Int. J. Dev. Biol. 53: 447-457 (2009)
doi: 10.1387/ijdb.082587rs

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Theoretical exploration of blastocyst morphogenesis

REBECCA J. SHIPLEY[1], MICHAEL B. BONSALL[2], DAVID J. ALLWRIGHT[1] and CHRIS F. GRAHAM*[,2]

[1]Mathematical Institute and [2]Department of Zoology, University of Oxford, UK

ABSTRACT A theoretical exploration of cell distribution on the mouse blastocyst is conducted. A model ball of cells represents the morula which develops into a 32-cell model blastocyst that is enclosed in a spherical surface with a hemispherical cavity at one end. In the combinatorial analysis it is assumed that each cell of the 2-cell embryo forms 16 cells in the blastocyst and that these 16 cells touch each other. The results of the analysis identify a tendency for one set of 16 cells to contribute twice as many cells to the basal solid end of the blastocyst than the other set, a developmental bias that is also found by some observers of natural blastocysts. In the geometric analysis, half the volume of the inner group of cells of the morula and blastocyst and half the volume of the surrounding shell cells, the trophectoderm, is assumed to be formed from the progeny of each 2-cell stage cell. Making various assumptions about morphogenesis, it is found that there is a tendency for a curved frontier between the volumes from each 2-cell stage cell, the clonal volumes, to lie at an angle of 43.4° to the equator of the blastocyst and for the bulk of the frontier circumference to lie on either side of the equator. These tendencies are also found by some observers of real blastocysts.

KEY WORDS: *blastocyst, morphogenesis, theory, combinatorial, geometry*

Introduction

Frequent embryological events can be described as developmental routines and their control amongst vertebrates with spherical eggs and complete cleavage is partly understood in a few organisms. In *Xenopus laevis*, for instance, the repetition of some events is sufficiently common that fate maps can be constructed, albeit with wide variation in the fate of early blastomeres. Despite this variation, cells in identified regions of the early embryo more often than not contribute to particular organs of the tadpole, and this observation demonstrates that cell lineages must have a degree of consistency from one embryo to the next. One fully worked example is the lineage from the 32-cell stage (Dale and Slack, 1987) and for *X. laevis* these routine events depend on intrinsic discontinuities in the oocyte and further asymmetries directed by both gravity and sperm entry. Embryonic tissue architecture emerges by signalling between the heterogeneous cells that contain different parts of the late zygote.

Unfortunately the situation is not so clear for mammalian development. For mice, four aspects of early developmental routines have been questioned: "Do they exist?", and if they are real "Do they depend on zygote organization?", "Are they related to cell lineage?" and "Do they matter?" The role of early development routines has been the subject of recent intense study (Gray *et al.*, 2004, Alarcon and Marikawa, 2005, Motosugi *et al.*, 2005, Plusa *et al.*, 2005, Chazaud *et al.*, 2006, Gardner, 2006, Hiiragi *et al.*, 2006a, Hiiragi *et al.*, 2006b, Hiiragi and Solter, 2006, Motosugi *et al.*, 2006, Plusa *et al.*, 2006, Wakmundzka *et al.*, 2006, Zernicka-Goetz, 2006, Dietrich and Hiiragi, 2007, Gardner, 2007, Kurotaki *et al.*, 2007, Torres-Padilla *et al.*, 2007, Bischoff *et al.*, 2008). In this article we consider, theoretically, how these patterns might occur during the formation of the mouse blastocyst.

The aim of the present paper is to explore where early cell lineages and the second polar body (2PB) are likely to end up in and on a model blastocyst. It has been suggested that these two features mark potential sources of developmental organization that to some degree steer blastocyst morphogenesis and this paper considers what would happen if this is not so during the morula to blastocyst transition. The role of chance in the allocation of cells and the 2PB to different parts of the blastocyst is the focus of our theoretical models. Chance distributions provide the null

Abbreviations used in this paper: ICM, inner cell mass; PB, polar body.

***Address correspondence to:** Chris F. Graham. Department of Zoology, University of Oxford, South Parks Rd, Oxford OX1, 3PS, UK.
Fax: +44-0-186-5931-0447. e-mail: chris.graham@zoo.ox.ac.uk

Electronic Supplementary Material for this article is available at: http://dx.doi.org/10.1387/ijdb.082587rs

Accepted: 8 May 2008. *Published online:* 29 January 2009.

ISSN: Online 1696-3547, Print 0214-6282

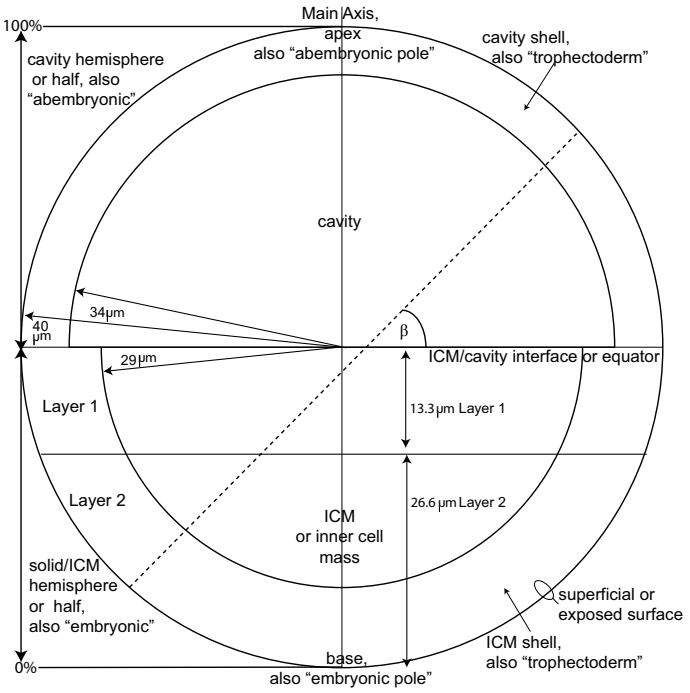

Fig. 1. Section of the invented blastocyst, bisected along its main axis. *The dimensions of the model 32-cell stage blastocyst are given on the Figure. The dimensions are based on the data sets in Supplementary Material 1 & 2 which has been extracted from the literature. The topological terms used in the Figure and text will make the analysis accessible to those outside the field of preimplantation mouse development. The historic embryology terms are between inverted commas in the Figure. The ICM or inner cell mass fits both conventions. The cavity is not described as a blastocoel because it does not match up with similar structures and the field can not decide which part of its surrounds are the floor and which its roof (Eyal-Giladi, 1997, O'Farrell* et al.*, 2004). Layer 1 is similar to the Boundary Zone (Piotrowska* et al.*, 2001). Supplementary Material 3 records the terms used for staging the blastocyst.*

hypotheses by which the influence of pre-existing organization on observed distributions can be judged. The discovery of appropriate null hypotheses relevant to morphogenesis routines is a general problem and the suggested approach may have wide application even though the results in this paper are limited to local solutions. Predictions from combinatorial probability models and models of morphogenesis geometry are used to obtain these null hypotheses. The basis of the article is that natural selection is concentrated on the form and function of the mammalian blastocyst and that embryological organization and cell lineages are expected to be bound by this evolutionary process.

Results

The models

We take blastocyst morphogenesis to start from a 35 μm radius solid ball (model morula) with inner and outer cell populations that do not interchange during the formation of the blastocyst. That is to say that the whole of the blastocyst's inner cell mass (ICM) is formed from the inner cells of the morula and its outside layer, the trophectoderm or shell, is formed from the exposed superficial cells of the morula. In the absence of contradictory evidence, every outside cell occupies the same area of the model morula's exposed surface, an assumption adopted for simplicity. The total cellular volume and cell number are assumed to remain the same throughout the transformation (references in Supplementary Material 3). The idealized blastocyst consists of a hemispherical cavity contained inside one half of an otherwise solid ball with the dimensions shown in Fig. 1. In this structure the shell over the ICM has been allocated more cells and volume than that over the cavity: this representation accommodates the greater number of shell cells that are found over the ICM (see Supplementary Material 1). No real morula or blastocyst has ever looked exactly like these geometrical conveniences even though they are closely related to data about embryos at or near the 32-cell stage (Supplementary Materials 1 & 2). Here the final blastocyst is called a 50% blastocyst because the ICM/cavity interface is half way along the main axis, starting from 0% at the base of the ICM shell solid hemisphere and running to 100% at the apex of the cavity shell: the ICM/cavity interface is on the plane of the equator. This model therefore represents a brief stage in the whole process of blastocyst morphogenesis in nature. Synonyms for the "50% blastocyst" and other stages of blastocyst morphogenesis are shown in Supplementary Material 3.

