The direct measurement of embryogenic volume and nucleo-cytoplasmic ratio during mouse pre-implantation development

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Abstract

After fertilization, the mammalian conceptus undergoes cleavage, a process of cell proliferation in the absence of interphase growth. It is not known when cleavage ends and gives way to fully replicative cell cycles with a stable nucleo-cytoplasmic ratio. We have used two-photon excitation and confocal microscopy to measure directly volumes and nucleo-cytoplasmic ratios of whole murine concepti and their individual constituent blastomeres during pre-implantation development up to the early uterine attachment stage (day 5). We show that the total cytoplasmic volume of the conceptus remains constant during pre-implantation development, and that the average nucleo-cytoplasmic ratio increases exponentially throughout the same period. Data from individual blastomeres show that both volume and nucleo-cytoplasmic ratio diverge in the inner and outer subpopulations evident from the 16-cell stage (fifth developmental cycle) onwards. Cells from emergent outer trophoblast populations are larger and have smaller nucleo-cytoplasmic ratios than those from emergent inner pluriblast populations. Moreover, the nucleo-cytoplasmic ratio of the trophoblast appears to be stabilizing, suggesting that for this subpopulation cleavage may end at the 16–32-cell transition. Putative hypoblast and epiblast cell subpopulations within the pluriblast were not distinguishable by volume or nucleo-cytoplasmic ratio. Embryonic stem cell volume was higher than that of either cell subpopulation of expanded blastocysts, and their nucleo-cytoplasmic ratio was similar to that of trophoblast cells.


Introduction

Most proliferative cell cycles involve cell growth and chromosomal replication during interphase followed by division to smaller daughter cells during M-phase, such that both cell numbers and total cell mass increase (O’Farrell 2004). Recent work has explored how growth is coupled to cell cycle progression so as to control the size of the maternal cell and thereby its mitotic offspring (Kellogg 2003, Fingar et al. 2004). However, some mitotic cells do not show interphase growth, for example the period of post-fertilization development in many animal species, traditionally called ‘cleavage’ cell cycles. Cleavage is a process by which a large oocyte, laden with developmental information and energy supplies, is converted to a multi-cellular embryo or larva. It is not known how growth is suppressed during cleavage. Cleaving cells in some organisms are characterized by absence of the G1 and G2 phases, and it has been suggested that this feature is important as it offers no opportunities for growth checkpoints to function. However, during mouse cleavage, although the S phase does occupy most of each of the first five developmental cell cycles, G phases are present, even though short and variable, at least some cdk:cyclin oscillations occur, and there is evidence for some checkpoint activity (Streffer et al. 1980, Howlett et al. 1983, Smith & Johnson 1986, Chisholm 1988, Moore et al. 1996, Fulka et al. 1999, Bemark et al. 2000, Takai et al. 2000, Waclaw & Chatot 2004). Cleavage in the mouse also differs from other organisms in that the oocyte is smaller than many others (60–100 μm diameter), early cleavage cell cycles are longer (12–24 h), transcriptional activation occurs soon after fertilization (Telford et al. 1990, Schultz 2002), development occurs within the nutritive female genital tract, and early development is primarily concerned with the elaboration of a system of membranes for tapping maternal resources (Johnson & Selwood 1996, Johnson & Day 2000, Johnson & McConnell 2004).

In this paper, we derive quantitative data on cleavage for the pre-implantation period of development. We describe the direct measurement of total conceptus volume and nucleo-cytoplasmic ratio and examine
whether the emergent cell lineages differ in cell volume and nucleo-cytoplasmic ratio.

