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Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus

II. Relationships from $4\frac{1}{4}$ to $9\frac{1}{2}$ days

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SUMMARY

Each of the three primary axes of the primitive streak ($6\frac{3}{4}$ days *p.c.*) to C-shaped ($9\frac{1}{2}$ days) stage mouse embryo has a specific relationship to the uterine horn axes. By a retrograde analysis of younger sectioned embryos it has been possible to construct an axis fate map for the implanting $4\frac{1}{4}$-day blastocyst and to show how its implantation in one or the other of two specific orientations to the ends and walls of the horn leads to these embryo–horn relationships. The implanting blastocyst axis fate map can be related to an axis fate map of the attached blastocyst (Smith, 1980) since these too are in one or the other of two orientations to the ends and walls of the horn. It is suggested that the asymmetries of the attached and implanting blastocysts that allowed the distinctive attachment and implantation orientations to be recognized, are the initial expressions of a three-dimensional system of positional information that is present in the attached blastocyst.

INTRODUCTION

Three regularities in the relationship of the $6\frac{3}{4}$- to $9\frac{1}{2}$-day mouse embryo's primary axes to the ends and walls of the uterine horn have been described (Snell & Stevens, 1966). Between the primitive streak and S-shaped embryo stages, the embryo's dorsal–ventral (D–V) axis is more or less parallel to the horn's mesometrial–antimesometrial (M–antiM) axis with the dorsal pole toward the mesometrium. Between the same stages, the embryo's anterior–posterior (A–P) axis is more or less perpendicular to the horn's long axis with the primitive streak toward either the right or left wall of the horn. Then at the beginning of the C-shaped stage, the embryo's right–left (R–L) axis becomes parallel to the M–antiM axis of the horn with right toward the placenta. As is evident, the orientation of two of these axes (D–V and R–L) is invariable with respect to the poles of the axes of the horn at any one stage while that of the third (A–P) is in one or the other of two orientations at 180° from each other.

The orientation of the embryonic D–V axis can be traced back to a unique relationship of the $4\frac{1}{4}$-day blastocyst to the uterus. The embryonic–abembryonic (E–abE) axis of the implanting blastocyst is oriented parallel to the M–antiM axis

Key words: mouse, conceptus, blastocyst, fate map, asymmetries, primary axis, axis orientation, uterine orientation.

of the horn with the embryonic pole directed toward the mesometrium. This orientation clearly anticipates the orientation of the primitive streak to S-shaped embryo's D–V axis in space since in this orientation the epiblast (a marker of dorsal) is toward the mesometrium and the hypoblast (a marker of ventral) is toward the antimesometrial surface of the horn. Described here is a similar relationship between another asymmetry of the implanting blastocyst and the future location of its embryonic A–P axis. The mechanism responsible for the 180° difference in the orientation of the A–P axis within the horn is also described. The following interpretation of these data is discussed. By the time of attachment the mouse blastocyst contains a three-dimensional system of positional information that first causes it to implant in one or the other of two orientations to the horn and eventually leads to the localization of its D–V and A–P axes. Spatial cues provided by uterine asymmetries at its implantation site are responsible for its R–L visceral asymmetries.

MATERIALS AND METHODS

The mouse conceptuses in this study come from two strains and overlap in developmental stage. Most of the $4\frac{1}{4}$- to $6\frac{1}{2}$-day conceptuses are from the Q strain; CF1 embryos make up the bulk of the $6\frac{3}{4}$- to $9\frac{1}{2}$-day olds. The times indicated for the stages described here are the times of sacrifice of the CF1 female after mating; the Q conceptuses having been found to be developmentally less advanced (by approximately 5–10 h) at all ages examined. Mating was arbitrarily assumed to have occurred at 1:00 a.m. of the day a vaginal plug was found. Pregnant Sprague-Dawley females purchased from the Charles River Laboratory provide data on $11\frac{1}{2}$-day rat embryos.

Excised uterine horns were pinned to paraffin wax, then fixed in Carnoy's fluid (1 glacial acetic acid: 3 absolute ethanol) for 2 h. The horns, recorded as being left or right and with the oviduct end marked, were cut in transverse, frontal or sagittal section at 10 μm. Almost all sections were stained with Azure B by the method of Flax & Himes (1952). Camera-lucida drawings were made of successive sections of the conceptuses and the surrounding implantations sites. In the initial stages of this study, clay models of conceptuses in each developmental stage were made from these drawings. These models with proposed axes indicated were then oriented within three-dimensional models of the horn. Models made from sections of horn cut in one plane were then cut in one of the other two planes and the appearance of the model conceptus sections compared with conceptus sections in the horns cut in the same plane. Some embryos ($8\frac{1}{2}$- and $9\frac{1}{2}$-CF1 mice and $11\frac{1}{2}$-day rats) were examined after dissection of the uterine horns. These horns after fixation were transferred to 70 % ethanol and the right wall of the implantation chamber was cut away with iridectomy scissors. Twistable clay models of $8\frac{1}{2}$-day embryos were used as an aid in relating their orientation within the horn to that of the $9\frac{1}{2}$-day embryos.

Term usage

The ends of the long axis of the uterine horns are identified as their oviductal and cervical ends. The right side of both horns is toward the female's right side. The third horn axis, the M–antiM axis, runs from the mesometrial to the antimesometrial surface.

The terms conceptus and embryo are used in their narrowest sense; the conceptus consisting of both embryo and extraembryonic-membrane-forming cells. The long axis of the conceptus, therefore, is not the long axis of the embryo but instead is the same as the E–abE axis of the elongated blastocyst. An implanted conceptus is cut longitudinally by both transverse and sagittal section of the horn and transversely by frontal section.

The embryo is the organism proper after the primitive streak has appeared. At the primitive streak stage the embryo has been described by Snell & Stevens (1966) as cup-shaped; 18 h later as S-shaped in sagittal section and after rotation around its A–P axis as C-shaped. In its S-shaped

stage its trunk is concave toward its dorsal surface; in its C-shaped stage its trunk is convex toward its dorsal surface. The A–P axis in such embryos is of course curved rather than straight and so it is the line connecting their anterior and posterior ends that is said here to be perpendicular to the M–antiM axis of the horn and to the long axis of the conceptus.

The terms Type L and Type R orientation were used initially (Smith, 1980) to describe a hitherto unknown relationship of the implanting asymmetrical blastocyst-stage conceptus to the ends and walls of the horn. Now called *implantation* orientations, the terms have been extended to describe this relationship of horn and conceptus axes in stages as developmentally advanced as the early primitive streak stage. That is, as early as the implanting blastocyst stage the polar trophoblast–inner cell mass (PT–ICM) complex is ovoid rather than disc-shaped and its long axis is in the same plane as, but slightly tilted with respect to, the long axis of the blastocyst. In Type L implantation orientation most of the polar trophoblast surface of the complex is toward the right wall and oviductal end of the horn (Fig. 1). In Type R implantation orientation, it is toward the left wall and cervical end of the horn. Classification of the older conceptuses with respect to these two implantation orientations depends on the fact that the polar trophoblast derivatives, extraembryonic ectoderm and trophoblast and ectoplacental cones also are asymmetrical and tilted with respect to the long axis of the conceptus (Fig. 1).

The terms Type R and L *axis* orientation describe the orientation of the A–P axis of the embryo to the ends and walls of the horn. In Type L axis orientation, the embryo's anterior is toward the left wall and oviductal end; in Type R orientation, toward right wall and cervical end.

