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# SUPPLEMENTARY MATERIAL

**corresponding to:**

# Theoretical exploration of blastocyst morphogenesis

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**SUPPLEMENTARY MATERIAL**

Supplementary Material 1 Data Sets

Data Set: Blastocyst Cell Number Range 28-34, taken from the literature

63 blastocysts with cell numbers ranging from 28-34 were selected from published work and influenced the form of the invented blastocyst (Piotrowska *et al.*, 2001, Piotrowska-Nitsche *et al.*, 2005, Piotrowska-Nitsche and Zernicka-Goetz, 2005). These papers contained much larger data sets and a greater range of cell numbers. In contrast to the analysis of the original authors, this Table combines data from "reversed" distributions with those from regular distributions, combines data from all embryos whatever the apparent geometry or order of clone cell division, combines data irrespective of how many cells each 1/2 stage blastomere contributed to the blastocysts and irrespective of the position of the sperm entry point. Nine embryos in this number range were excluded because the clones appeared to have intermingled (no angle of tilt recorded by the authors).

Supplement 1, Table S1.1

| Cell number | Mean | Median | SD | Range |
|---|---|---|---|---|
| Whole blastocyst[1,2] | 31.2 | 31.0 | 1.81 | 28-34 |
| Cavity shell | 9.1 | 9.0 | 1.50 | 6-12 |
| Layer 1/ Boundary zone | 9.0 | 9.0 | 1.28 | 7-13 |
| Layer 2 | 13.1 | 13.0 | 1.33 | 10-16 |
| Tilt | 37.6 | 32.0 | 26.0 | 3-90 |

In 12 of 55 blastocysts in this data set (21.8%), the cavity shell was formed from one 1/2 stage clone: it had a monoclonal origin.

[1] The distribution of total cell numbers was 5 at 28 cells, 8 at 29, 9 at 30, 12 at 31, 12 at 32, 9 at 33, 8 at 34.

[2] The clones often contributed different numbers of cells to the blastocyst: 14 blastocysts no difference, 18 cases 1 cell difference, 13 cases 2 cell difference, 6 cases 3 cell difference, 6 cases 4 cell difference, 4 cases 5 cell difference, 1 case 7 cell difference, 1 case 8 cell difference.

Further features of this data set are set out in Table 2 and in the body of the text.

Data Sets: Distribution of Second Polar Body (2PB) on the blastocyst

There are some reports that the 2PB tends to be located on the surface of the median or middle third of the blastocyst (Gardner, 1997, Ciemerych *et al.*, 2000). This is a horizontal slice containing the surface and volume between 33 and 66% of the main axis of the blastocyst and in a 50% model blastocyst it is 26.6μm thick and corresponds to Layer 1 (13.3μm thick) and a slice taken from the cavity shell immediately above the equator (13.3μm thick). Slightly different scoring methods were used in each study and the extent of the cavity in the real blastocysts was judged by eye to be near 50%.

Gardner observed that intact 2PB were on the surface of this region in 64% (n=75) freshly isolated blastocysts and they were also here in 55% (n=63) blastocysts developing in culture (Gardner, 1997). This observation is supported by the finding that the frequency ranged from 43-53% (n=44) in another study, with the higher figure including cases where the 2PB overlapped the borders between the median region on both sides (Ciemerych et al., 2000).

This median or middle region is not quite the same as the midzone which was used to score the associations with the blastocyst equator in another paper (Gardner, 2001).

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

Supplementary Material 2 Derivation of Models

The model blastocyst is geometrically defined in Figure 1 and the text.
These following proportions of this invention were related to observations on real blastocysts in the literature as set out below.

1. The number of outside cells at the 32-cell stage is taken to be 21 with 11 inside constituting the ICM (this Supplementary Material, Table S2.2).

2. The number of cells in the cavity shell is taken to be 8. The cavity shell cell number is about 7 based on regression analysis of all the data in one paper (Piotrowska et al., 2001) and about 9 for embryos in the cell number range 28-34 inclusive (Supplementary Material 1, Table S1.1) (Piotrowska *et al.*, 2001, Piotrowska-Nitsche and Zernicka-Goetz, 2005).