**Materials and Methods**

**Recovery and handling of eggs and concepti**

Female CD1 and MF1 mice (Harlan, Bicester, UK) of 3–6 weeks were housed in groups of between five and ten. Procedures were carried out under Home Office license and after local ethical review. Ovulation was induced by intraperitoneal injection of PMS (10 iu; Folligon; Sigma) followed after 46–48 h by intraperitoneal injection of human chorionic gonadotropin (10 iu; Chorulon; Sigma). Females were paired with individually housed CD1 studs males (Harlan) and mating assessed by inspecting for a vaginal plug. Concepti were retrieved from the oviducts or males (Harlan) and mating assessed by inspecting for a vaginal plug. Concepti were recovered from the oviducts or uteri between day 0 (day of positive vaginal plug) and day 5 (when attachment is initiated) by flushing with PBS or (mostly) phytohaemagglutinin (0.1–0.2 mg/ml; Sigma) followed by washing with PBS. After concepti had firmly adhered to the coverslip, 1 ml M2 + 4% BSA was added. Whole concepti were imaged with their zonae intact at 37 °C using a Leica TCS-SP-MP two-photon excitation and confocal microscope. Visualization was by sequential two-photon excitation and confocal microscopy, using a picosecond pulsed tunable Tsunami laser (Spectra Physics, Mountain View, CA, USA), tuned to 775 nm to excite Hoechst and Calcein and the 568 nm line of a Krypton laser to excite FM4-64. A water immersion ×63 lens with a numerical aperture of 1.2 and a cover-glass correction collar were used. Emitted fluorescent light was captured in discrete windows (Hoechst 400–480 nm, FM4-64 600–700 nm, and Calcein 505–545 nm). The confocal pinhole was set to 1 Airy disk equivalent for the objective lens in both confocal and two-photon imaging. A Z-series of images was collected from each subject, capturing an image every 1 μm. Every image was used for three-dimensional (3D) reconstruction, while every second image was used for stereology.

**Analytical procedures**

The Cavalieri principle (Gunderson & Jenson 1987) was used to estimate volumes of concepti, ICMs, single cells and their nucleo-cytoplasmic components. Images on screen (521 × 521 mm) were overlain with quadratic grids with a spacing of 20 mm for cytoplasm and 10 mm for nuclei. The number of intersections overlying nuclei and cytoplasm was counted. The near-spherical shape of the concepti meant that less than the accepted maximum of 200 points per object from 10–15 sections could be counted, giving an error coefficient of 5–10% (Roberts et al. 1993, 1994). The volume of the conceptus (excluding blastocoelic volume) or its constituent cytoplasm or nuclei could then be calculated. For single-cell analysis, Z-stacks were captured through the conceptus and individual cells within these images were chosen for analysis. Where cell subpopulations were analysed, random selection of cells was attempted. However, not all cells within the total image were delineated clearly enough to obtain an accurate volume. Data from these cells are not included, and so a degree of non-random selection cannot be excluded. The nucleo-cytoplasmic ratio was also calculated for isolated single cells, although these are not comparable directly to those obtained through whole conceptus analysis due to problems with different levels.

**Imaging concepti**

Nuclei were stained by incubating concepti in M2 + 4% BSA containing 10 μg/ml Hoechst 33258 (Sigma; 1 mg/ml stock) for 2–4 h. Cytoplasm was stained by a 15 min incubation in the calcium dye Calcein AM (0.5 μg/ml in M2 + 4% BSA; Molecular Probes, Eugene, ON, USA). Membranes were stained with the styryl dye FM4-64 (3.025 μg/ml; Molecular Probes), which was added to the...
Gamma correction and a median filter were applied to sectional images to achieve a satisfactory 3D image. Volume estimates of the same concepti made using the Cavalieri method and 3D reconstructions did not differ significantly ($P < 0.0001$). 3D reconstruction using Imaris proved impossible to use reliably for calculation of the nucleo-cytoplasmic ratio, for technical reasons. Thus, the degree of variability in staining between individual nuclei prevented reconstruction adequate for measurement purposes (see Fig. 1c).

**Statistical tests**

Data from whole conceptus analysis have been plotted such that each data point represents a single conceptus ($n = 86$). Pearson correlation coefficients were calculated, and are included in the figure legends. For single-cell analysis, cells from concepti of each developmental stage were grouped together and the mean ± 95% confidence interval plotted. Sample sizes ($n$ values) are given in Table 1 and the figure legends.