Most of the photographs here are from horns sectioned transversely or sagittally. In these illustrations conceptuses in Type R and L implantation orientation appear to be mirror images of each other. This is not the case: they are at 180° from each other. For example, the photographed surface of a section of a conceptus in Type R implantation orientation in a transverse section of a horn cut from its cervical to its oviductal end is the surface toward the 'right' side of the conceptus. It is the surface toward the 'left' side in a conceptus in Type L implantation orientation.

The statistical analyses

The standard test of heterogeneity was applied to the numerical data on the distribution of $6\frac{3}{4}$-, $8\frac{1}{2}$- and $9\frac{1}{2}$-day CF1 embryos in Type R and L axis orientation in right and left horns. ($\chi^2 = 1{\cdot}043$ d.f. = 6). Since this figure supports the conclusion that these embryos come from the same population with respect to the observed asymmetrical frequency of orientation types in these horns, the CF1 numerical data were lumped in further analyses. Chi-square tests were used for most of these. However in an attempt to demonstrate that the orientations of adjacent embryos were independent of each other, the number of runs (a group of embryos of like-orientation type) of length 1 to 6 was determined (Table 3). The number of runs decreases by one-half as the length of the runs increases from 1 to 2 to 3. This is what would be expected in an infinitely long horn if the probability of a blastocyst's implanting in each implantation type is the same and is unaffected by the implantation type of its neighbour.

ABBREVIATIONS

af,	amniotic fold, posterior	*h,*	hypoblast
b,	blastocoel	*le,*	luminal epithelium
cf,	cranial limiting furrow	*mt,*	mural trophoblast
e,	epiblast	*pA & pP,*	poles, putative anterior and posterior of complex
ec,	ectoplacental cone	*pD & pV,*	poles, putative dorsal and ventral of complex
ee,	embryonic ectoderm	*pa,*	proamniotic cavity
em,	embryonic mesoderm	*pp,*	prechordal plate
eee,	extraembryonic ectoderm	*ps,*	primitive streak
eem,	extraembryonic mesoderm	*pt,*	polar trophoblast
ep,	endoderm, parietal	*tc,*	polar trophoblast cone
ex,	exocoelom	*tcc,*	polar trophoblast cone cavity
gc1 & gc2	giant cells, primary and secondary	*tco,*	polar trophoblast cone opening
ICM,	inner cell mass	*sR & sP*	sides, rounded and pointed

RESULTS

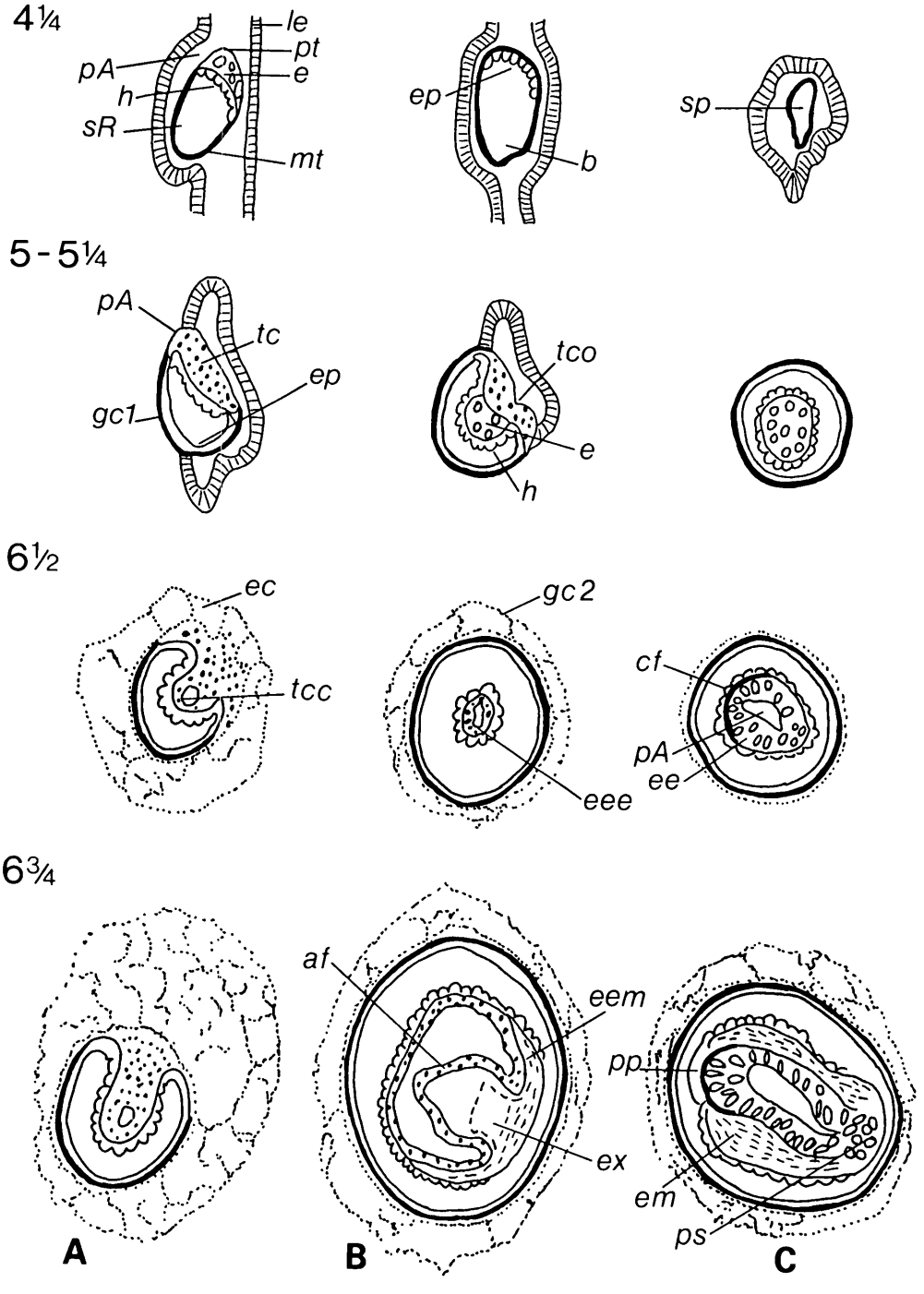
The 4¼-day conceptus

By 4¼ days the blastocyst clearly is no longer radially symmetrical (Fig. 1:4¼): its now ovoid PT–ICM complex is slightly tilted with respect to the long axis of the conceptus. As is shown in Fig. 2 the direction of the tilt is such that one pole of the long axis of the complex is slightly closer to the mesometrial surface of the horn than is the other pole. Furthermore, the longer wall of the blastocoel (where the complex is closer to the mesometrium) is 'rounded': the shorter wall, 'pointed'. As is strikingly apparent in frontal sections of the uterine horn, such blastocysts are in either Type R or Type L implantation orientation to the ends and walls of the horn (Fig. 1:4¼). Also indicated in Fig. 1:4¼ are the asymmetries of the walls of the implantation chamber. These are complementary to those of the blastocyst.