Plan of experiments

There are three thought experiments. Thought Experiment 1 is a cell lineage analysis and the position of progeny of each cell of the 2-cell stage (1/2 stage clones) is determined using several assumptions about clone shape in the morula and blastocyst. In Thought Experiment 2 a conceptual bead is placed on the surface of the morula and its blastocyst position is computed on the assumption that it lies on the circumference of the frontier between the 1/2 stage clones. In the final study (Thought Experiment 3) the position of the bead is calculated as if it behaved independent of the frontier. In each case the results obtained with the models are compared with the data in Supplementary Material 1. In both these data sets and other studies in the literature it is clear that there are extensive differences between one blastocyst and the next and so the single model blastocyst described in the present study can only be a guide to the properties of the whole population of 50% blastocysts found in nature. In addition, the information required to validate or reject the following models

TABLE 1

DISTRIBUTION OF CELLS ALONG THE MAIN AXIS

Position in Blastocyst	Cell Distribution along Main Axis 3 Sections			Cell Distribution along Main Axis 4 Sections		
Cavity apex	Total 32 cells	Inner 11 cells	Outer 21 cells	Total 32 cells	Inner 11 cells	Outer 21 cells
Cavity shell	5	0	5	4	0	4
				4	0	4
Layer 1	3+ 12	7	3+ 5	17	10	7
Layer 2	12	4	8			
Base				7	1	6

TABLE 2

COMPARISON OF DATA SET WITH MODEL CALCULATIONS

Proportion of Embryos with:	Published Data[2]	Probability by Cells in the Model
Monoclonal cavity shell	19.0 % n=63	18.4% n=87
One or both clones confined to 2 adjacent regions[1]	35.0% n=63	25.3% n=87
Both clones confined to 2 adjacent regions	9.5% n=63	2.3% n=87
One clone contributes twice as many cells to Layer 2 when compared with other clone	76.2% n=63	69.0% n=87
Monoclonal Layer 2	20.6% n=63	9.2% n=87

requires a more formal statistical comparison with the data as in no case is it shown that that the form of any of the natural blastocysts sufficiently conforms to those of the models.

Thought Experiment 1: combinatorial analysis of cell lineage

Developmental routines that have been described

Clone distribution on blastocyst form has been reported to follow routines and here we focus on the tendency of one 1/2 stage clone to contribute disproportionately to Layers 1 & 2 that make up the ICM hemisphere of the blastocyst so that the frontier appears to align with the equator (Tables 1 & 2, Supplementary Material 1, Table S1.1). In the literature, this constraint has been recognized by several measures of which three are relevant to Experiment 1. In the first measure the position of the clone is described by the frequency that its frontier with the other clone is excluded from the cavity shell which is then entirely formed from one 1/2 stage clone and has a monoclonal origin. In the data set this occurs in about 19% of blastocysts. The second measure of clone position is the frequency at which one or both clones are confined to two of the adjacent regions shown in Fig. 1 and Table 1 (Cavity shell and Layer 1 or Layer 1 and Layer 2). These regions lie perpendicular to the main axis of the blastocyst and when a clone is in two adjacent regions then the frontier is more closely aligned with the equator than when it is in three regions. From these data about 35% of embryos have one clone confined to two regions and in this sub-group about a third have both clones restricted to two adjacent regions, the third measure. Additional methods of scoring clone position in nature are set out in Table 2 and two additional methods are discussed later in Thought Experiment 2. Models are next used to explore if these cell distributions are similar to those expected by chance.

Combinatorial lineage analysis of the blastocyst

Clone shape & coherence. In this thought experiment, the two 1/2 stage clones are granted exactly the same cell number and each cell has the same volume in both the morula and the blastocyst. Under this cell based analysis of the probable position of the two 1/2 stage clones on the blastocyst it is clear that clone shape and coherence will influence the distribution. For instance, a solid spherical shaped clone (a ball) is too large to fit into the inner core of the morula and with this shape such a clone must inevitably contribute to the two cell types formed from the inner and outer cells of this stage and later it could not fit anywhere into a blastocyst because its diameter is larger than any uninterrupted span of cellular material. There are also constraints on the position of a clone with a monolayer form because it could only lie in the shell of the morula and blastocyst and it would be cup shaped at both stages. A simple illustration of the importance of clone coherence is that the small pieces of split clones can fit into more places. In the next analysis the clones are allowed to adopt a number of shapes but all the cells in each clone must cohere, meaning that all the cells in one clone must touch at least one cell in the same clone. The effect of a single cell moving away from one clone is also noted.

Clone distributions in the cavity shell. The structure of the blastocyst is taken as fixed and the cell clones must fit into these constraints; the probability calculations explore the relationship between clone shape and position. To understand the distribution of the 1/2 stage clones in a 50% 32-cell blastocyst, sections through the 3D structure taken parallel to the main axis are collapsed to a single plane so that all the cells of each clone can be seen (Fig. 2). The arrangement of 1/2 stage clones in the 8 cell cavity shell is a constrained combinatorial solution of the likely arrangement of the clones in the whole structure (see Material & Methods). The cells of each clone are distinguished from each other by the labels 'b' and 'w' and the minimum constraints are that each 'b' cell should touch another 'b' cell, that the 'w' cells should also cohere, and that all these cells are in a single linear array. The complete constrained combinatorial pattern that is possible in the cavity shell is then:

$$b^8 + b^7w^1 + b^6w^2 + b^5w^3 + b^4w^4 + b^3w^5 + b^2w^6 + b^1w^7 + w^8$$

This patterning suggests that there are 9 ways in which the cells from the 1/2 stage clones can be arranged in a cavity shell of 8 cells. Given this patterning, the probability of the cavity shell consisting of cells from one clone is 2/9 (b^8 plus w^8 make up 22% of the possibilities). If the cavity shell happened to contain 9 cells then there would be 10 possibilities and two of these would contain cells from a single clone (b^9 or w^9), and so on with the proportion of monoclonal cavity shells decreasing as the proportion of cells of 32-cell stage that are in the cavity shell increases.

A simple way of noting the impact of clone coherence is to consider the frequency of monoclonal cavity shells when a single cell becomes isolated from all other members of the same clone in the cavity shell. Let the previous 9 combinations persist and to

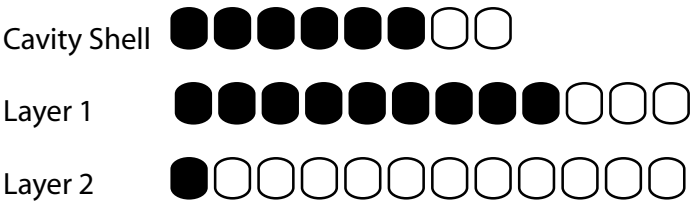

Fig. 2. Regions used in probability calculations. *The distribution of cells in the blastocyst is represented as 3 tiers of cells. These tiers or regions are the Cavity Shell, Layer 1 & Layer 2 and cell numbers in each region are the same as those in Table 1. In all cases there are 32 cells and those from each clone are represented by 16 closed and 16 open rectangles.*

these add a further twenty six as the single wandering cell is placed in any position amongst the cells of the other clone, isolated from the linear array of the same clone. The frequency of the monoclonal cavity shells becomes 6% (2/35) rather than 22%.

Clone distributions in the whole blastocyst. Layer 1 and Layer 2 are next introduced into the combinatorial analysis so that the ICM cells are included to make three stacked linear arrays with the cavity shell as the top deck (Fig. 2). In these arrays clone shape and position have a substantial influence on the probability that the cavity shell is formed from a single 1/2 cell clone. A simple measure is used to quantify shape and position and that is to count the number of cells of any particular clone that are in Layer 1, either as part of a clone confined to two contiguous regions of the model blastocyst (Cavity shell + Layer 1 or Layer 1 + Layer 2) or as a Layer 1 isthmus between a clone spread to all three regions. Amongst these possible distributions, 18.4% of the model embryos display a cavity shell made of the progeny of only one 1/2 stage clone when "loose" or dispersed clones with only 1 cell in a Layer 1 isthmus are included in the sample (n = 87 embryos).

The influence of clone shape on the chance that the clone will contribute to the whole of the cavity shell can be illustrated by noting the number of cells from a clone that are in Layer 1. If a quarter or less of a 16-cell clone are in Layer 1 then 13% of the cavity shells are entirely formed from that clone (n = 30). If more than a quarter of the cells are in Layer 1 then 7% of the cavity shells are entirely formed from the cells of that clone (n = 57) as the clone compacts into either the cavity shell + Layer 1 or Layer 1 + Layer 2. These calculations suggest that coherent clones must have a relatively loose form to deliver the highest observed frequencies of monoclonal cavity shells (Table 2). To summarize, the frequency at which the cavity shell is monoclonal demonstrates that clone position and shape can be major determinants on the dispositions of clones by chance.