3. The volume of single ICM cells is taken to be c. 72 % of the outside cells. The relative volumes of the shell (trophectoderm) cells and the ICM cells were calculated from the Table 1 of Aiken and colleagues (Aiken et al., 2004). As the authors point out, not all the cells in each conceptus were delineated clearly enough to obtain an accurate volume and "Data from these cells are not included and so a degree of non-random selection can not be excluded." The authors also emphasise that the regression analysis in their Table 3 may give a more reliable guide to the relative cell numbers because it is based on more examples (M.H. Johnson, personal communication). Their Table 1 data is easier to extract from the publication: at the 28-cell stage the individual ICM cell volumes were 69% of the shell cell volumes and at the 32-cell stage the ICM cell volumes were 60% of the shell cell volumes. A high figure has been chosen for the model because this reduced the rounding up required to fit cells and volumes in different regions.

4. Supplementary Material 2, Table S2.1.
The volumes of cellular material in different regions of the model blastocyst was obtained by progressively adjusting cell volumes (above) and the cell distributions in the data set (Supplementary Material 1) to arrive at a plausible form.

| Region | Volume $\mu m^3$ | % total volume | No. of cells | Cell volume $\mu m^3$ | Bounding radii μm |
|---|---|---|---|---|---|
| Cavity | 82,324 | - | - | - | 34 |
| Cavity Shell | 51,723 | 27.8 | 8 | 6,466 | 34 & 40 |
| ICM | 51,084 | 27.5 | 11 | 4,644 | 29 |
| ICM Shell | 82,966 | 44.7 | 13 | 6,382 | 29 & 40 |
| Total solid hemisphere | 134,050 | 72.2 | 24 | - | 40 |
| Total shell cells (trophectoderm) | 134,692 | 72.0 | 21 | - | NA |

| | | | | | |
|---|---|---|---|---|---|
| Total cells | 185,776 | 100 | 32 | | 40 |

Note: Rounding up has been used in calculating the cell distributions.

The cell distribution in the invented blastocyst is also shown in text Table 1 and it bears some relationship to published data. The values in the literature cover a wide range: for instance in the selected data set the mean number of cells in the Boundary Zone is 9 (Supplementary Material 1, Table S1.1), while in the group of early blastocysts with a greater range of total cell numbers a mean of 11.4 cells (range 9-13) was observed in this region (Piotrowska et al., 2001). A similar region in the model (Layer 1) contains 12 cells made up of 5 outer and 7 ICM cells (text Table 1). Note that when the model is sliced perpendicular to the main axis into 4 horizontal parts then the segment immediately below the ICM/cavity interface contains 17 cells, considerably more than those reported (Piotrowska et al., 2001). This is because the slice is 20 μm deep.

Working backwards in development, the spherical morula is assumed to contain the same cellular volume as the blastocyst (discussed Supplementary Material 3). In the absence of a cavity the total volume of 185,776 $\mu m^3$ can be contained in a solid sphere of radius c. 35.43 μm and within that sphere the inner cells maintain the volume of the future ICM (51,084 $\mu m^3$) in a 23 μm radius sphere. This structure is the invented morula.

**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.

**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. *Mechanisms of Development* **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.