**Results**

**Total conceptus volume and averaged nucleo-cytoplasmic ratio over the pre-implantation period**

Examples of concepti used for analysis are shown in Fig. 2. The total volume of concepti (excluding blastocoelic volume) increases slightly during the pre-implantation period, and can be accounted for entirely by the increasing total nuclear volume with each successive cell cycle (Fig. 3a), while whole embryo cytoplasmic volume remains constant. Mean cytoplasmic volume per cell was calculated by division from whole conceptus data and found to decrease exponentially throughout the pre-implantation period (Fig. 3b and c), consistent with cleavage divisions continuing throughout this period. The data from Fig. 3a were also used to calculate the changing mean nucleo-cytoplasmic ratio, which was found to increase during pre-implantation development (Fig 3d). Together with data showing that cytoplasmic volume remains constant (Fig 3a), these results imply that within...
the conceptus as a whole, interphase growth has not occurred and that cleavage divisions have not terminated by the time of attachment.

The cytoplasmic and nuclear volumes of clusters of outer trophoblast and inner pluriblast cells of 3.5-day blastocysts were measured in order to calculate average nucleo-cytoplasmic ratios for each component tissue. Trophoblast measurements were made from whole conceptus images, but pluriblast measurements were made from whole immunosurgically isolated ICM images, since isolation of ICMs improved penetration of nuclear staining at later stages. The mean nucleo-cytoplasmic ratios from each subpopulation differed significantly (Fig. 3; \( P = 0.0015 \)). No difference was seen between mural and polar trophoblast subpopulations (\( P = 0.122 \)).

**Volumes and nucleo-cytoplasmic ratios from individual cells over the first seven developmental cell cycles**

The averaged data from whole volume analysis suggest continuance of cleavage throughout pre-implantation development. However, the data from the late blastocyst stage indicate that nucleo-cytoplasmic ratios of subpopulations differ. To explore when such differences might emerge, direct measurement of individual blastomere
Volume and nucleo-cytoplasmic ratio of mouse conceptus at different stages of development was undertaken. Whole conceptus optical images were taken and individual blastomeres within these selected for cell-by-cell volumetric analysis. Concepti with total numbers approximating a serial doubling of blastomere numbers were used to reduce problems of blastomere asynchrony. Data were collected during the first seven developmental cell cycles and, from the 16-cell stage onwards, cells were categorized according to their inner and outer positions within the conceptus (Table 1).

For early cell cycles (first to fourth), distribution plots of cell volumes calculated in this way were found to be normally distributed and unimodal, whereas subsequent cell cycles (fifth to seventh) were spread in an increasingly bimodal distribution. Cell volume was again found to decline by approximately half with each cell cycle (Fig. 4a), and was not significantly different to values calculated from whole conceptus analysis. Converting these data to a linear plot, it is clear that from the fifth (16-cell stage) developmental cell cycle onwards that the volume of outer cells was significantly greater than that of inner cells ($P = 0.0131$). A significant difference was also found in the nucleo-cytoplasmic ratio of the trophoblastic population appearing to stabilize. ES cell volume (Fig. 4a) was also measured and found to be higher than that of either inner or outer cells of 3.5-day blastocysts. Within the pluriblast cell population, no difference in volume or nucleo-cytoplasmic ratio of juxta-coelic putative hypoblast precursors and deeper putative epiblast precursors was found (Fig. 4a and b). ES cell nucleo-cytoplasmic ratio (Fig. 4b) was found to be similar to that of trophoblast cells from 3.5-day blastocysts.

Figure 3 (a) Measured volumes throughout pre-implantation development of whole conceptus (φ; $R^2 = 0.2526$), cytoplasmic compartment (□; $R^2 = 0.0012$) and nuclear compartment (grey triangles; $R^2 = 0.7951$) plotted against total number of nuclei per conceptus. Each point represents a single mouse conceptus ($n = 86$ in panels a–d). (b) Calculated mean cell volume, generated by division of each conceptus volume by the number of nuclei within it. Each point represents a single conceptus. (c) Data from (b) shown as a logarithmic plot; $R^2 = 0.9651$. (d) Calculated mean nucleo-cytoplasmic ratio (shown as a percentage) throughout the pre-implantation period in relation to total cell number. Each point represents a single conceptus and was generated by dividing the summed volume of nuclei by the total cytoplasmic volume; $R^2 = 0.8494$. (e) Nucleo-cytoplasmic ratio (shown as a percentage) from whole subpopulations within 3.5-day blastocysts. ICM data are from isolated ICMs ($n = 15$); trophoblast data are from intact conceptus images ($n = 10$ concepti for polar trophoblast and $n = 15$ concepti for mural trophoblast). The total cytoplasmic volume of each subpopulation from each embryo was divided by its subpopulation total nuclear volume.
Discussion