The extent to which the model blastocyst mimics the properties of natural blastocysts in the data set is outlined in Table 2 and the match is not exact (see *Plan of Experiments)*. Nevertheless, these results from the model 32-cell stage provide appropriate starting null hypotheses for interpreting clone distribution on the blastocyst in future observations of this stage.

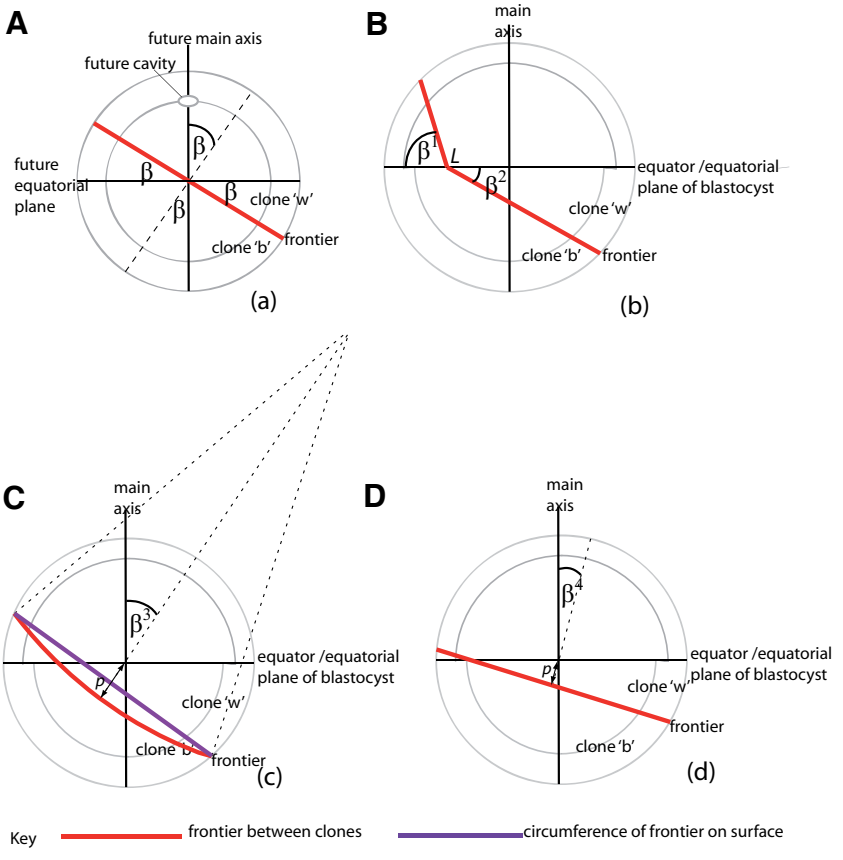

Fig. 3. Illustrations of the geometric models. (A) *The morula with a* single planar *frontier. An incipient cavity is shown and this is taken to define the apical end of the main axis of the future blastocyst. This arrangement is the starting stage for the development of the blastocyst models. The angle β is the acute angle between the frontier and the future equator. The angles that are equal to β are shown.* **(B)** *Blastocyst with a* two plane *frontier that divides both the shell and the ICM volumes in half. The kink along the line* L *normal to the paper becomes extreme as* β^2 *approaches 0º and the line L moves out of the structure of the blastocyst breaking the 'b' clone into two pieces and this clone no longer coheres.* **(C)** *Blastocyst with a* curved *frontier that divides both the shell and the ICM volumes in half. The frontier is a spherical cap whose dimensions are defined by the radius of a notional sphere (dotted lines) centered outside the blastocyst and by the angle* β^3. **(D)** *Blastocyst with a* single planar *frontier. The illustrated situation of the frontier is only possible when the constraints of dividing both the ICM and shell volumes in half are relaxed (they are met when* $\beta^4 = 90^o$*). However the relaxed condition that the frontier should halve the total volume of the blastocyst (shell plus ICM) is retained.*

Conclusion 1

To interpret the influence of prior organization on a morphogenetic event it is important to develop combinatorial models of this type or to explain why they are not appropriate.

Thought Experiment 2: the geometry of frontier disposition

It is clear that combinatorial analysis is a convenient method because it relates directly to the unit of development, the cell, and in experimental papers it is often cell numbers that have been counted in different parts of the blastocyst. Despite the advantages of using biological units there is a loss of spatial information in the three deck form shown in Fig. 2. In several studies, geometry is used to describe the position of both the inter-clonal frontier and objects on the blastocyst surface, and in the next experiment geometry is used to discover how the change in form between the morula and the blastocyst will influence the relationship between the inter-clonal frontier and the equator of the blastocyst.

Developmental routines that have been described

In this thought experiment morphogenesis from a solid morula is taken into account and the relative volumes of ICM and shell cells extend the analysis

by considering information that contributes to the distribution of volume in the blastocyst (Fig. 1, Supplementary Material 2). The position of the frontier has been described as non-random by reports of two different routines in natural blastocysts (Supplementary Material 1). In both cases the models are not expected to display identical routines to those of the empirically observed blastocysts: these blastocysts were not chosen for their match to the model. First, the mean angle between the irregular outline of the frontier and the equator (a β angle, Fig. 3) is seen to be 37.6º rather than the expectation of 57.3º (see below and Materials & Methods). Second, the 2PB is found on the surface of the horizontal middle slice between 33% and 66% of the blastocyst main axis in 43-64% of blastocysts rather than in one third of blastocysts. This second observation provides information about the position of the frontier if the 2PB sits on the inter-clonal frontier in the blastocyst.

Planar frontier models that divide both the ICM and Shell into equal volumes

The starting state is a solid ball (morula) in which it is assumed that the frontier is at a random angle to the future positions of the cavity, main axis, and equator of the blastocyst and that the eventual position of these landmarks can be anticipated (Fig. 3A). In the initial models the clones are taken to have identical volumes in the morula and blastocyst and the frontier is on a diametral single plane running through the centre of the morula to make the angle β^{morula} to the future equator: by this origin each clone contains half the volume of the ICM and half the volume of the shell. Initially the search is to find a biologically plausible model to calculate the likely position of the frontier on the blastocyst when it develops from such a morula (Fig. 3 B,C).

With the assumption that the cavity forms in the morula at a position independent of the frontier, β^{morula} will have a probability density function sin β, and the mean angle between the frontier and the equator will be 57.3º (1 radian), see Materials & Methods.

In the analysis it is not appropriate to treat the blastocyst frontier as a single plane even if later it becomes helpful to describe a curved frontier by its planar tangent (below). There are several reasons for rejecting a single plane treatment. First, if the frontier in the blastocyst were to be treated as a single plane that bisected both the ICM and shell volumes then its only possible position is meridional, i.e. a plane passing through the main axis perpendicular to the equator. This position would be expected if the cavity always formed on the frontier and $\beta^{blastocyst}$=90º, a situation that occurs at a frequency of 1/69 blastocysts in the data set (Supplementary Material 1) and at a frequency of 2/41 in a recent report (Kurotaki *et al.,* 2007). Second, morphogenesis will introduce a deformation into any single plane frontier that traverses the inner cells of this morula and such a frontier could not survive the transformation (Fig. 4). Multiplanar or curved treatments of the blastocyst frontier must be developed to accommodate the change in form.

One possibility is a folded plane in the blastocyst. For instance a plane that lies at angle β^2 to the equator in the ICM hemisphere, which is folded along the line L in the equatorial plane to then lie at an angle β^1 to the equator in the cavity shell, as illustrated in Fig. 3B. This situation is explored by calculating β^2 as a function of β^1 and the shell and cavity radii, and using the volume of an obliquely sliced hemisphere (Materials & Methods). Both the ICM and the shell volumes are halved in this model but it remains an unsatisfactory model because at some angles the cells of a clone become separated from each other: an increasingly sharp fold develops as β^2 reduces, and when β^2 becomes very small the line L moves outside the blastocyst entirely and the clonal continuity is lost. The single and two plane models do not mimic nature and they are not discussed again until the rule of volume parity is discarded (Supplementary Material 4).

Curved frontier models that divide both the ICM and Shell into equal volumes

A plausible analysis must accommodate blastocyst morphogenesis in which the whole configuration of the morula changes with the formation of an extensive cavity and the relative movement of about half the apical hemisphere cellular volume of the morula to the ICM/solid hemisphere of the blastocyst. There are no records of the intermediate steps in these shape changes *in vivo* and therefore there is no detailed mechanical model of this deformation and the final position of the frontier can not be accurately predicted. The next aim is therefore to make geometrical models that are simple but biologically probable and explore their consequences.