The 4¾-day conceptus

By the end of this stage, proliferation of the polar trophoblast cells and a change in the shape of some of them from squamous to columnar has produced a mass of cells organized into a stubby trophoblast cone. This mass of cells is called extraembryonic ectoderm by Snell & Stevens (1966) and polar trophoblast by Gardner (1978). The cone is asymmetrical. It has a long axis coinciding with that of the ovoid PT–ICM complex and its apex lies over the portion of the complex located most mesometrially in the chamber not at its centre. Early stages of the cone's development are illustrated in Figs 3 and 4. The columnar shape of the cone cells located most mesometrially in the lumen and on the side where the blastocoel is rounded (pA and sR, Fig. 3) and the lesser height of those closest to the antimesometrial side of the horn and where the blastocoel is pointed (pP and sP) are evident. Also evident in this conceptus is the asymmetry of the mass of its epiblast cells: it is rounded on one side (pA); flattened on the other side (pP). Its shape is quite obviously complementary to that of the base of the cone. Fig. 4 is

Fig. 1. Stylized camera-lucida drawings of 4¼-, 5- to 5¼-, 6½- and 6¾-day transversely sectioned conceptuses in frontally sectioned horns. The oviductal end of the horns is toward the top of the page; the left wall is toward the left. All conceptuses are in Type L implantation orientation. If the drawings but not the horn orientations are rotated 180°, they will illustrate sections through conceptuses in Type R orientation. The (A) sections are closest to the mesometrium and the most mesometrially located cells are found on the side of the sections labelled pA. In the 4¼ (A) section, the characteristic difference in the shape of the walls of the implantation chamber is seen. In the 5- to 5¼-day (A&B) sections, the presence of luminal epithelium at the level of and at the trophoblast cone side is characteristic. The 6½- and 6¾-day (A) sections show the continuing asymmetry of the trophoblast cone and extraembryonic ectoderm to the ends and walls of the horn. The 4¼-day (B) section is at the level of the junction of polar and mural trophoblast. The 5- to 5¼-day (B) section is at the level of the junction of extraembryonic ectoderm and epiblast and shows the direction in which the epiblast is 'pushed' into the blastocoel. The remaining (B) sections are through the extra-embryonic ectoderm. The 4¼-day (C) section is through the abembryonic mural trophoblast. The 5- to 5½-day (C) section is through the ICM. The 6½- and 6¾-day (C) sections are through the epiblast near its junction with the extraembryonic ectoderm.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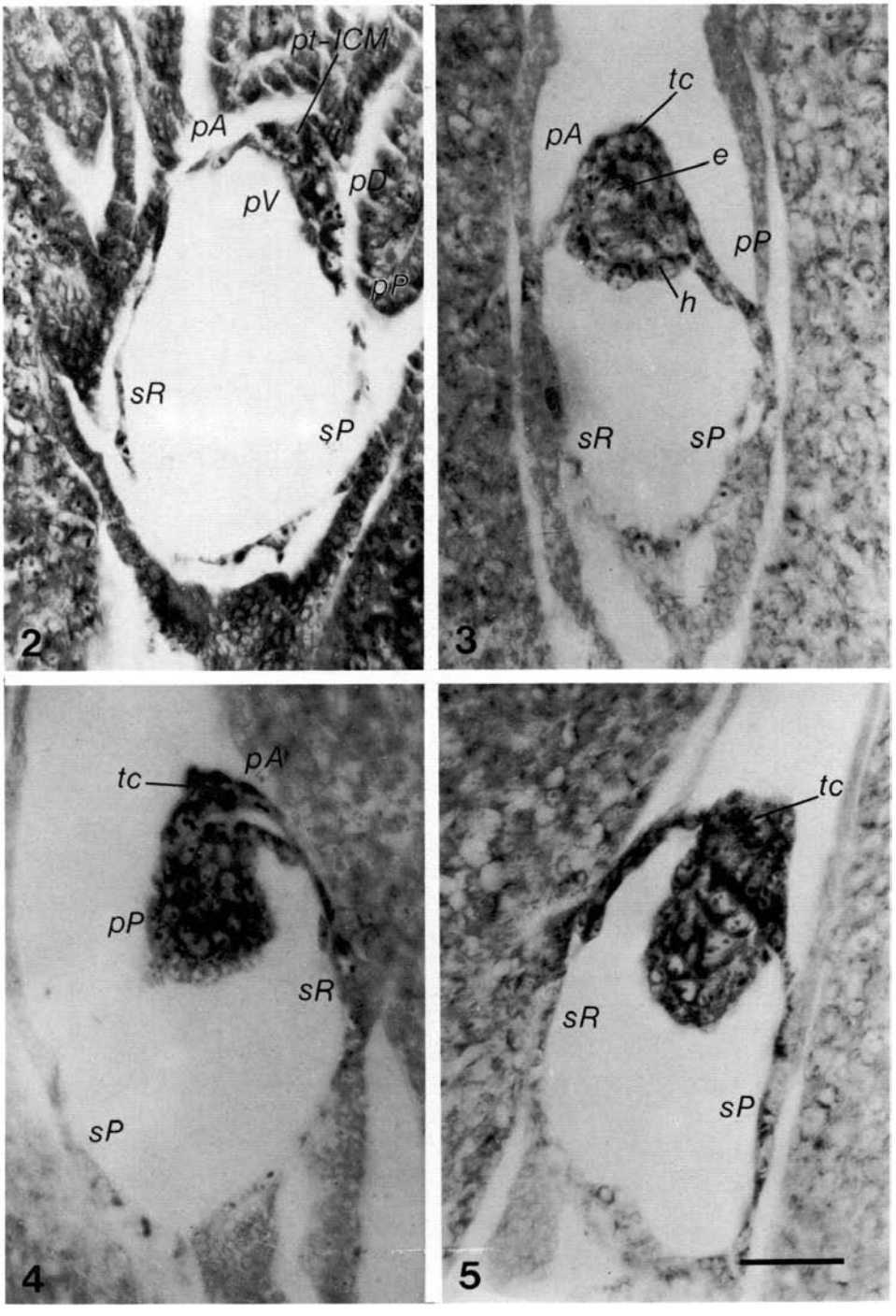
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from a developmentally more advanced hornmate in the alternative implantation orientation. The asymmetry of its cone and mass of epiblast cells is more obvious and is again correlated with the rounded and pointed asymmetry of the blastocoel walls. A section containing the thickest part of a large cone (Fig. 5) illustrates the stage in cone development just before the future extraembryonic ectoderm cells appear. The outer surface of this cone is smoothly convex. Note also the asymmetries of the cone at its junction with the mural trophoblast.