Supplementary Material 2, Table S2.2 Inside cells at different stages

| Conceptus Mean Cell Number in these Ranges | | | | | | | Total cell no. Mean (range), n = sample size | Reference |
|---|---|---|---|---|---|---|---|---|
| 16 cells | 17-27 | 28-32 cells | 33-57 cells | 58-64 cells | 64-110 cells | 111 + cells | | |
| 0.17 | | | | | | | 15 ± 2 | (Dietrich and Hiiragi, 2007) |
| 1.70 * (1-2) | | | | | | | 16 ± 0 n = 14 | (Graham and Lehtonen, 1979) |
| 5.0 (3-7) | | | | | | | 16 ± 0 n = 20 | (Kimber et al., 1982) |
| 6.00 | | | | | | | 16 ± 0 n = 27 | (Reeve, 1982) |
| 3.00 (3-4) | | | | | | | 16 ± 0 1/8, n = 134 | (Pedersen et al., 1986) |
| 5.22 (2-7) | | | | | | | 16 ± 0 n = 65 | (Fleming, 1987) |
| | 5.50* (2-10) | | | | | | ? (17-32) n = 20 | (Barlow et al., 1972) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 8.56 ± 0.89 | | | | | | 22.50 ± 0.89 n = 59 | (Surani and Barton, 1984) |
| | | 6.79* | | | | | 30.83 (25-37) n = 24 | Graham, quoted in (Handyside, 1978) |
| | | 12.93 ± 2.79 | | | | | 30.24 ± 4.37 n = 25 | (Handyside, 1978) |
| | | 13.9 ± 3.78 | | | | | 28.76 ± 5.12 n = 17 | (Handyside, 1978) |
| | | 8.0 ± 1.6 | | | | | 29.3 ± 3.9 n = 25 | (Chisholm et al., 1985) |
| | | 9.1 ± 2.7 | | | | | 30.5 ± 6.2 n = 32 | (Chisholm et al., 1985) |
| | | 10-12 | | | | | 32 | (Pedersen et al., 1986) |
| | | 12.3 ± 2.6 | | | | | 31.1 ± 3.6 n = 45 | (Handyside and Hunter, 1986) |
| | | 11.90 | | | | | 29.8 (26-31) n = 42 | (Fleming, 1987) |
| | | 12.40 | | | | | 32 (32) n = 65 | (Fleming, 1987) |
| | | 11.90 (6-17) | | | | | 29.43 (23-37) n = 21 | (Hardy and Handyside, 1993) |
| | | | 10.86* (9-13) | | | | ? (33-64) n = 7 | (Barlow et al., 1972) |
| | | | 9 ± 2 | | | | 34.4 ± 4 n = 17 | (Dietrich and Hiiragi, 2007) |
| | | | 10.8* ± 2.4 | | | | 34.7 ± 6.4 n = 10 | (Chisholm et al., 1985) |
| | | | 17.56 ± 5.63 | | | | 36.92 ± 8.33 n = 12 | (Handyside, 1978) |
| | | | 13.60 | | | | 38.60 (33-53) n = 62 | (Fleming, 1987) |
| | | | 13.0 ± 3.8 | | | | 43.1 ± 7.3 n =19 | (Chisholm et al., 1985) |
| | | | 14.3 ± 5.5 | | | | 50.8 ± 8.8 n = 32 | (Chisholm et al., 1985) |
| | | | 13.60 | | | | 38.60 (33-53) n = 62 | (Fleming, 1987) |
| | | | 11.2* ± 0.7 | | | | 54.5 ± 1.7 n = 43 | (Tam, 1988) |
| | | | | 15.2* ± 1.3 | | | 57.7 n = 12 | (Rands, 1985) |
| | | | | | 24.04* (12-37) | | ? (65-128) n = 27 | (Barlow et al., 1972) |
| | | | | | 24.00 ± 4.36 | | 82.50 ± 7.78 n = 2 | (Handyside, 1978) |
| | | | | | | 51.00* (44-59) | ? (129-256) n = 3 | (Barlow et al., 1972) |
| | | | | | | 36.20 ± 10.33 | 141.25 ± 11.30 n = 4 | (Handyside, 1978) |
| | | | | | | 45.00 | Est. (130-139) n = 5 | (Copp, 1978) |

Supplementary Material 2, Table S2.2 Legend

Table S2.2 contains information gleaned from cultured embryos and those that have been analyzed soon after removal from the mother. Culture does not seem to be a major source of variation between studies. Most of the variation of inside cell numbers at a particular stage is probably due to technique and scoring method and the variation that is explained by these factors can be found both within and between studies. Serial sectioning of embryos appears to increase the total number of cells that are found in the embryo (Chisholm et al., 1985) but underestimate the number of inside cells up to c. 64 cell stage (see studies marked with an asterisk).

