about half of which also formed an extra heart (Bouwmeester et al., 1996). If an inductive interaction between the endoderm and the mesoderm is also critical for heart differentiation in the mouse embryo, the early spatial association of the heart mesoderm and the gut endoderm during gastrulation would be important for initiating and perpetuating such a tissue interaction.

In summary, the present study has provided new insights into the process of lineage specification in the gastrulating mouse embryo. Specifically, cellular ingression and cell movement in the mesoderm through the primitive streak are associated with a restriction in mesodermal potency. During normal development, such a morphogenetic repertoire may be essential to establish an early association of the heart mesoderm and the gut endoderm. This will ensure that tissues involved in the inductive interaction are properly sited during cardiac differentiation. We have shown that different epiblast cell populations have equivalent developmental potency, which enables them to respond to the cardiogenic signals in the gastrulating embryo following heterotopic transplantation. We have further demonstrated that the initial morphogenetic cell movement associated with germ layer formation is not critical for the specification of heart mesoderm. The necessary signal(s) for myocardial specification may be found only after the cells have reached the cardiogenic field.

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