ICM deformation. A major deformation during the morula to blastocyst transition is the distortion of the spherical inner cell population into the hemispherical ICM (Materials & Methods, Fig. 4). In the ICM, this deformation necessarily flattens out most angles of the frontier to the equator, reducing the value of β^{morula}. This change can be estimated quantitatively since the aspect ratio (width:height ratio) of the ICM alters from 1 to 2, and as an approximation an angle β^{ICM} in the blastocyst will be related to an angle β^{morula} by tan β^{ICM} = 1/2 tan β^{morula} (Materials & Methods). This relationship introduces a curve into the part of any interclonal-frontier that runs through the ICM. Despite this curve in the blastocyst's ICM frontier, the mean angle of this frontier to the equator can be exactly described by a single plane ($\beta^{ICM)}$ that refers to a plane that is a tangent to the red curved frontier that runs parallel to the purple line across the ICM (for instance in Fig. 3C). This reduced aspect ratio of the deformation results in β^{ICM} having a mean value of 43.6º, which is noticeably much closer to the observed mean frontier tilt angle of 37.6º than the mean of

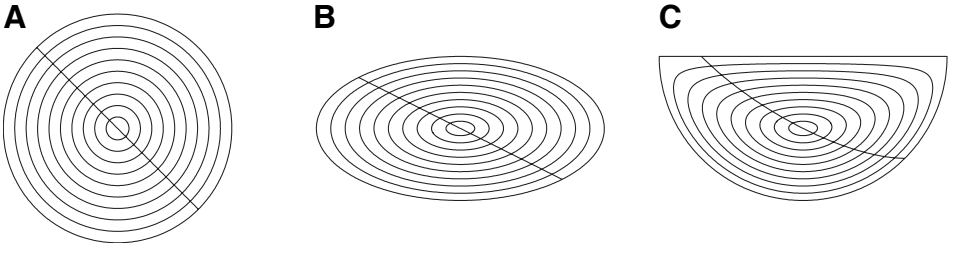

Fig. 4. Illustration of inner cell distortion during formation of the blastocyst shown as cross sections. *A spherical group of inside cells in the morula is transformed into a hemispherical ICM in the blastocyst. The method is derived in Materials and Methods.*

β^{morula}. However this calculation is only about the deformation of the ICM during morphogenesis and the analysis is next extended to the frontier position in the whole blastocyst.

Assumptions about blastocyst morphogenesis. Lacking detailed observations, two assumptions are next made about the geometry of the frontier in the shell of the blastocyst. First it is taken that the frontier retains continuity where it crosses from the ICM to the shell, i.e. there is no significant shearing or separation of the cells except where the blastocyst cavity intervenes. A consequence of this assumption is that β^{ICM} has a dominant influence on β for the whole blastocyst ($\beta^{blastocyst}$). The second assumption is that since a single plane frontier in the morula bisects the volumes of both the inner and outer cell populations, it similarly must bisect the volume of both the ICM and the shell in the blastocyst. The initial search is for a biologically plausible frontier position that will vary with β and bisect both volumes.

Angle of tilt. The frontier in the blastocyst is now treated as a single curved surface that includes both the ICM and the shell. The frontier is a spherical cap with its centre somewhere on a line at angle β^3 to the main axis, as illustrated in Fig. 3C. Thus β^3 is the mean angle between the curved frontier and the equator. It turns out that this choice gives a viable frontier for each positive acute angle β^3: that is each clone contains both half the volume of the shell and half the volume of the ICM and the inter-clonal frontier runs smoothly from one to the other as illustrated by the 10º steps in β^3 shown in Fig. 5. To complete the analysis some assumption must be made about the distribution of the random angle β^3. Since β^3 is the angle of the frontier in the ICM, it is natural to propose that β^3 has a mean value of 43.6º, close to the observed mean value of 37.6º in this data set.

Conclusion 2

The analysis demonstrates that the angle of frontier tilt to the equator depends on assumptions about the movements of morphogenesis and that these assumptions influence null hypotheses for monitoring the significance of the tilt.

Distribution of the frontier circumference

In some papers, the 2PB has been reported to remain at the inter-clonal boundary during blastocyst formation. In the following analysis a conceptual bead, a conbead, represents such a 2PB with the difference that the conbead is initially randomly distributed on the model morula's frontier circumference (the inter-clonal boundary on the surface of the morula). The previous analysis of $\beta^{blastocyst}$ can then be used to discover where a frontier based conbead would tend to be found on the blastocyst surface. The mean angle between conbead position and the equator can be estimated and for each 1º step between 0 and 90º the length of the frontier circumference in three horizontal slices of the blastocyst calculated. Assuming that the conbead will distribute on the blastocyst surface in proportion to the length of these circumferences in each slice, the most likely position of the conbead is on the surface of middle horizontal slice between 33 and 66% along the main axis of the blastocyst where 45.8% of conbeads will be found in a population of blastocysts. The other conbeads are on the basal slice (31.1%) and apical slice (23.1%).

It should be noted that in this model the probability that the whole of the frontier circumference is contained in Layers 1 & 2 is very low (2.1% of blastocysts) and consequently the frequency of monoclonal cavity shells is much lower than that in the data set (2.1% compared to 22%). Changing some of the assumptions of the model can substantially change the distributions described above (see Supplementary Material 4).

Conclusion 3

With this favoured model (Fig. 3C), frontier based conbeads are expected to cluster on the surface of the middle slice of the blastocyst.

Thought Experiment 3: independent surface markers

In Thought Experiment 2 the distribution a 2PB or conbead was calculated with the assumption that they accurately reported on the position of the frontier circumference. There are some reports that the 2PB does not remain on this boundary and to simulate these cases a third thought experiment is conducted. Assume that the conbead point is passed down from parent cell to one daughter but it is otherwise allowed to engage in a random walk around the cell surface from the zygote onwards. In this case, there are several

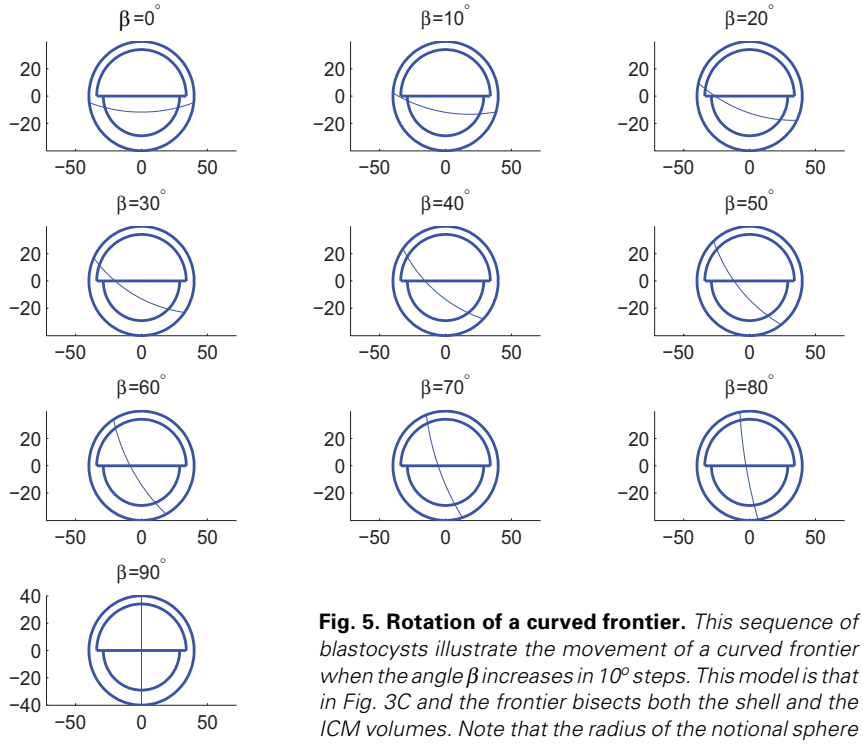

Fig. 5. Rotation of a curved frontier. *This sequence of blastocysts illustrate the movement of a curved frontier when the angle β increases in 10º steps. This model is that in Fig. 3C and the frontier bisects both the shell and the ICM volumes. Note that the radius of the notional sphere centered outside the conceptus changes with each step and thus the curvature of the frontier changes with each step. When β is less than 60º the curvature of the frontier is within 14% of its value for β =0º and so the flattening of the frontier is only visible in these figures when β exceeds 60º. Eventually there is a* single planar *frontier at β = 90º.*