The total volume of the mouse conceptus remains broadly constant throughout pre-implantation development, but the serial increase in total volume of nuclei leads to an exponential increase in the average nucleo-cytoplasmic ratio. This result suggests that cleavage, as defined by the absence of interphase growth, has not ceased before day 5 and the beginning of attachment. The absence of any net increase in cytoplasmic volume occurs despite the presence of transport systems for the uptake of metabolic substrates (Gardner et al. 2000, Lane & Gardner 2000) and the machinery to use them for macromolecular synthesis (Epstein 1975, Wales & Hunter 1990). Previous work (Turner et al. 1992) indicated that embryonic dry mass actually decreased during the pre-implantation period, which is consistent with our findings.

However, the use of averaged whole conceptus data masks variation in emergent subpopulations. The most evident subpopulations to emerge over the pre-implantation period are the trophoblast and pluriblast cell populations that make up the outer transporting epithelium and the ICM of the blastocyst respectively (Johnson & Selwood 1981). Our quantitative analysis of individual cell volumes has confirmed these earlier observations. Thus, between the one-cell and eight-cell stages, volume distributions are unimodal, and suggest an approximate halving of cell volume with each division. At the 16-cell stage and later, there is a significant difference between the volume of inner and outer cells. By the expanding blastocyst stage, ICM cells continue to reduce in size and so become smaller than those from trophoblastic populations. These volume differences are also reflected in diverging nucleo-cytoplasmic ratios for trophoblast and pluriblast. Thus, the trophoblast nucleo-cytoplasmic ratio appears to be plateauing (Figs 3e, 4a and b). There was no significant volume difference between cells from mural and polar trophoblast subpopulations, nor between ICM cells adjacent to the blastocoel (potential hypoblast) and those deep within the ICM (potential epiblast; Fig. 4; Chisholm et al. 1985, Rossant 1986, Johnson & McConnell 2004).

The size differential between inner and outer cell subpopulations could arise in a number of ways. The differential could reflect an eccentricity of the cleavage planes at the 8- to 16-cell and 16- to 32-cell divisions. Qualitative observations have already suggested that this is the case (Johnson & Ziomek 1981, Ziomek et al. 1982, Reeve & Kelly 1983), and it is known that these divisions do generate cell subpopulations that differ in phenotype and potential (reviewed in Johnson & McConnell 2004). Thus, differential division is likely to provide at least part of the explanation. An alternative explanation might be that a growth increase in cytoplasmic volume occurs during the cell cycle in outer cells, indicating that cleavage ends earlier in this subpopulation. This seems to us unlikely given the overall average stability of cytoplasmic volume and the absence of net total growth, since it would require inner cells to shrink during the cell cycle.