The 5- to 5½-day conceptus

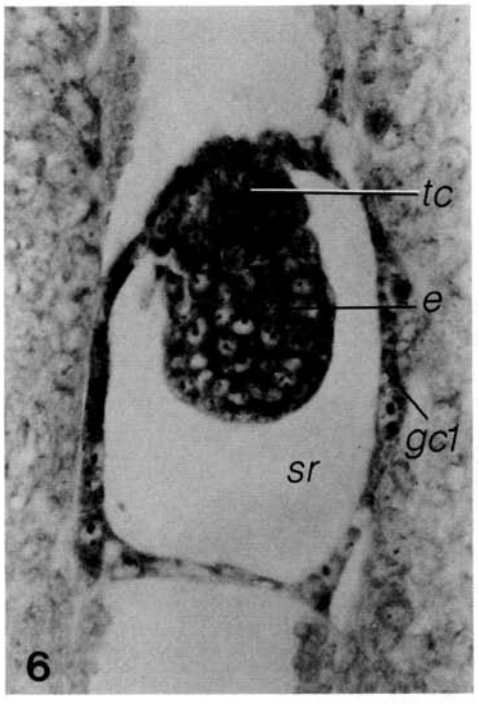
The egg cylinder begins to form when the cells of the trophoblast cone that will become extraembryonic ectoderm cells (Copp, 1979) start to involute into the blastocoel. That involution is beginning is first suggested by the presence of blebs on the outer surface of the cone (Fig. 6) or a slight depression in the surface of the cone (Fig. 1, 5–5¼: B). Also apparent in Fig. 6 is the same asymmetry of the cone at its junction with the mural trophoblast as is seen in Fig. 5. Clear too is the inclination of the apical surface of the cone toward one wall of the horn (see also Fig. 1, 5–5¼: A). By 5½ days with the continued involution of cells, the depression has become a funnel-shaped cavity within the cone and elongating mass of extraembryonic ectoderm cells (Fig. 7). In this study these cavities were found routinely although Snell & Stevens (1966) say they were present only rarely in their material. Presumably the use of different fixatives in the two studies accounts for this discrepancy. Both the depression and the opening into the cavity are located along the long axis of the trophoblast cone and are closer to the right wall and oviductal end of the horn in blastocysts whose cone and blastocoel asymmetries show them to be in Type L implantation orientation to the horns (Fig. 1, 5–5¼: A and B). They are closer to the left wall and cervical end of the horn in blastocysts in Type R orientation (Fig. 7). Furthermore, the tube of extraembryonic ectoderm cells juts into and slants across the blastocoel in the direction of the left to right wall of the horn in conceptuses in Type R orientation (Fig. 7) and in the direction, right to left wall of the horn in those in Type L orientation (Fig. 1, 5–5¼: B). The epiblast also is asymmetrically positioned within the blastocoel (Fig. 1, 5–5¼: C) presumably because it is pushed into the blastocoel by the involution of the extraembryonic ectoderm cells. It is closer to the rounded than the pointed side of the blastocoel. It often appears rounded itself on the side toward the rounded wall of the blastocoel and flattened on the other side (Fig. 1, 5–5¼: C).

Fig. 2. 4¼-day conceptus in Type L implantation orientation (Type L). Sagittal section of horn, oviduct to the right. Bar = 50 μm.

Fig. 3. 4¾-day conceptus (Type L). Polar trophoblast cells at (*pA*) are columnar; at (*pP*) are squamous. Transverse section of horn (TSH) with right wall to the right. Bar = 50 μm.

Fig. 4. Developmentally more advanced hornmate of conceptus in Fig. 3 (Type R). Asymmetry of trophoblast cone at (*pA*) and (*pP*) ends more distinct. (TSH). Bar = 50 μm.

Fig. 5. 4¾-day conceptus (Type L) with fully developed trophoblast cone. (TSH). Bar = 50 μm.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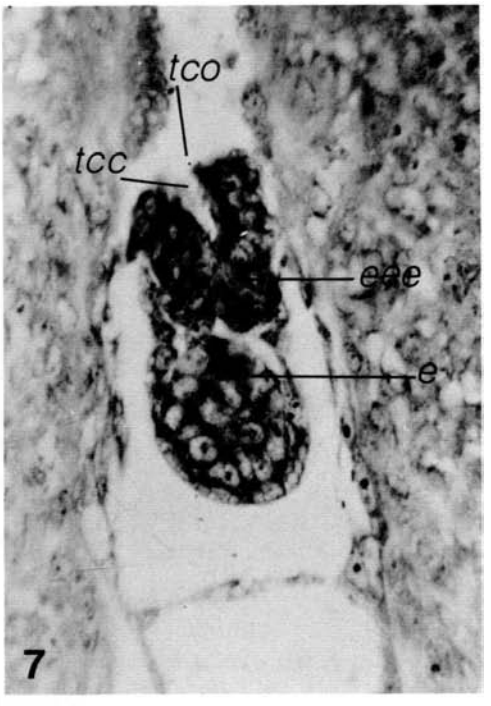
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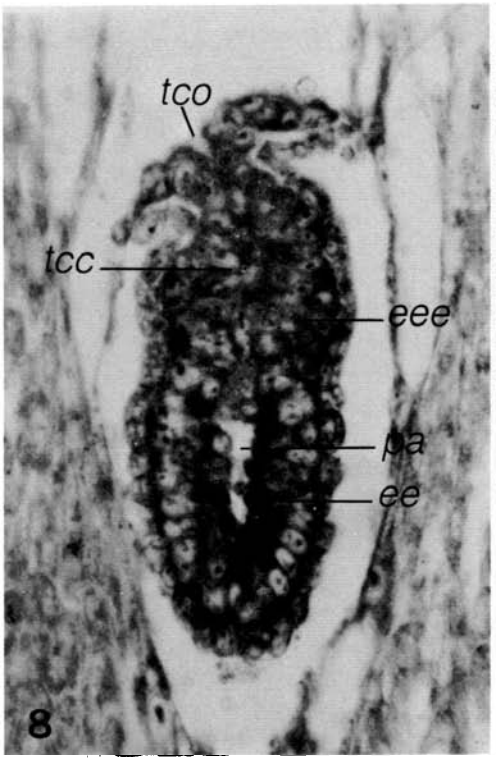
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The 5¾- to 6-day conceptus

At the beginning of this stage, a proamniotic cavity is present and the epiblast cells around it appear to be organized into an epithelium (Fig. 8). During this time the tube of extraembryonic ectoderm that continues to be inclined toward one wall of the horn increases in length dramatically apparently partly as a result of continued involution of cells from the trophoblast cone. In addition, other cells of this cone (still recognizable as such by the continuing greater cytoplasmic basophilia of its cells) have migrated away from it and are differentiating into secondary giant and ectoplacental cone cells (Fig. 9). The latter cells are recognizable by virtue of the reduced basophilia of their cytoplasm as early as 5¾ days. As their number increases they form a lacunae-filled cone whose base arches over and eventually obstructs the opening into the cavity of the extraembryonic ectoderm (Fig. 10). The ectoplacental cone too is clearly asymmetrical at 6 days (Fig. 10), its asymmetry coinciding with that of the trophoblast cone. Note also that the trophoblast and ectoplacental cones of the conceptus in Fig. 9 are inclined toward one horn wall, those of the conceptuses in Figs 6 and 7 toward the other. It is implanted in Type L orientation; they, in Type R.