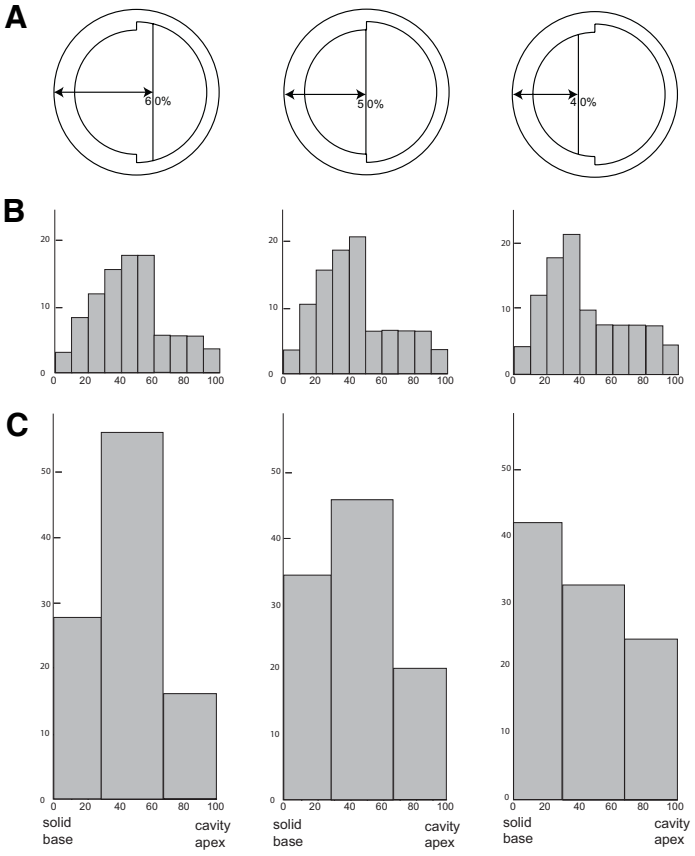

Fig. 6. Distribution of conbead on the surface of the blastocyst in relation to volume of cellular material. *The volume distribution in the 50% blastocyst is described in Supplementary Material 2, while those in the 40% & 60 % blastocysts were arrived at by respectively increasing or decreasing the volume of the ICM i.e. the volume of the outer cells was the same in each blastocyst.* **(A)** *The type of blastocyst is indicated by the position of the ICM/cavity interface on the main axis (superficial cavity apex is at 100% on this axis).* **(B)** *The distribution of volume along the main axis at 10% intervals.* **(C)** *The distribution of volume along the main axis at 33.3% intervals. It is noticeable that the visual impression of blastocyst structure is well represented at 10% intervals and not at 33.3% intervals. By comparing the same data presented in (B) and (C) it is also clear that the frequency of volume sampling makes a major difference to the perception of volume distribution, a well known problem in presenting statistical data in histograms.*

circumstances which could order the distribution of the conbead on the blastocyst shell. For instance, if it is mapped by the concentration of trophectoderm cells then the conbead would be most frequently found over the ICM/solid hemisphere (62% of cases, Table 1).

It is also possible that this lineage restricted but wandering conbead is distributed by total cellular volume (Fig. 6 B,C). Of course, it could be argued that distribution by total cells or total cellular volume is biologically implausible but this may not be the case. For instance, if the conbead is attached to a cell that will divide to form part of the ICM and part of the shell, then it is the position of that parental cell's volume that may determine the future distribution of the conbead located on one daughter cell. Figure 6 shows that if this constraint is allowed then the conbead will be found on the surface of the middle slice in 46% of the model blastocysts. Note that the proportion of the blastocyst occupied by the cavity substantially alters the distribution of a conbead whose position reflects that of underlying cellular volume. This is particularly clear when that distribution is scored in thirds along the main axis (Fig. 6C).

Discussion

The effect of pre-patterning, cryptic preformation and regulative mosaicism on blastocyst development is attracting increasing and often conflicting opinions (see references in Introduction). This paper solely addresses opinions based on the distribution of 1/2 cell clones and the polar body on blastocyst structure. In the present study the impact of clone coherence, shape, volume, and the geometry of morphogenesis from morula to blastocyst has been explored in three thought experiments and the theoretical methods incorporate sufficient biological details to provide null hypotheses against which future experimental evidence can be evaluated. To justify this opinion we critically discuss these methods for studying morphogenesis.

Why the 32-cell stage?

The case for finishing this analysis at the 32-cell stage must be made. Events that occur from the earliest cleavage stages make it progressively harder to detect developmental features directly inherited from the zygote because later cell interactions and other distortions mask inherited organization. After the 32-cell stage, these events include: relatively rapid increase of cells in the cavity shell compared to cells in and surrounding the ICM (Supplementary Material 2), migration of outer cells from around the ICM to the cavity shell, preferentially in one direction (Copp, 1979, Cruz and Pedersen, 1985, Gardner, 1998, Gardner and Davies, 2002), and extensive cell death in the ICM and possibly the trophectoderm (Copp, 1978, Handyside and Hunter, 1986).

These processes of local cell death, cell migration, cell multiplication, and cell interactions become prominent features of later mouse development and it is therefore likely that the 50% 32-cell stage blastocyst provides the best and last opportunity for detecting the transmission of zygote order. It may also be the best opportunity for observing such transmission because the asymmetry introduced by the blastocyst cavity is the most prominent, persistent, noticeable, and universal of all those asymmetries that have been reported.

Status of thought experiments

The value of theoretical models in developmental biology must be examined. Simple thought experiments or models may be redundant as more accurate, detailed and extensive biological information about the transition from one developmental stage to another becomes available. One set of models simulates compaction and cavity formation as driven by the minimization of cellular surface energy and other surface forces (Goel *et al.*, 1986, Lewis *et al.*, 1988, Honda *et al.*, 2008). Our approach has been to provide theoretical tools for modelling blastocyst morphogenesis irrespective of the physical and biochemical forces that are involved. The importance of the present approach is at an intermediate stage of understanding when models can constrain and clarify interpretation: thus the pattern of cell contacts on some

metazoan epithelia is now seen as the consequence of progressive developmental mechanisms rather than a packing problem at a single stage (Gibson *et al*., 2006, Farhadifar *et al*., 2007). For mouse such a "real time" emergent analysis of the morula to blastocyst transition is not yet available because the obscured form of the cell membranes in living material makes it impossible to follow the exact time-dependent dynamics of this major regulator of mitotic direction and cell polarization. Thought experiments help the study of blastocyst morphogenesis while knowledge is scarce.

Types of analysis

It is clear that these models are caricatures and represent development of a putative blastocyst. Combinatorial analysis focuses on the biological unit of the cell but as used here it does not represent the variation in cell volumes between shell and ICM and it lacks precision about spatial distribution. The utility of the method is that it can accommodate irregular shapes. Such shape analysis leads to the conclusion that there is a decreasing frequency of monoclonal cavity shells as the contribution of the same clone to Layer 1 increases (see, Thought Experiment 1). Combinatorial analysis is useful when new data sets emerge: for instance if the mean number of cells in the cavity shell is 9 rather than 8, as illustrated in a recently published study, then the methods can be readily adjusted to take this into account (Bischoff *et al.,* 2008)(Zernicka-Goetz, personal communication). The influence of a variety of cell distributions on clone characteristics is explored in Supplementary Material 4. Combinatorial analysis is also suitable for assessing the impact of clone shape and coherence. We have assumed clone coherence in most of our analysis. This assumption is supported by some data sets but not others because the frequency of clone coherence varies widely, from c.94% to 48% (Piotrowska *et al*., 2001, Motosugi *et al*., 2005, Kurotaki *et al*., 2007).

In contrast to combinatorial analysis, the accuracy of spatial location in a geometric analysis of model blastocyst form is more realistic in the sense that the models of the blastocyst and its morula precursor have been designed to take into account the smaller volume of ICM cells compared to those of the shell (Aiken *et al.,* 2004). However, converting the geometry back into cell distributions involves rounding up and assumptions about the movements of morphogenesis and cell packing. This reconversion has been avoided. The geometric analysis depends on two biological assumptions. First that ICM and shell cell volumes remain constant from the morula to the blastocyst stage and second that each 1/2 clone contributes half its volume to each of these two regions. The first assumption was built into the model from the biological data reviewed in Supplementary Material 1. The second assumption was built into the model for the same reason and is further discussed in Supplementary Material 3. There appears to be no data on the distribution of 1/2 cell clone volume between these two cell populations in either the morula or the blastocyst and these assumptions are adopted for their simplicity. In Supplementary Material 4 we explore the effect of allowing one clone to overgrow the other in the morula to blastocyst transition. Overgrowth is represented by alterations in volume in these calculations.