Figure 4 (a) Logarithmic plot of mean values of individual blastomere volumes, separated into inner and outer cells. Cells were designated inner or outer on the basis of examination of optical sections. 95% confidence intervals are shown; n values for individual blastomeres are shown in Table 1. Note that the data conform to an approximately straight line during the early cell cycles. From the fifth cell cycle (16-cell stage), however, a significant difference in cell volume is shown between inner cells (smaller; ◆; R² = 0.9762) and outer cells (larger; ◆; R² = 0.9822). Values for ES cells (△; n = 40), epiblast (□; n = 17) and hypoblast (■; n = 16) are also shown. (b) Mean values of nucleo-cytoplasmic ratio of individual blastomeres; n values for individual blastomeres are shown in Table 1. Pluriblast/inner cells (R² = 0.9764), outer cells (R² = 0.8265). ES cells (n = 40), epiblast (n = 17), hypoblast (n = 16); symbols as in (a).
A third possibility is that at later stages some cells in each of the two subpopulations are in different developmental cell cycles and so of different sizes (assuming that non-growth cleavage divisions are indeed continuing). This is a possible explanation, given that there is an increasing asynchrony of blastomere divisions with each successive developmental cell cycle, such that cycles might overlap (Chisholm et al. 1985). In addition, there is evidence of differences in cell cycle length between inner and outer cells and of mitotic arrest at later expanded blastocyst stages (Barlow et al. 1972, Johnson & Ziomek 1981, MacQueen & Johnson 1983, Surani & Barton 1984). We have attempted to minimize the problem of overlapping developmental cell cycles by identifying for single-cell analysis concepti with exactly 16, 32 or 64 cells. However, it is possible that trophoblast cells as a population are delayed compared with pluriblast cells and so this may account for part of the size difference between them. A fourth explanation of volume differences between cell subpopulations at blastocyst stages is that the mid-32-cell stage onwards, blastocoele expansion occurs (Smith & McLaren 1977, Chisholm et al. 1985). Fluid transport across the mural trophoblast cells (Borland et al. 1977, Wiley 1984) might lead to increases in their volume, although the presence of membrane ion channels, which regulate trophoblastic cell volume and prevent swelling or shrinkage, make this unlikely (Kolajova et al. 2001), as does the observation that there is no significant difference in volume between mural and polar trophoblast (Fig. 3e). Finally, it is possible that the difference in sizes between the two subpopulations might be an artifact of the non-random selection of cells for measurement within each subpopulation in the second part of the study. Whereas this cannot be excluded formally, it seems unlikely, since similar differentials were observed when averaged estimates from whole ICM and total trophoblast were made in the first part of the study.

Overall, the most likely interpretation of the subpopulation differentials, given the data on volumetric constancy of the whole conceptus, is that they arise initially by asymmetric division, but developmental cell cycle overlap may account for some of the later divergence. If this conclusion is correct, then cleavage has not ended for either cell subpopulation by the time of attachment. It is reasonable to think that cell volume should not occur until after a secure nutritional contact with the maternal uterine epithelium has been achieved. Interestingly, the volume of the ES cells was measured as being of the same order as day 4–5 trophoblast or pluriblast. ES cells are thought to be equivalent to the late pluriblastic population, so the observation suggests that cleavage may have ceased prior to attachment.

Do data in the literature support a nucleo-cytoplasmic ratio as a form of developmental timer as proposed for a number of developmental systems (reviewed in Johnson & Day 2000) including the mouse (evidence reviewed in Day et al. 2001). The usual mechanistic model proposed is the titration of a cytoplasmic factor in the oocyte against increasing amounts of DNA (Prioleau et al. 1994). Our observations suggest that such a timing mechanism could (i) operate until attachment and possibly beyond in some or all cells of the conceptus, and (ii) operate differentially in inner and outer cells prior to implantation to activate developmental programmes at different times in the two emergent lineages, perhaps contributing to the initiation of different transcriptional patterns.

Second, the notion of what constitutes cleavage may need to be clarified. We have operated a strict definition based on absence of net growth and the parcelling out of cytoplasm into cells of decreasing size and increasing nucleo-cytoplasmic ratio. A series of other developmental characteristics has been identified in association with cleavage in a range of organisms and this association has sometimes lead to their elision into the definition of cleavage (O’Farrell 2004). For example, in Xenopus, the mid-blastula transition (MBT) occurs during a specific developmental cell cycle and is associated with characteristic changes to cell cycle length, the appearance of G1 and G2 phases, cell division asynchrony and motility, changes in protein turnover, and the initiation of transcriptional and apoptotic activity (Newport & Kirschner 1982a, 1982b, Newport & Kirschner 1984, Sible et al. 1997, Stack & Newport 1997). The MBT is often implicitly taken to mark the end of cleavage, even though further reductions in cell volume and nucleo-cytoplasmic ratio may occur. The developmental properties newly expressed at the MBT have also been taken as the mark of a non-cleavage phenotype, even though this is clearly not a straightforward case even in Xenopus (Sible et al. 1997). It is certainly not the case in the mouse since transcriptional activation, long and asynchronous cell cycles with checkpoints and G phases, cell motility and cyto-differentiation are all occurring during, and not after, the period of cleavage. Analogies between different organisms on the basis

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of cleavage similarities and differences may therefore require some qualification (O’Farrell 2004).

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