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.
BARLOW, P., OWEN, D.A.J. and GRAHAM, C.F. (1972). DNA synthesis in the preimplantation mouse embryo. *J. Embryol. Exp. Morphol.* 27: 431-445.
CHISHOLM, J.C., JOHNSON, M.H., WARREN, P.D., FLEMING, T.P. and PICKERING, S.J. (1985). Developmental variability within and between mouse expanding blastocysts and their ICM's. *J. Embryol. Exp. Morphol.* 86: 311-336.
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.
FLEMING, T.P. (1987). A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. *Dev. Biol.* 119: 520-531.
GRAHAM, C.F. and LEHTONEN, E. (1979). Formation and consequences of cell patterns in preimplantation mouse development. *J. Embryol. Exp. Morphol.* 49: 277-294.
HANDYSIDE, A.H. (1978). Time of commitment of inside cells isolated from preimplantation mouse embryos. *J. Embryol. Exp. Morphol.* 45: 37-53.
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.
HARDY, K. and HANDYSIDE, A.H. (1993). Cell allocation in twin half embryos bisected at the 8-cell stage: Implications for preimplantation diagnosis. *Mol. Reprod. Dev.* 36: 16-22.
KIMBER, S.J., SURANI, M.A. and BARTON, S.C. (1982). Interaction between blastomeres suggest that changes in cell surface adhesiveness during the formation of the inner cell mass and trophectoderm in the preimplantation mouse embryo. *J. Embryol. Exp. Morphol.* 70: 133-158.
PEDERSEN, R.A., WU, K. and BAŁAKIER, H. (1986). Origin of the inner cell mass in mouse embryos: Cell lineage analysis by microinjection. *Dev. Biol.* 117.
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.

RANDS, G.F. (1985). Cell allocation in half- and quadruple-sized preimplantation mouse embryos. *J. Exp. Zool.* 236: 67-70.
REEVE, W.J.D. (1982). The distribution of ingested horseradish peroxidase in the 16-cell mouse mebryo. *J. Embryol. Exp. Morphol.* 66: 191-207.
SURANI, M.A.H. and BARTON, S.C. (1984). Spatial distribution of blastomeres is dependent on cell division order and interactions in mouse morulae. *Dev. Biol.* 102: 335-343.
TAM, P.P.L. (1988). Postimplantation development of mitomycin-c treated mouse blastocysts. *Teratology* 37: 205-212.

Supplementary Material 3 Blastocyst Morphogenesis

Blastocyst from Morula, Cell Behaviour

There are no detailed descriptions of the cell and volume movements of the morula to blastocyst transition. The mouse blastocyst has a rapidly changing form and two nearly identical studies can obtain different results because there are differences in blastocyst shape between the two samples. However, the major transformations can be pieced together. The morula is a solid ball of cells with lipid drops and mitochondria concentrated near the surfaces of cell apposition (Ducibella and Anderson, 1975, Wiley and Eglitis, 1980). Vesicles about 1-2 μm in diameter accumulate in these regions and intercellular clefts develop between the cells, as if the vesicles or their contents had been expelled between the cells (Calarco and Brown, 1969, Wiley and Eglitis, 1980, Wiley and Eglitis, 1981). Clefts lead into one or two larger cavities beneath the outside cell layer and a single cavity becomes dominant and for a period it is almost entirely lined by trophectoderm cells, as judged by TEM and SEM (Wiley and Eglitis, 1981, Fleming *et al.*, 1984).

Scoring under dissecting microscopes, embryologists say that the blastocyst stage begins when the first one or two 5-10μm diameter extracellular cavities can be easily seen under the dissecting microscope, close together at one side of the ball and separated from the exterior by a monolayer: the cell numbers of these "nascent" blastocysts can vary widely but they tend to be in the range 28-33 cells (Smith and McLaren, 1977, Handyside and Hunter, 1986, Dietrich and Hiiragi, 2007). The spatial relationship between the earliest spaces and the final major cavity of the later blastocyst is not known with precision and unfortunately there is little agreement about criteria for staging the subsequent morphogenesis of the blastocyst (Table S3.1 see below).