Biological variation

These three thought experiments with a model morula and blastocyst can be used to predict where chance would distribute cells and frontiers on the form of this ideal model blastocyst. The chance distributions of the favoured model of morphogenetic events provide the null hypotheses against which future observations could be tested to identify developmental routines in blastocyst morphogenesis (Table 2, Figures 2 & 6). It is important to emphasise that none of the presently available data sets have been collected with the aim of validating or falsifying the models that have been proposed in the present theoretical paper. Specifically, the detailed position of cells and cell volumes are not included in the published data sets and without this information it is difficult to justify any null hypotheses in this field. What our models suggest are ways for formulating appropriate null hypotheses and they should motivate clear empirical approaches in the studies of blastocyst morphogenesis.

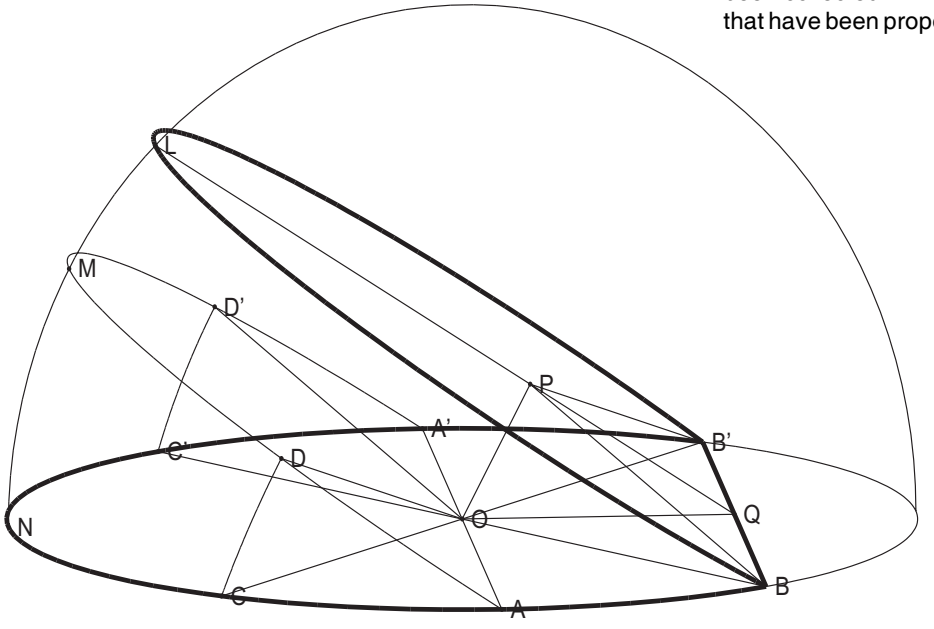

Fig. 7. Method for calculating the volumes on either side of a plane cutting through a hemisphere. *The method is derived in Materials and Methods.*

Interpreting developmental routines

If blastocyst architecture emerges from the morula without reference to preceding organization and in accordance with the models, then the conbead and 2PB and clone frontier will regularly and routinely tend to be over-represented in or on the middle slice that includes Layer 1 of the blastocyst. In addition, the angle between the interclonal frontier and the equator will tend to be 43.4° if the movements of morphogenesis are in accord with the models (Fig. 4). The explanation of these distributions is that they

are directed by the allocation of 8 or so cells in the morula to the formation of a shell around a hemispherical cavity and by the deformation of the inner cells from a sphere to a hemisphere. The conclusion must be that regular patterns of development at the frequencies found in this theoretical study do not by themselves establish that any plan of future development is specified before the morula stage. The results of the three thought experiments make it necessary to choose biologically plausible null hypotheses to be the touchstone of the statistical test of randomness in mouse preimplantation development and other areas of biology (Siegel, 1988).

The results of these thought experiments also highlight the problems of interpreting spatial distributions in development. If the 2PB and the frontier show no tendency to line up with Layer 1 and the equator, then such observations suggest that chance depositions have been lost: several studies have either not recorded these alignments or challenged the evidence for their existence, for example (Chroscicka *et al.*, 2004, Alarcon and Marikawa, 2005, Motosugi *et al.*, 2005, Waksmundzka *et al.*, 2006, Kurotaki *et al.*, 2007). One possibility is that significant developmental organization has been transmitted by the zygote to offset the distributions expected from our models, while another possibility is that although a chance disposition is established the biological variation in small data sets makes it difficult to find. A final explanation of the failure to find the distributions we propose is that the form or cell number of the studied blastocysts was widely different from those of the model. On the other hand several studies have described these alignments, for example (Gardner, 1997, Ciemerych *et al.*, 2000, Gardner, 2001, Piotrowska *et al.*, 2001, Piotrowska-Nitsche and Zernicka-Goetz, 2005, Gardner and Davies, 2006, Gardner, 2007, Bischoff *et al.*, 2008). To support the interpretation that these distributions are due to the transmission of significant developmental organization from the zygote it must be shown that the 2PB and the frontier occupy regions of the blastocyst at a higher frequency than that expected by the models in the present paper. These authors might also identify alternative models to those that we propose here.

The present thought experiments show that it is early days in the search for organization in the mouse zygote that influences the position of the blastocyst cavity let alone the anatomy of the fetus. It is also the case that while an alternative full epigenetic explanation of blastocyst formation is both experimentally demanding and slow to emerge and thought experiments will for some time be essential for guiding interpretation.

Materials and Methods

Morula and blastocyst models

We assume that model morula gives rise to a model 32-cell blastocyst (Fig. 1) with negligible change in cell number or cell volume. These models were devised by reviewing the experimental literature that is summarized in Supplementary Material 1, 2, and 3.

Combinatorial analysis

To understand the origins of cells in the 32-cell blastocyst stage two approaches are taken: the first is a combinatorial analysis and the second is a geometric analysis of cell organization. In each case the aim is to obtain probable distributions of cells in the putative (32-cell) blastocyst.

The 'patterning' of the cells in the blastocyst can be thought of as a constrained combinatorial problem. Combinatorial problems are those associated with discrete or finite objects. A simple combinatorial problem is to ask how many different ways are there of ordering n objects. As cell organization is a combinatorial problem, the rule of sum can be used (it broadly states that if there are 'b' ways of doing one process and 'w' ways of doing another process then the two processes are mutually exclusive such that there are the w+b ways of choosing one of the processes). Using this approach, the calculation is the number of ways that two 16-cell lineages can be arranged to form a blastocyst. The combinatorial problem has been constrained by the rule that cell lineages must be coherent (cells from the same lineage must touch). This method is an extremely simple and powerful approach for understanding the probable distributions of the 2 cell-lineage types.

In the present analysis, the distribution of cells in the mouse blastocyst was represented by three stacked linear arrays, and with the constraints described in the text, all 87 possible distributions of two coherent 16-cell clones were drawn out. The horizontal linear arrays were given identical polarity and the cells arranged to maximise intra-clonal cell contacts in each distribution. There were no other geometrical constraints within a linear array. In the vertical "main axis" of these arrays there is an explicit asymmetry: the top layer (representing the cavity cap) has fewer cells than the other layers and so shape and position are related to both the horizontal and vertical axes. The figures in Table 2 were arrived at by scoring the distributions in each class.

Geometric analysis

The numerical relationships of parts of well known geometric forms were taken from pre-university textbooks and a reference work (Zwillinger, 2003). The bulk of the analysis depends on calculating the volumes on either side of a frontier that runs through the blastocyst with these two volumes representing the progeny of each cell at the 2-cell stage (Figs. 1, 3 & 5). The form of the blastocyst is such that a single frontier plane that cuts total cellular volume in half must run at an off centre oblique angle to the equator (a β type angle) except when the plane runs through the main axis (a meridional plane) or parallel to the equator.

Frontier plane through a hemisphere

Slicing a hemispherical solid: The methods for calculating the volumes on either side of a frontier plane through a hemispherical shape is set out (Fig. 7). The basic volume calculation that underlies each of the volumes we need to calculate is the volume of an obliquely sliced hemisphere. In Fig. 7 we illustrate a hemisphere of unit radius with centre *O*, sliced by a plane that cuts the base of the hemisphere in the line *BB'*. The volume we wish to calculate is the volume of that part of the hemisphere that lies below this slicing plane. So it has two flat faces outlined in bold: the segment *BNB'* of the base circle, and the segment *BLB'* of the circle formed by the intersection of the slice plane with the hemisphere; and it has one curved face, the curved surface of the hemisphere lying between the arcs *BLB'* and *BNB'*. We shall let *p* denote the perpendicular distance *OP* of the slice plane from *O*, and α denote the angle $\angle OQP$ of the slice plane to the base. Then we shall show that the volume of this region is

$$V_1 = \frac{p^2 r\cos\alpha}{3\sin^2\alpha} + \left(p - \frac{p^3}{3}\right)\operatorname{atan2}(r, -p\cos\alpha) + \frac{2\operatorname{atan2}(r,\cos\alpha)}{3},$$