The 6½-day conceptus

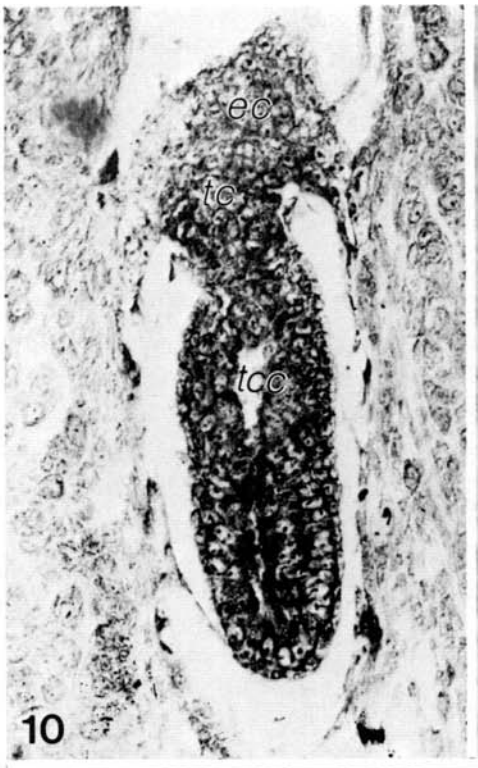
Using the relationship between the extraembryonic and embryonic ectoderm portions of the egg cylinder found at 6¾ days (next section) as a guide, histological differences that might be related to the putative prechordal plate and primitive streak sides of the 6½-day egg cylinder were looked for in good transverse sections of its embryonic portion (Fig. 1, 6½: A,C and Fig. 12). Near the epiblast's junction with the extraembryonic ectoderm, more of the epiblast's nuclei were found to have an oval outline on the side predicted to become the prechordal plate side; more to have a circular outline on the side predicted to become the primitive streak side. Moreover, the apical cytoplasm of the cells with nuclei with the oval outline often appears to be more basophilic than that of the cells whose nuclei are circular in outline. A gap was often found between the more basophilic epiblast cells and the overlying hypoblast. However, a localized difference in the hypoblast cells seems to be a better indicator of anterior at this stage because not all the conceptuses were perfectly sectioned. Such hypoblast cells that appear to be 'unhealthy' because of their ragged rather than vacuole-filled apical border are probably those in the region Theiler (1972) calls the cranial limiting furrow.

Fig. 6. 5-day conceptus (Type R). Blebs at apex of cone. (TSH). Bar = 50 μm.

Fig. 7. 5¼- to 5½-day conceptus (Type R). The opening into the cavity of the trophoblast cone is evident. (TSH). Bar = 50 μm.

Fig. 8. 5½- to 5¾-day conceptus (Type R). The proamniotic and polar trophoblast–extraembryonic ectoderm cavities are shown. Extraembryonic ectoderm juts into the blastocoel. (TSH). Bar = 50 μm.

Fig. 9. 5¾- to 6-day conceptus (Type L). Secondary giant and ectoplacental cone cells have migrated out from the trophoblast cone. The latter is still oblique to the long axis of the implantation chamber. (TSH). Bar = 50 μm.



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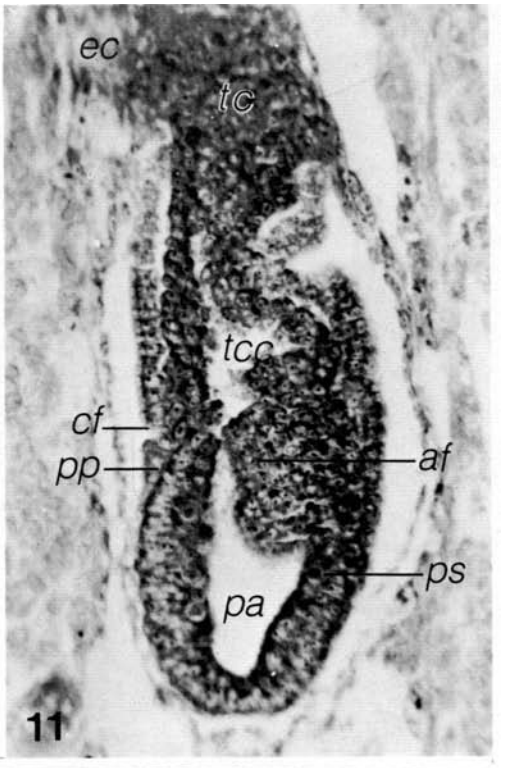

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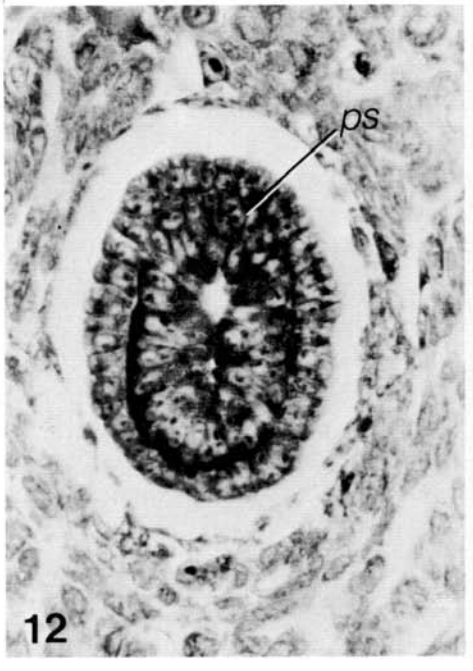

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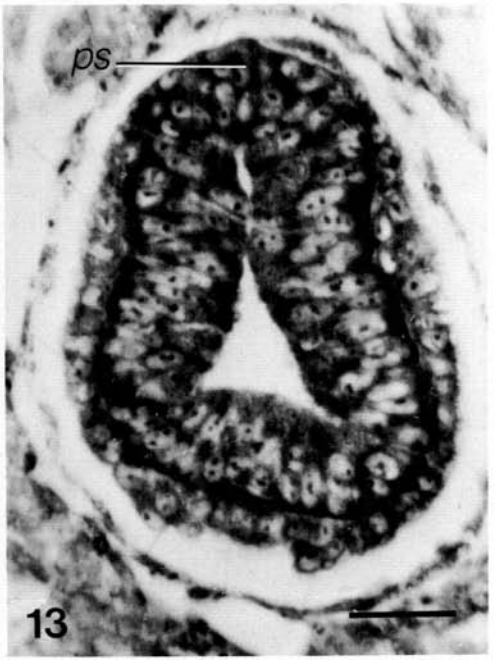

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Conceptuses in both horns of three Q females and cut in transverse section were classified both for implantation orientation type and for embryonic axis orientation type using the four criteria for anterior and posterior described. These conceptuses timed as $6\frac{3}{4}$ days old but with no mesoderm cells present were previously included in the lumped data from $6\frac{3}{4}$- to $7\frac{1}{4}$-day litters (Smith, 1980). It must be emphasized that it was extremely difficult to classify some of these embryos with respect to embryonic axis orientation and repeated classifications did not always agree. The data are given in Table 1. As can be seen, only 29 of the 32 conceptuses that could be classified as to axis orientation also could be classified as to their implantation orientation. Both embryonic axis and implantation orientation was the same in 27 conceptuses: 12 in Type R orientation; 15, in Type L. It is assumed that the discrepancy in the orientations in the other two is not real but instead is the consequence of the difficulty in determining the orientation of the embryonic axis.

The $6\frac{3}{4}$-day conceptus

At this stage there is no doubt as to A–P axis orientation: a primitive streak and at least extraembryonic mesoderm cells are present. All $6\frac{3}{4}$-day embryos also have distinctive foregut endoderm or prechordal plate mesoderm cells underlying and closely applied to a small midline region of anterior embryonic ectoderm (Fig. 1, $6\frac{3}{4}$: C, Figs 11, 13). The egg cylinder and trophoblast cone are still asymmetrically oriented with respect to the ends and walls of the horn so the conceptus can still be classified as to implantation type although the ectoplacental cone is becoming symmetrical.