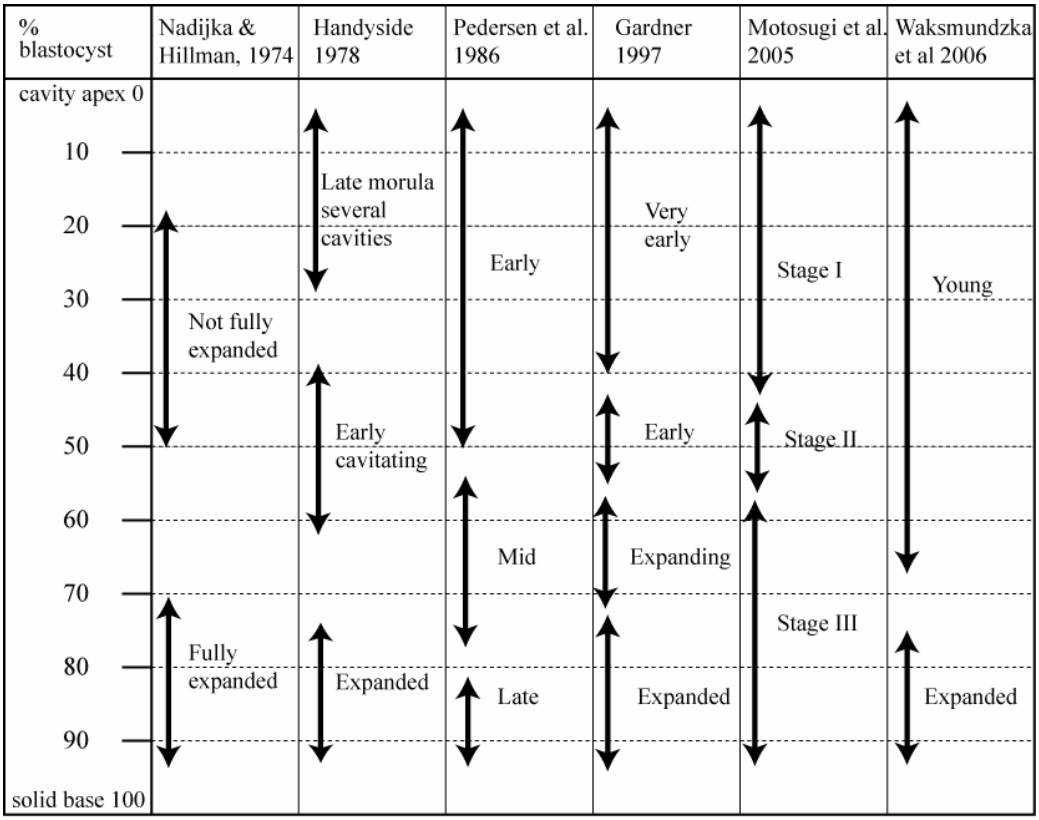
Over about 12 hours the cavity expands and forms a 50-60% blastocyst. It is worth noting that while we have adopted the rule that no volume of cellular material is lost during the transition this view is not universally accepted (Wiley and Eglitis, 1981). The rapid change in form is not tightly linked to cell number and is probably not dependent on cell division. Later changes in form are accompanied by but not dependent on differential growth, survival, and directed cell migration (Table S2.2). In this late period, there is a disproportionate increase in trophectoderm cell number when compared to those of the ICM with very little interchange between these two populations (Dyce *et al.*, 1987, Fleming, 1987). Starting with a slight preponderance of trophectoderm/shell cells in c.32-cell blastocysts, there is more than a three fold increase of these over the 30 h of development to the last recoverable implantation stages containing c.110-139 cells (Copp, 1978, Handyside & Hunter, 1986). In sharp contrast and for most of this interval, the increase of ICM cells is slower, doubling or nearly doing so and the explanation appears to be cell death. The ICM shows peaks of 8% or 10% dead cells as judged by nuclear morphology and the trophectoderm over the ICM also displays nuclear remnants (Copp, 1978, Handyside and Hunter, 1986). Additional data about the relative sizes of these two cell populations support these studies (Handyside, 1978, Reeve, 1982,

Chisholm *et al.*, 1985, Rands, 1985, Hardy and Handyside, 1993); (Table S2.2). The exception to this rule is a study from the author's laboratory where it was found that the proportion of shell cells progressively declined between the 17-32 cell stage and the implanting 129-256 cell blastocyst (Barlow et al., 1972). The discrepancy is unexplained. In summary, the consensus view is that shell cells increase in number much faster than those of the ICM.