Equation (1)

where $r = \sqrt{\sin^2\alpha - p^2}$ and atan2(*y*,*x*)=arg(*x*+*iy*) is the usual two-argument inverse tangent function defined as the anticlockwise angle of rotation from the positive *x*-axis to the point (*x*,*y*). We can show this formula from the diagram. First, the tetrahedron with vertices *O*, *P*, *B*, *B'* has volume equal to the first term in (1). Then, consider the plane through *O* parallel to the slice plane, so it intersects our volume in the semicircle *AMA'*. Then consider the point *C* that is the reflection of *B* in the diameter *AA'*, and let *D* be the point of the arc *AM* that is closest to *C*, so *CD* is an

arc of great circle, perpendicular to *AM* at *D*. If we now imagine slicing our volume along the sectors *OCD* and *OAD* then the piece with vertices *OACD* can be rotated through 180º about *OA* so that *C* coincides with *B*, and the face *OCD* then becomes coplanar with *OPB*. If we do the same to the corresponding points *B', C', D'* in the other half of the diagram, we obtain a region that is the part of the unit sphere contained between the slice plane and the parallel plane but with the sector of angle $\angle BPB'$ removed. This therefore has volume

$$(p-\frac{1}{3}p^3)(\angle LPB)$$

which is the second term in (1). Finally we have to calculate the remaining volume, which is the part of the hemisphere bounded by the sectors *OCD*, *ODD', OD'C', OC'C*. This will be one third of the area of the unit sphere bounded by *CDD'C'*. But since each of those arcs is an arc of a great circle, the area can be found by spherical trigonometry and this gives the third term in (1).

We now show how this case can be used to calculate all the volumes we need in order to find a sphere *S* that bisects the volumes of the ICM and of the shell. The volume of a hemispherical shell contained within some other sphere *S* is the difference between the volumes of the outer and inner hemispheres contained within *S*, so it is enough to be able to calculate the volume of a hemisphere contained within some other sphere *S*. However, the intersection of *S* with the hemisphere is a circle, and by splitting up the volume we want into the volumes either side of the plane of that circle we decompose what we want into one obliquely sliced hemisphere and a region of *S* bounded by two planes. If the line where those two planes meet is called λ and we consider the diametral plane of *S* containing λ, then we see that the volume of *S* that we want can itself be written as the sum or difference of two obliquely sliced hemispheres.

Now, if the centre of our bisecting sphere *S* is on a line at angle α to the main axis at distance ξ from the centre of the cell, and its radius is *r*, then the two equations we wish to solve are

(vol. ICM contained within *S*) = 1/2 (total vol. ICM) (2)
(vol. shell contained within *S*) = 1/2 (total vol. shell) (3)

The results above enable us to program explicit calculations for the left-hand-sides of these two equations as functions of ξ and *r*. Then standard numerical methods enable the equations to be solved for (ξ,*r*), and this leads to the results given. Although these are nonlinear equations, we did not find multiple solutions for any β.

ICM deformation rule: As explained in the text, models with a curved frontier between the two volumes were also required. A single plane frontier that runs through the ball of inner cells of the morula curves and flattens as these cells are deformed into the blastocyst's ICM hemisphere. The deformation of the inner cells of the morula to the hemispherical ICM of the blastocyst involves the transforming a sphere of radius *r* into a hemisphere of equal volume, so that the radius of the hemisphere is *rb* where $b=\sqrt[3]{2}\approx 1.26$. One way to illustrate what happens during a deformation like this is to think of it occurring in two stages, the first squashing the sphere of radius *r* to an oblate spheroid with semi-major axes *rb* and semi-minor axis r/b^2. This preserves its volume and gives the right axial and equatorial dimensions of the hemisphere. It converts a given diametral plane in the sphere (a β type angle) to a plane across the spheroid at a smaller angle to the equator as illustrated by the cross sections in Fig. 4.

The second stage is to deform the oblate spheroid by moving material parallel to the main axis to produce the hemisphere. This stage bends that plane, but its average angle to the equator is not affected: some parts become steeper and others shallower (Fig. 4C). So the change in angle is entirely due to the first stage, and since this shrinks by a factor b^2 along the axis and expands by *b* perpendicular to the axis, the combined effect is to reduce the aspect ratio by a factor of $b^3=2$, and so $\tan\beta^{ICM}=1/2\tan\beta^{morula}$. Note that we are not assuming that the inner cells of the morula actually deform like this — it will be much more complex, and will depend on the physical stresses acting and the viscoelastic properties of the cells during the process. All we are intending to gather from these diagrams is that (a) in any axisymmetric deformation of the sphere into a hemisphere there is a flattening out; and (b) a reasonable quantitative measure of the flattening is to take $\tan\beta^{ICM}=1/2\tan\beta^{morula}$. We are also not assuming any particular way that this illustrative deformation of the inner cells might extend continuously to the outer.

The model with a curved frontier that halves ICM volume and halves trophectoderm (shell) volume is studied by treating the frontier between the halves as a spherical cap of a sphere with a diameter that is substantially larger than the blastocyst and with a centre external to the blastocyst (Fig. 3C). A tangent to this spherical cap is used to describe frontier orientation. Note that in order to accommodate the volume constraint both the cap radius and its distance from the midpoint of the main axis change as it rotates around the main axis and so the shape of the inter-clonal frontier changes (Fig. 5).

The probability that a frontier will be oriented at a particular angle to the equator (a β type angle) depends on the distribution of surface area of a sphere rather than that of a circle. We are assuming that the future cavity forms at a certain radius (say *r*) out from the centre of the morula (Fig. 3A), but that its position is uniformly distributed over the surface area of the morula sphere of radius *r*. The frontier intersects the surface of that sphere in a great circle because that is the assumption of the model. Half the surface area of that sphere is within *r*/2 of the frontier plane and so the angle α between the cavity and the frontier has a median of 30 degrees (i.e. there is probability 1/2 that $\alpha<30^o$and 1/2 that $\alpha>30^o$). $\beta=90-\alpha$ and so the median angle to the equator is 60º. In fact by generalizing the argument showing that the median is 60º, it follows that the probability that β^{morula} lies between β_1 and β_2 is $\cos(\beta_1)-\cos(\beta_2)$, where $0<=\beta_1<=\beta_2<=90^o$. Using this it can be shown by integration that the mean value of β^{morula} is 57.3º(1 radian, i.e. $180/\pi^o$). Furthermore, when β^{morula} has this distribution, and β^{ICM} is modelled by the earlier deformation rule, the resulting mean value of β^{ICM} is 43.4º.

NOTE ADDED IN PROOF:
The following references mark the steady progress of understanding blastocyst formation as a series of developmental events.

JEDRUSIK, A., PARFITT, D.-E., GUO, G., SKAMAGKI, M., GRABAREK, J.B., JOHNSON, M.H., ROBSON, P. and ZERNICKA-GOETZ, M. (2008). Role of cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. *Genes Dev.* 22: 2692-2706.

SUWINSKA, A., CZOLOWSKA, R., OZDZENSKI, W. and TARKOWSKI, A.K. (2008). Blastomeres of the mouse embryo lose totipotency after the fifth cleavage division: Expression of *cdx2* and *oct4* and developmental potential of inner and outer blastomeres of the 16- and 32-cell embryo. *Dev. Biol.* 322: 133-144.

Acknowledgments

C.F.G. raised the embryological problems and compiled the supplementary materials. R.J.S. and D.J.A. undertook the geometrical analysis. M.B.B. undertook the combinatorial analysis. All authors contributed to writing the manuscript. We thank Jens-Erik Dietrich, Toshihiko Fujimori, Richard Gardner, Alan Grafen, Takashi Hiiragi, Alex Kacelnik, John Ockendon, David-Emlyn Parfitt, Davor Solter, and Magdelana Zernicka-Goetz for access to unpublished data and some extensive discussions: M.B.B. is a Royal Society Research Fellow.

References

AIKEN, C.E.M., SWOBODA, P.P.L., SKEPPER, J.N. and JOHNSON, M.H. (2004). The direct measurement of embryogenic volume and nucleo-cytoplasmic ratio

during mouse pre-implantation development. *Reproduction* 128: 527-535.

ALARCON, V.B. and MARIKAWA, Y. (2005). Unbiased contribution of the first two blastomeres to mouse blastocyst development. *Mol. Reprod. Dev.* 72: 354-361.

BISCHOFF, M., PARFITT, D.-E. and ZERNICKA-GOETZ, M. (2008). Formation of the embryonic-abembryonic axis of the mouse blastocyst: Relationships between orientation of the early cleavage divisions and pattern of symmetric/asymmetric divisions. *Development* 135: 953-962.

CHAZAUD, C., YAMANAKA, Y., PAWSON, T. and ROSSANT, J. (2006). Early lineage segregaton between epiblast and primitive endoderm in mouse blastocysts through the grb2-mapk pathway. *Dev Cell* 10: 615-624.