Numerical data on the agreement of implantation and A–P axis orientation in conceptuses from the frontally sectioned horns of four CF1 females are to be found in Table 1. Of the 45 classifiable conceptuses, 21 were in Type R A–P embryo axis orientation (prechordal plate toward the right wall); 24, in Type L A–P axis orientation (prechordal plate toward the left wall). In all but three of these, embryo axis and implantation type corresponded. One of those in Type R axis orientation appeared to be in Type L implantation orientation and two in Type L axis orientation appeared to be in Type R implantation orientation. Again it is assumed that these discrepancies are not real but due to difficulties in classification. A summary of the remaining portion of the numerical data from

Fig. 10. 6- to $6\frac{1}{2}$-day conceptus (Type R) (TSH). Proliferation of ectoplacental cone cells has cut off the cavity in the trophoblast cone and eee from the lumen. Bar = 100 μm.

Fig. 11. $6\frac{3}{4}$-day conceptus (Type L). Tilted trophoblast cone. Most ectoplacental cone cells not shown (TSH). Bar = 100 μm.

Fig. 12. $6\frac{1}{2}$-day conceptus in frontal section of horn (FSH). Section of embryonic ectoderm near its junction with extraembryonic ectoderm. Bilateral symmetry evident. Putative primitive streak side toward top of page. Bar = 50 μm.

Fig. 13. $6\frac{3}{4}$-day conceptus. Section of embryonic ectoderm near its junction with extraembryonic ectoderm. A–P asymmetry of both ectoderm and endoderm evident. Primitive streak toward top of page. Two mesoderm cells at right. (FSH). Bar = 50 μm.

$6\frac{3}{4}$- to $7\frac{1}{4}$-day Q litters can also be found in Table 1. One of the litters reported on earlier (Smith, 1980) and for which only one horn was available has been replaced by two litters for which both horns were available.

The $8\frac{1}{2}$-day embryo

The S-shaped embryo of this age is beginning the clockwise (when viewed from anterior to posterior) rotation around its A–P axis that transforms it into a C-shaped one. The brain neural folds of the vast majority of the $8\frac{1}{2}$-day embryos were found to have rotated to such an extent that they seemed to be twisted at almost a right angle to the trunk–tail region. The right side of the head was always closer to the right side of the trunk; the left side of the head was always farther from the left side of the trunk. Since the foregut region of such embryos is still tightly fixed to the yolk sac it is highly unlikely that the A–P orientation of this region can be reversed without its attachment to the yolk sac being broken. The relationship of the anterior and posterior ends of these embryos in the two orientations to the ends and walls of the horn then should be the same as at $6\frac{3}{4}$ days.

An embryo was considered to be in Type L embryonic axis orientation therefore, if its head was toward the cervical end of the horn. The right side of the trunk of such an embryo lies against the left wall of the horn as it does in $6\frac{3}{4}$-day conceptuses in Type L orientation. An embryo was classified as in Type R axis orientation if its head was toward the oviductal end of the horn. The right side of the trunk of such an embryo is toward the right wall of the horn as it is at $6\frac{3}{4}$ days in conceptuses in Type R axis orientation.

The dissection data are summarized in Table 1. As was the case for the younger CF1 embryos, the number in Type L (51) and in Type R (50) was almost identical.

Table 1. The same embryos are ordered in two ways so that the frequency differences between embryos in Type R and L embryonic axis orientation in right and left horns are more easily seen. In columns (C) 9–14 the number of (upper figure for CF1) embryos in Type R or L axis orientation in each horn is given. Here note that although the total number of Type R and L CF1 embryos found was the same (C11 & 14) the frequency (lower figure) of Type L embryos in the right horn (C13) is different from the other three frequencies. In C15–18, the number of Type R and L embryos in each horn is given. Note that although the number of embryos in right and left horns is very different (C6 & 7) ($\chi^2 = 11{\cdot}23$ d.f. = 1), the frequency of Type R in left horn (C15) and Type L in right horns (C18) are the same and different from (higher) than those of the other two combinations ($\chi^2 = 5{\cdot}095$ d.f. = 1).

[1] angle of section made four unclassifiable for axis orientation

[2] cones of three symmetrical and unclassifiable

[3] cone and embryonic axis orientation of one do not agree

[4] 18 resorbed or section angle poor

[5] three resorbed (one LH; one RH): one tiny twins: one with two cones

[6] one Type L implantation orientation

[7] two Type R implantation orientation

[8] five LH, three RH resorbed: one LH, four RH tiny: one LH, one RH destroyed in dissecting

[9] three LH, six RH resorbed: two RH destroyed in dissecting

[10] two resorbed

Table 1. *Embryonic axis orientation of mouse and rat embryos in right and left horns*

Age	No. & kind	Capsules			Classifiable embryos			Type R			Type L			Left horn Type		Right horn Type	
		Left horn	Right horn	Total	Left horn	Right horn	Total	Left horn	Right horn	Total	Left horn	Right horn	Total	R	L	R	L
6½	3	17	19	36[1]	16	16	32	6	8	14	10	8	18	6	10	8	8
	Q	47·22	52·78		50·00	50·00											
					13	16	29[2]	5	7[3]	12	8	7[3]	15	5	8	7	7
					44·83	55·17											
6¾ to 7½	5	22	28	50[4]	17	15	32	8	9	17	9	6	15	8	9	9	6
	Q	44·00	56·00		53·12	46·88											
6¾	4																
	CF1	22	28	50[5]	19	26	45	10	11[6]	21	9[7]	15	24	10	9	11	15
		44·00	56·00		42·22	57·78		22·22	24·22	46·67	20·00	33·33	53·33	52·63	47·67	42·31	57·69
8½	10																
	CF1	48	68	116[8]	41	60	101	25	25	50	16	35	51	25	16	25	35
		41·37	58·63		40·60	59·40		24·75	24·75	49·50	15·85	34·65	50·50	60·98	39·02	41·67	58·33
9½	11																
	CF1	65	86	151[9]	62	78	140	35	36	71	27	42	69	35	27	36	42
		43·05	56·95		44·29	55·71		25·00	25·71	50·71	19·29	30·00	49·29	56·45	43·55	46·15	53·85
Total																	
CF1	25	135	182	317	122	164	286	70	72	142	52	92	144	70	52	72	92
		42·59	57·41		42·66	57·34		24·48	25·17	49·65	18·18	32·17	50·35	57·38	42·62	43·90	56·10
11½	5																
	rat	26	31	57[10]	24	31	55	13	16	29	11	15	26	13	11	16	15
		45·61	54·39		43·64	56·36		23·64	29·09	52·73	20·00	27·27	47·27	54·17	45·83	51·61	48·39

Table 2. *Order of Type R and L axis orientation types within the uterine horns*

	Left horn Female number											Total R Total	Right horn Female number											Total R Total
Capsule number	1	2	3	4	5	6	7	8	9	10	11		1	2	3	4	5	6	7	8	9	10	11	
1	R	R	R	R	R	R	R	L	L	L	L	7/11	R	R	L	R	L	R	L	R	L	R	L	6/11
2	R	R	R	R	rr	R	L	R	L	L	L	7/11	R	¢	R	R	*	L	L	R	L	L	L	4/9
3	R	R	R	*	L	L	L	*	L	R	L	4/9	R	R	L	L	R	L	R	L	L	L	L	4/11
4	R	R	L	R	R	L	R		R	R	R	8/10	L	L	*	R	R	L	L	L	R	L	R	4/10
5	R		L	*	L	R	L		L	L	L	2/8	R	*	L	R	R	L	L	L	R	L	R	5/10
6	R			R	R	R	R			L	L	5/7	R	L	L	L	L	*	R		¢		L	2/7
7				L			L					0/2	R	R	R		*	L	rr				R	5/6
8				R								1/1	L	L			L	R	L				R	2/6
9				R								1/1		R			*	L	R				L	2/4
10				L								0/1						L					R	1/2
11				L								0/1											R	1/1
12																							L	0/1
												35/62												36/78

The right and left horns of females 1–11 are identified by the female number. The order of the 9½-day embryos within the horns is indicated by the capsule number: Capsule 1 is at the oviductal end. R and L indicate the orientation types of the embryos. * is a resorbed embryo: ¢, an embryo destroyed in dissection: rr, Type R twins.