The excess shell cells are not evenly distributed and they change the form of the blastocyst. As the blastocyst total cell numbers increase from 31 to 69 so there is a disproportionate increase in the number of cells in the cavity shell after short periods in culture: regression analysis of all data in a particular paper gives 7 cells in the cavity shell at a total cell number of 30 and 18 cells in this position when the total cell number is 60 (Piotrowska et al., 2001). As growth proceeds so does this excessive development of the cavity shell so that the between c. 32 cell stage and implantation (110-149 cells) the number of cells in the cavity shell has increased x7 while those over the ICM no more than x3 (Copp, 1978). There is currently no evidence that cell multiplication is faster in the cavity shell and there is evidence that the progeny of trophectoderm/shell cells over the ICM are an additional source of cells for this region (Copp, 1979, Cruz and Pedersen, 1985, Gardner, 1998, Gardner and Davies, 2002).

Describing Blastocyst Form

Table S3.1: Historical descriptions of blastocyst form, selected to illustrate the range of descriptions (Nadijcka and Hillman, 1974, Handyside, 1978, Pedersen *et al.*, 1986, Gardner, 1997, Motosugi *et al.*, 2005, Waksmundzka *et al.*, 2006).




Legend Supplementary Material 3, Table 3.1 Blastocyst Form
The left hand column records the extent of the blastocyst cavity expressed as percentage of the distance between the cap apex and the ICM/cavity interface (Fig 1). The meaning of the terms used to describe these stages has been estimated from illustrations and text of the published papers. The purpose of this Figure is to demonstrate that words should be replaced by quantitative measures. No attempt has been made to illustrate the following: "and sorted into expanded, semi-expanded, nascent, and pre-nascent blastocysts". The Table is a remarkable illustration of mammalian embryologist's ability to disagree about the simplest things.

BARLOW, P., OWEN, D.A.J. and GRAHAM, C.F. (1972). DNA synthesis in the preimplantation mouse embryo. *J. Embryol. Exp. Morphol.* 27: 431-445.
CALARCO, P.G. and BROWN, E.H. (1969). An ultrastructural and cytological study of preimplantation development of the mouse. *J. Exp. Zool.* 171: 253-284.
CHISHOLM, J.C., JOHNSON, M.H., WARREN, P.D., FLEMING, T.P. and PICKERING, S.J. (1985). Developmental variability within and between mouse expanding blastocysts and their icm's. *J. Embryol. Exp. Morphol.* 86: 311-336.
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.
DIETRICH, J.-E. and HIIRAGI, T. (2007). Stochastic patterning in the mouse pre-implantation embryo. *Development* 134: 4219-4231.
DUCIBELLA, T. and ANDERSON, E. (1975). Cell shape and membrane changes in the eight-cell mouse embryo: Prerequisite for morhogenesis of the blastocyst. *Dev. Biol.* 47: 45-58.
DYCE, J., GEORGE, M., GOODALL, H. and FLEMING, T.P. (1987). Do trophectoderm and inner cell mass cells in the mouse blastocyst maintain discrete lineages? *Development* 100: 685-698.
FLEMING, T.P. (1987). A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. *Dev. Biol.* 119: 520-531.
FLEMING, T.P., WARREN, P.D., CHISHOLM, J.C. and JOHNSON, M.H. (1984). Trophectodermal processes regulate the expression of totipotency within the inner cell mass of the mouse expanding blastocyst. *J. Embryol. Exp. Morphol.* 84: 63-90.
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. and DAVIES, T.J. (2002). Trophectoderm growth and bilateral symmetry of the blastocyst in the mouse. *Hum. Reprod.* 17: 1839-1845.
HANDYSIDE, A.H. (1978). Time of commitment of inside cells isolated from preimplantation mouse embryos. *J. Embryol. Exp. Morphol.* 45: 37-53.
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.
HARDY, K. and HANDYSIDE, A.H. (1993). Cell allocation in twin half embryos bisected at the 8-cell stage: Implications for preimplantation diagnosis. *Mol. Reprod. Dev.* 36: 16-22.
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.
NADIJCKA, M. and HILLMAN, N. (1974). Ultrastructural studies on mouse blastocyst substages. *J. Embryol. Exp. Morphol.* 32: 675-695.
PEDERSEN, R.A., WU, K. and BAŁAKIER, H. (1986). Origin of the inner cell mass in mouse embryos: Cell lineage analysis by microinjection. *Dev. Biol.* 117.
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.
RANDS, G.F. (1985). Cell allocation in half- and quadruple-sized preimplantation mouse embryos. *J. Exp. Zool.* 236: 67-70.
REEVE, W.J.D. (1982). The distribution of ingested horseradish peroxidase in the 16-cell mouse mebryo. *J. Embryol. Exp. Morphol.* 66: 191-207.
SMITH, R. and MCLAREN, A. (1977). Factors affecting time of formation of the mouse blastocoele. *J. Embryol. Exp. Morphol.* 41: 79-92.