CHROSCICKA, A., KOMOROWSKI, S. and MALESZEWSKI, M. (2004). Both blastomeres of the mouse 2-cell embryo contribute to the embryonic portion of the blastocyst. *Mol. Reprod. Dev.* 68: 308-312.

CIEMERYCH, M.A., MESNARD, D. and ZERNICKA-GOETZ, M. (2000). Animal and vegetal poles of the mouse egg predict the polarity of the embryonic axis, yet are not essential for development. *Development* 127: 3467-3474.

COPP, A.J. (1978). Interaction between inner cell mass and trophectoderm of the mouse blastocyst i. A study of cellular proliferation. *J. Embryol. Exp. Morphol.* 48: 109-125.

COPP, A.J. (1979). Interaction between inner cell mass and trophectoderm. Ii.Fate of polar trophectoderm. *J. Embryol. Exp. Morphol.* 51: 109-120.

CRUZ, Y.P. and PEDERSEN, R.A. (1985). Cell fate in the polar tropectoderm of mouse blastocysts as studied by microinjection of cell lineage tracers. *Dev. Biol.* 112: 73-83.

DALE, L. and SLACK, J.M.W. (1987). Fate map of the 32-cell stage of *xenopus laevis*. *Development* 99: 527-551.

DIETRICH, J.-E. and HIIRAGI, T. (2007). Stochastic patterning in the mouse pre-implantation embryo. *Development* 134: 4219-4231.

EYAL-GILADI, H. (1997). Establishment of the axis in chordates: Facts and speculation. *Development* 124: 2285-2296.

FARHADIFAR, R., ROPER, J.-C., AIGOUY, B., EATON, S. and JULICHER, F. (2007). The influence of cell mechanics. Cell-cell interactions and proliferation on epithelial packing. *Curr. Biol.* 17: 2095-2104.

GARDNER, R.L. (1997). The early blastocyst is bilaterally symmetrical and its axis of symmetry is aligned with the animal-vegetal axis of the zygote in the mouse. *Development* 124: 283-301.

GARDNER, R.L. (1998). Clonal analysis of growth of polar trophectoderm in the mouse. *Hum. Reprod.* 11: 1979-1984.

GARDNER, R.L. (2001). Specification in embryonic axes begins before cleavage in normal mouse development. *Development* 128: 839-847.

GARDNER, R.L. (2006). Weaknesses in the case against prepatterning in the mouse. *Reproductive BioMedicine Online* 12: 144-149.

GARDNER, R.L. (2007). The axis of polarity of the mouse blastocyst is specified before blastulation and independently of the zona pellucida. *Hum. Reprod.* 22: 798-806.

GARDNER, R.L. and DAVIES, T.J. (2002). Trophectoderm growth and the bilateral symmetry of the blastocyst in the mouse. *Hum. Reprod.* 17: 1839-1845.

GARDNER, R.L. and DAVIES, T.J. (2006). An investigation of the origin and significance of bilateral symmetry of the pronuclear zygote in the mouse. *Hum. Reprod.* 21: 492-502.

GIBSON, M.C., PATEL, A.B., NAGPAL, R. and PERRIMON, N. (2006). The emergence of geometric order in proliferating metazoan epithelia. *Nature* 442: 1038-1041.

GOEL, N.S., DOGGENWEILER, C.F. and THOMPSON, R.L. (1986). Simulation of cellular compaction and internalization in mammalian embryo as driven by minimization of surface free energy. *Bull. Math. Biol.* 48: 167-187.

GRAY, D., PLUSA, B., PIOTROWSKA, K., NA, J., TOM, B., GLOVER, D.M. and ZERNICKA-GOETZ, M. (2004). First cleavage of the mouse embryo responds to change in egg shape at fertilization. *Curr. Biol.* 14: 397-405.

HANDYSIDE, A.H. and HUNTER, S. (1986). Cell division and death in the mouse blastocyst before implantation. *Roux's Archive for Developmental Biology* 195: 519-526.

HIIRAGI, T., ALARCON, V.B., FUJIMORI, T., LOUVET-VALLEE, S., MALESZEWSKI, M., MARIKAWA, Y., MARO, B. and SOLTER, D. (2006a). Where do we stand now?-mouse early embryo patterning meeting in freiburg, germany (2005). *Int. J. Dev. Biol.* 50: 581-586.

HIIRAGI, T., LOUVET-VALLEE, S., SOLTER, D. and MARO, B. (2006b). Embryology: Does prepatterning occur in the mouse egg? *Nature* 442: E3-E4.

HIIRAGI, T. and SOLTER, D. (2006). Fatal flaws in the case for prepatterning of the mouse egg. *Reproductive BioMedicine Online* 11: 150-152.

HONDA, H., MOTOSUGI, N., NAGAI, T., TANEMURA, M. and HIIRAGI, T. (2008). Computer simulation of emerging asymmetry in the mouse blastocyst. *Development* 135: 1407-1414.

KUROTAKI, Y., HATTA, K., NAKAO, K., NABESHIMA, Y. and FUJIMORI, T. (2007). Blastocyst axis is specified independently of early cell lineage but aligns with the zp shape. *Science* 316: 719-723.

LEWIS, H.W., GOEL, N.S. and THOMPSON, R.L. (1988). Simulation of cellular compaction and internalization in mammalian embryo development - ii models for spherical embryos. *Bull. Math. Biol.* 50: 121-142.

MOTOSUGI, N., BAUER, C., POLANSKI, Z., SOLTER, D. and HIIRAGI, T. (2005). Polarity of the mouse embryo is established at blastocyst and is not prepatterned. *Genes Dev.* 19: 1081-1092.

MOTOSUGI, N., DIETRICH, J.-E., POLANSKI, Z., SOLTER, D. and HIIRAGI, T. (2006). Space asymmetry directs preferential sperm entry in the absence of polarity in the mouse oocyte. *PLoS Biology* 4.

O'FARRELL, P.H., STUMPF, J. and SU, T.T. (2004). Embryonic cleavage cycles: How is a mouse like a fly? *Curr. Biol.* 14: R35-R45.

PIOTROWSKA-NITSCHE, K., PEREA-GOMEZ, A., HARAGUCHI, S. and ZERNICKA-GOETZ, M. (2005). Four-cell stage mouse blastomeres have different developmental properties. *Development* 132: 479-90.

PIOTROWSKA-NITSCHE, K. and ZERNICKA-GOETZ, M. (2005). Spatial arrangement of individual 4-cell stage blastomeres and the order in which they are generated correlate with blastocyst pattern in the mouse embryo. *Mech. Dev.* 122: 487-500.

PIOTROWSKA, K., WIANNY, F., PEDERSEN, R.A. and ZERNICKA-GOETZ, M. (2001). Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. *Development* 128: 3739-3748.

PLUSA, B., HADJANTONAKIS, A.K., GRAY, D., PIOTROWSKA-NITSCHE, K., JEDRUSIK, A., PAPAIOANNOU, V.E., GLOVER, D.M. and ZERNICKA-GOETZ, M. (2005). The first cleavage of the mouse zygote predicts the blastocyst axis. *Nature* 434: 391-5.

PLUSA, B., HADJANTONAKIS, A.K., GRAY, D., PIOTROWSKA-NITSCHE, K., JEDRUSIK, A., PAPAIOANNOU, V.E., GLOVER, D.M. and ZERNICKA-GOETZ, M. (2006). Embryology: Does prepatterning occur in the mouse egg? Reply. *Nature* 442: E4-E4.

SIEGEL, A.F. (1988). *Statistics and data analysis*. John Wiley & Sons, New York.

TORRES-PADILLA, M.E., PARFITT, D.-E., KOUZARIDES, T. and ZERNICKA-GOETZ, M. (2007). Histone arginine methylation regulates pluripotency in the early mouse embryo. *Nature* 445: 214-219.

WAKMUNDZKA, M., WISNIEWSKA, A. and MALESZEWSKI, M. (2006). Allocation of cells in the mouse blastocyst is not determined by the order of cleavage of the first two blastomeres. *Biol. Reprod.* 75: 582-587.

WAKSMUNDZKA, M., WISINIEWSKA, A. and MALESZEWSKI, M. (2006). Allocation of cells in mouse blastocysts is not determined by the order of cleavage of the first two blastomeres. *Biol. Reprod.* 75: 582-587.

ZERNICKA-GOETZ, M. (2006). The first cell-fate decisions in the mouse embryo: Destiny is a matter of both chance and choice. *Curr. Opin. Genet. Dev.* 16: 406-12.

ZWILLINGER, D. (2003). Crc standard mathematical tables and formulae, (ed. Boca Raton: CRC Press LLC.