Surprisingly, the distribution of the orientation types was not the same in the right and left horns. There were 16 Type L in left horns; 35, in right and 25 Type R in left horns; 25, in right.

The 9½-day embryo

A typical 9½-day embryo is C-shaped with the left side of its tail or posterior trunk lying against the right side of its head (a right-handed helix). The brain neural folds seem closed or closing and the optic cups are beginning to form. The ventricular loop of the heart is bent sharply toward the right side of the body but the atrium has not yet sacculated nor moved dorsal to the ventricle. Forelimb buds are present.

All embryos were found to be in one or the other of two uterine positions at 180° from each other. Either their head was toward the cervix and their dorsal surface was toward the right wall or their head was toward the oviduct and their dorsal surface, toward the left wall. In these two positions as Snell & Stevens (1966) point out, the right side of the embryo is toward the placenta. These orientations are clearly the result of the clockwise rotation of Type L and R 8½-day S-shaped embryos respectively. The data from these dissections are summarized in Table 1. In Table 2 the embryos are listed in the order in which they were arranged in the horns. The same kind of information was recorded for the 6¾- and 8½-day embryos (Tables 3 & 4).

The 11½-day rat embryo

Since S-shaped rat embryos *in vitro* were reported to undergo counterclockwise rotation (when viewed from anterior to posterior) in becoming C-shaped (Deuchar, 1971), it was thought worthwhile to investigate the orientation of C-shaped rat embryos at the same developmental stage as that of 9½-day mouse embryos. Although the 11½-day rat embryos were slightly more advanced developmentally than the 9½-day mice (they had hindlimb buds) their orientation was identical to that of the mice. Moreover, all had the same right-handed helical shape as the mice indicating that they too rotate in a clockwise direction. These data are given in Table 1.

DISCUSSION

A mouse blastocyst fate map

From the morphological descriptions and the numerical data given here it is evident that there is a direct correlation between A–P differentiation within the embryonic portion of the 6¾-day egg cylinder and asymmetries of its extra-embryonic ectoderm portion and trophectoderm and ectoplacental cones. It is also evident that the asymmetries of the 6¾-day derivatives of the polar trophoblast can be traced back to the asymmetries of the 4¼-day implanting blastocyst. These relations are summarized in Fig. 1.

An axis fate map for the PT–ICM complex of the implanting blastocyst has been constructed based on these correlations. The anterior pole of the A–P axis is located at the mesometrially directed end of the long axis of the complex, the end toward the rounded side of the blastocoel; the posterior pole, at the most antimesometrially directed end. The D–V axis is perpendicular to the A–P axis, the epiblast and hypoblast marking its dorsal and ventral poles. The right and left sides of the complex are then fixed.

A similar fate map was proposed earlier for the younger attached blastocyst (Smith, 1980). However, since the attached blastocyst may or may not appear morphologically different along its proposed A–P axis, it is a fate map for the attached blastocyst *in utero* only. That is, in the absence of morphological asymmetry along the presumptive A–P axis, presumptive anterior and posterior can be designated only by virtue of their relationship to the mesometrial and antimesometrial surfaces respectively of the lumen. The conclusion with respect to this relationship is based on an interpretation of the way the attached blastocyst with E–abE axis more or less horizontal (perpendicular to the M–antiM axis of the horn) comes to be more or less vertical (parallel to the M–antiM axis) during implantation (Smith, 1980). This is that the blastocyst implanted and fixed first at its abembryonic pole rotates 'upward' 90° into the lumen and away from the 'floor' (the antimesometrial surface) of the implantation site as implantation continues. As the consequences of such undirectional rotation, the cells that are later found at the most mesometrially directed (the anterior) side of the oval PT–ICM complex

of the vertical blastocyst are those that were at the most mesometrially directed (anterior) side of the disc-shaped PT–ICM complex of the horizontal blastocyst. That this interpretation of the relationship between the PT–ICM cells of the attached and implanting blastocyst is correct was reinforced by the discovery that the attached blastocyst may display, in miniature, the same asymmetries that characterize the implanted blastocysts (Smith, 1980).

What have been proposed here are, of course, fate maps not maps of committed cells. That this is the case for the ICM cells is clear from experiments showing that descendents of an ICM cell from a young blastocyst transplanted into a young blastocyst has the potential to contribute to many tissues of the foetus (Gardner, 1978). Gardner's results, nevertheless, do not preclude the possibility that the attached blastocyst *as a whole* contains a three-dimensional system of positional information (Wolpert, 1969). For example, Johnson & Pratt (1983) have proposed a cell contact mechanism whereby the totipotency of young ICM cells may be reversibly suppressed. An early expression of one component of such a three-dimensional system may be the trophoblast cells' changing ability to attach to and invade the luminal epithelium. That is, the polar trophoblast cells begin to invade the epithelium approximately two days later than do mural trophoblast cells and transformation of mural trophoblast cells into invasive cells spreads progressively from the abembryonic pole toward their junction with the polar trophoblast (Dickson, 1966). The second gradient may have its first morphological expression in the asymmetry seen along the presumptive A–P axis of some attached blastocysts. Localization of the centre of the postulated gradient expressed first in the trophoblast seems to be related to the position of the ICM since Gardner (1978) has shown that the presence of ICM cells is necessary for the continued division of and probably suppresses the invasiveness of trophoblast cells. The centre of the other gradient may become localized as the result of a response of the horizontal blastocyst to microenvironmental differences mesometrial and antimesometrial to it (Smith, 1980). Such positional information could then be transformed into a stable pattern of unique determinants of cell types characteristic of different levels along the embryonic D–V and A–P axes by a sequence of interactions among and between trophoblast and ICM cells and their derivatives. The proposal that the uterine horn provides the mouse conceptus with positional cues that it somehow uses to orient its cell differentiation mechanisms in space may seem unreasonable in view of the ability of young mouse blastocysts to develop into apparently normal C-shaped embryos in culture (Hsu, 1982). However it is possible that the position of the A–P axis of these embryos is related to environmental differences in the culture environment. That is, the primitive streak consistently develops on the side of the egg cylinder facing the fluid phase not the solid phase of the medium (Hsu, personal communication). Moreover, examination of the photographs of Libbus & Hsu (1980) show many similarities between the asymmetries of the cultured blastocysts and those described here. For example, the PT–ICM complex of the blastocyst with blastocoel walls still intact (Fig. 3) is asymmetrically inclined with respect to the bottom of the dish and the

long axis of the blastocyst. Furthermore, the side of the blastocyst toward the bottom of the dish appears to correspond to the longer, rounded 'anterior' side of the 4¼-day blastocyst *in utero*; that toward the fluid phase, to the shorter 'posterior' wall since most of the PT–ICM complex is found there. The relationship of the asymmetries of the extraembryonic ectoderm and the ectoplacental cone is also the same as that described here.