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.
WILEY, L.M. and EGLITIS, M.A. (1980). Effect of colcemid on cavitation during mouse blastocoele formation. *Exp. Cell Res.* 127: 89-101.
WILEY, L.M. and EGLITIS, M.A. (1981). Cell surface and cytoskeletal elements: Cavitation of the mouse preimplantation embryo. *Dev. Biol.* 86: 493-501.

Supplementary Material 4 Model Variants

Combinatorial Analysis

In text Table 2 we compared a particular data set with the cell distribution of text Table 1. In Table S4.1 below we have maintained the total cell number of the blastocyst at 32 and included additional distributions of cells between the regions of the 3 tier model shown in text Fig 2. Some clone characteristics in the blastocyst change frequency in response to the changes in cell distributions. The conclusion is that the cell distributions in different regions of the blastocyst must be known in order to identify the appropriate combinatorial model.

Table S4.1
Comparison of Data Set with 3 Tier Model Calculations
This Table extends text Table 2 and includes an additional possible cell number distributions on the 3 tier model (text Fig 2)

| | | Probability of blastocyst having particular characteristics with different cell distributions | | | | |
|---|---|---|---|---|---|---|
| Sample size | 63[2] | 87 | 87 | 72 | 75 | 78 |
| No. cavity shell cells | 8 | 8 | 8 | 8 | 9 | 9 |
| No. Layer 1 cells | 12 | 14 | 12 | 10 | 11 | 9 |
| No. Layer 2 cells | 12 | 10 | 12 | 14 | 12 | 14 |
| Monoclonal cavity shell | 19.0% | 18.4% | 18.4% | 12.5% | 10.7% | 19.2% |
| One or both clones confined to 2 adjacent regions[1] | 35.0% | 25.3% | 25.3% | 15.3% | 13.3% | 17.9% |
| Both clones confined to 2 adjacent regions | 9.5% | 2.3% | 2.3% | 2.8% | 2.7% | 2.6% |
| One clone contributes twice or more cells to Layer 2 when compared with other clone | 76.2% | 69.0% | 69.0% | 50.0% | 69.3% | 51.3% |
| Monoclonal Layer 2 | 20.6% | 13.8% | 9.2% | 5.5% | 10.7% | 5.2% |

[1]These are the regions described in Fig 1 and Table 1, and the two regions must be adjacent to avoid clone splitting in the model (Cavity shell plus Layer 1 or Layer 1 with Layer 2). The influence of clone splitting is considered in the Discussion.

[2]This is data about blastocysts in the cell number range 28-34 extracted from the following references (Piotrowska *et al.*, 2001, Piotrowska-Nitsche *et al.*, 2005, Piotrowska-Nitsche and Zernicka-Goetz, 2005). The extraction procedure is described in Supplements 1 & 2.