Embryonic axis orientation within the horn

Clearly the orientation of the embryonic D–V axis of all primitive streak and S-shaped mouse embryos to the horn is based on the blastocyst's implantation with its E–abE axis parallel to the M–antiM axis of the horn and with its PT–ICM complex toward the mesometrium. Also clearly, as has been shown here, the two orientations of the A–P axis at 180° from each other are related to yet another asymmetry of implantation: whether the blastocyst is in Type L or R orientation to the ends and walls of the horn. If in Type L implantation orientation, anterior is toward the left wall; if in Type R, toward the right wall.

These two asymmetrical implantation orientations have also been traced back to two asymmetrical orientations of the attached blastocyst to the ends and walls of the horn which are strikingly similar to those of the implanting blastocyst (Smith, 1980). Either their abembryonic pole is attached to the left wall and their PT–ICM complex is toward the right wall and oviductal end of the horn as in Type L implantation orientation or their abembryonic pole is attached to the right wall and their PT–ICM complex is toward the left wall and cervical end of the horn as in Type R orientation. Furthermore, the shape of the horn wall to which the abembryonic pole is attached is the same in both orientations and is different from that of the opposite wall as is the case for the horn walls forming the implantation chamber.

Table 3. *Total number of Type L and R runs of length 1–6 found*

Type of run	Number of embryos in run						
	1	2	3	4	5	6	Total
R	44	23	11	2		2	82
L	46	22	12	2	2		84
	90	45	23	4	2	2	166

Table 4. *Distribution of runs in right and left horns*

Location	Number of embryos in run						
	1	2	3	4	5	6	Total
Left horn	38	20	8	2		2†	70
Right horn	52	25	15	2	2*		96
	90	45	23	4	2	2	166

* Both Type L
† Both Type R

If the positions of the putative D–V and A–P axes in the attached and implanting blastocysts are used to locate their putative right and left sides in each of these orientations, the following is also evident. It is always the right side of the blastocyst which is toward the wall to which its abembryonic pole is attached and it is always the right side of the implanted blastocyst which is toward the wall faced by the anterior end of the oval PT–ICM. No blastocysts were found with left side against the horn wall to which their abembryonic pole was attached, i.e., the theoretically possible orientations that are mirror images of Type R and L attachment orientations were not found. A minimum of two asymmetries of the horizontal blastocyst therefore appears to be required if its two attachment and implantation orientations are to be explained. These are a difference along its E–abE axis that causes it to attach first at its abembryonic pole and a difference that causes it to be the wall toward its 'right' to which it attaches. An observation by Hsu (1981) suggests a mechanism to account for the latter. He reports that the E–abE axis of the 3½-day blastocysts in culture usually rotates in a counterclockwise direction. Such non-random rotation of blastocysts *in utero* could be the means by which the right and left walls of the horn could come to respond differently to the blastocyst. The response might be such as to cause the PT–ICM complex, no matter its orientation at the time of attachment, to become oriented toward a particular end of the horn. Which end would depend on whether attachment was to the right or left wall of the horn.

The significance of the two implantation orientations

As the result of clockwise rotation around its long axis an S-shaped embryo in either implantation orientation becomes a C-shaped embryo with left side against the floor of the implantation chamber. Such unidirectional rotation may be based on a differential response of a bilateral conceptus to spatial cues provided by the uterine asymmetries to its right and left. On the other hand, the young blastocyst attaches to the horn wall to its right. Unidirectional rotation around the A–P axis may be therefore yet another expression of the factor that causes cultured blastocysts to rotate in a counterclockwise direction and if such rotation occurs *in utero* to so attach. Such attachment, clockwise rotation around the A–P axis and the C-shaped embryo's resulting position may be necessary for the development of normal visceral asymmetry in the mouse. Both the heart (Nelson, 1953) and the stomach (Romer, 1970) asymmetries in many vertebrates seem to involve first an enlargement of the ventral wall of the organ which produces a ventrally directed loop. This is followed by a clockwise rotation (as viewed from anterior to posterior) of the organ around its long axis which produces a right-directed loop.

In discussing the extensive investigations on heart looping, Stalsberg (1970) and Manasek (1981) both point out that at the very least, several experiments show that mechanical changes in the immediate environment of the heart can easily influence the direction of its looping. Development with the embryo's right rather than left side against a solid surface may produce a mechanical impediment to right-directed looping. That whole body rotation and the direction of heart

looping are related is suggested by observations on both birds and mice. For example, the rare chicks that undergo counterclockwise rotation and so come to lie with right side against the yolk frequently display *situs inversus* (Hamilton, 1952). Also *situs inversus* is found in one-half of mice homozygous for the *iv* allele (Hummel & Chapman, 1959; Layton, 1976). When 10½-day *iv/iv* embryos were examined, one half were found to be in the form of a right-handed helix and to lie with left side against the floor of the implantation chamber; one-half to be in the form of a left-handed helix (the result of counterclockwise rotation) and to lie with right side against the floor of the chamber. The former showed normal right-directed heart looping, the latter, left-directed heart looping (Layton, 1976 and personal communication). Clearly some factor in addition to genotype is involved in determining the direction of heart looping in *iv/iv* mice. Perhaps the embryos undergoing counterclockwise rotation arise from blastocysts implanted in the missing mirror-image orientations.

The report that rat embryos *in vitro* rotate in a counterclockwise direction around their long axis (Deuchar, 1971) has since been corrected (Deuchar & Parker, 1975). As the data presented here indicate, 11½-day C-shaped rat embryos are also in the form of a right-handed helix and they too lie with the left side against the floor of the implantation chamber.

Again, the proposal that implantation with the right side of the conceptus against the wall to which its abembryonic pole attaches, clockwise rotation in becoming C-shaped and subsequent development with right side toward the placenta are necessary for the development of normal visceral asymmetry in the mouse may seem unreasonable in view of the ability of young mouse blastocysts to develop into apparently normal C-shaped embryos in culture. However, until more examples of such C-shaped embryos are intensively analysed with respect to their external and internal morphology, it cannot be concluded that they routinely undergo clockwise rotation in becoming C-shaped or that they routinely develop right-handed visceral asymmetries (Hsu, personal communication).

The numerical data

Clearly the differences in the frequency distribution of Type L and R embryos in right and left uterine horns are based on differences in the total number of embryos/horn and the difference in the number of Type L embryos in the two horns. That more capsules and more viable 6¾-, 8½- and 9½-day embryos are found in right than in left horns can be explained by assuming more eggs are ovulated from the right than the left ovary. This is the case for Q females (McLaren, 1963). Greater postimplantational loss of embryos in left horns is eliminated since the proportion of resorbed and very small embryos in the capsules in right and left horns appears to be the same.

The asymmetrical distribution of orientation types in the two horns is most simply explained as being due to preferential blastocyst attachment to the medial wall of the horns. The types present in higher frequency (ca. 55 %) in each arise from blastocysts whose abembryonic pole is attached to the median wall (Type R

orientation in left horns: Type L orientation in right horns). The orientation types found in runs of 5 and 6 (Table 4) are consistent with this explanation. That is, assuming independence in implantation orientation, the probability of finding such runs is 0·077. This is approximately four times greater than the probability of finding such runs if the frequency in each horn is assumed to be 0·50.

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