Geometric Analysis

In the geometric analysis we made various assumptions in generating the favoured model in which the volumes of both the ICM and the shell are halved (text Fig 5). Alternative models are explored here to discover the extent that the conclusions depend on particular assumptions.

*Median value of $\beta^{blastocyst}$*

The assumptions about blastocyst morphogenesis depicted in text Fig 5 might be awry and in that case the median value $\beta^{blastocyst}$ might be $57.3^{o}$ for the model, a very different value from the $37.6^{o}$ in the data set. The revised value of $\beta^{blastocyst}$ also changes the results in Table S4.3, with a three fold increase in the frequency of monoclonal cavity shells but slight (c. 1%) changes in the distribution of the circumference in any slice (neither shown).

*Other assumptions of the geometric models*

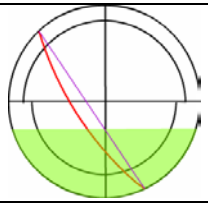
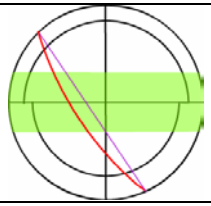
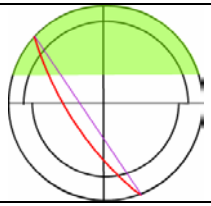
The consequences of discarding several assumptions have been considered (Tables S4.2 and S4.3). In each case morphogenesis begins with a morula divided into two identical hemispheres. Table S4.2 records the changed assumptions and Table S4.3 describes the consequence of these changes.

Table S4.2

All models start with a morula divided by a single frontier plane that bisects the shell and inner cell volumes. Starting from such a morula, the Table lists the constraints that influence the calculated position of the frontier in the blastocyst (Table S4.3).

| Shape of frontier in blastocyst | Ratio of volumes on either side of the frontier | Shell and ICM divided in the same ratio as each other | Interchange between the volumes of the ICM and shell | Consequences |
|---|---|---|---|---|
| Curved | 1:1 | Yes | No | Clones have equal cell numbers in both compartments |
| | 2:3 or 3:2 | Yes | Yes | One clone overgrows the other during blastocyst development |
| Single Plane | 1:1 | Not at all angles | No | Clones have equal cell numbers in both compartments |
| | 2:3 or 3:2 | Not at all angles | Yes | One clone overgrows the other during blastocyst development |

Table S4.3

| Shape of frontier and proportion of cellular volume occupied by each clone |  Circumferences in Basal Slice, green 0% to 33.3% |  Circumferences in Middle Slice, green 33.3% to 66.7% |  Circumferences in Apical Slice, green 66.7% to 100%, | Probability monoclonal cavity shell (i.e. whole circum. in range 0<z<40 µm) | Probability Layer 1 & Cavity Shell (i.e. whole circum. in range 26.6<z<80 µm) | Probability Middle Slice (i.e. whole circum. in range 26.6<z<54.4 µm) |
|---|---|---|---|---|---|---|
| Curved Frontier: One clone occupies 40% of Shell and ICM volumes above the frontier | 20.78% | 47.23% | 31.99% | 0.000 | 0.2842 | 0.1325 |
| Curved Frontier: Each clone occupies half the Shell and ICM volumes | 31.07% | 45.76% | 23.17% | 0.021 | 0.0797 | 0.0797 |
| Curved Frontier: One clone occupies 60% of Shell and ICM volumes above the frontier | 42.84% | 40.13% | 17.03% | 0.147 | 0.0006 | 0.0006 |
| | | | | | | |
| Single Plane Frontier: One clone occupies 40% of total volume (ICM and shell summed) above the frontier | 29.77% | 45.93% | 24.30% | 0.0214 | 0.0921 | 0.0921 |
| Single Plane Frontier: Each clone occupies half the total volume (ICM and shell summed) | 36.15% | 43.70% | 20.15% | 0.0680 | 0.0289 | 0.0289 |
| Single Plane Frontier: One clone occupies 60% of total volume (ICM and shell summed) above the frontier | 44.09% | 39.30% | 16.60% | 0.1469 | 0.0006 | 0.0006 |

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.