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Three-dimensional reconstruction of tetraploid↔diploid chimaeric mouse blastocysts

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ABSTRACT

Studies of tetraploid↔diploid (4n↔2n) mouse chimaeras have demonstrated unequal contributions of 4n cells to different tissues of the midgestation conceptus. Such a pattern has also been reported in chimaeras as early as E3.5d, which show an enhanced contribution of 4n cells to the mural trophectoderm (Everett & West, 1996). In this study, sectioned 4n↔2n and 2n↔2n control chimaeric blastocysts were digitised and reconstructed in 3 dimensions (3-D). The 3-D images revealed only limited mixing of cells from the 2 contributing embryos of individual blastocysts in both chimaera groups. Consequently, the distribution pattern of the 2 cell types was dependent on the spatial relationship between the orientation of the blastocyst and the boundary between the 2 clusters of cells. The distribution patterns observed were not strikingly different for 4n↔2n and 2n↔2n chimaeras, each showing some transgenic positive cell contribution in all 3 identifiable developmental lineages. It was notable, however, that in all 4n↔2n blastocysts at least some 4n cells were located adjacent to the blastocyst cavity. Such a consistent pattern was not evident in 2n↔2n chimaeras. This study has demonstrated the value of 3-D reconstructions for the analysis of spatial relationships of 2 cell populations in chimaeric mouse blastocysts.

Key words: Tetraploidy; blastocyst; chimaera; chimera; embryo.

INTRODUCTION

Tetraploid cells (4n) have been shown to be distributed nonrandomly in mouse postimplantation tetraploid/diploid (4n/2n) mosaics (Tarkowski et al. 1977) and 4n↔2n chimaeras (Lu & Markert, 1980; Nagy et al. 1990; 1993; James et al. 1995), readily contributing to the extraembryonic tissues but rarely to the fetus itself. Studies at E12.5 and E7.5 have revealed that 4n cells are not simply restricted to extraembryonic tissues but more specifically to trophectoderm and primitive endoderm derivatives (James et al. 1995). We also demonstrated that, as a group, 4n↔2n blastocysts show a statistically significant greater contribution of 4n cells to the mural trophectoderm than to the inner cell mass or polar trophectoderm (Everett & West, 1996). This phenomenon appears to be related to ploidy not cell size at aggregation and may contribute to the restricted distribution of 4n cells at later stages.

Three-dimensional (3-D) reconstruction is potentially an extremely powerful tool for visualising the distribution of cell types within embryos. We produced 4n↔2n aggregation chimaeras, in which the tetraploid component was hemizygous for the β-globin transgene *Tg*N(Hbb-1)83Clo (Lo et al. 1987), and analysed them at the blastocyst stage by computer-aided 3-D reconstruction of histological sections. The aims were (1) to evaluate the use of 3-D reconstruction for the analysis of cell distributions in chimaeras and (2) to test whether any consistent pattern could be distinguished for the distribution of 4n cells in individual E3.5 4n↔2n chimaeric blastocysts. Preliminary results from this study have been published in abstract form (Everett et al. 1996).

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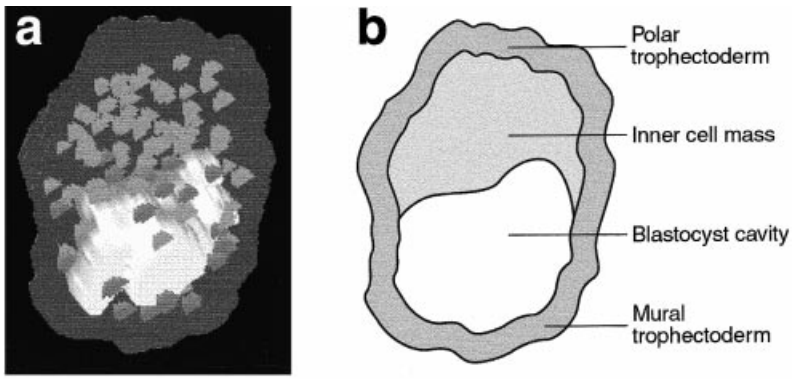

Fig. 1. (*a*) 2-dimensional view of a 3D computer reconstruction of the blastocyst shown in Fig. 2*a*. The outline of the blastocyst is shaded grey, the blastocyst cavity is white and the positions of individual nuclei are indicated by dots. The nuclei overlap one another because the 3-dimensional reconstruction has been collapsed to 2 dimensions. (*b*) Conventional diagram of the different parts of the blastocyst represented in (*a*).

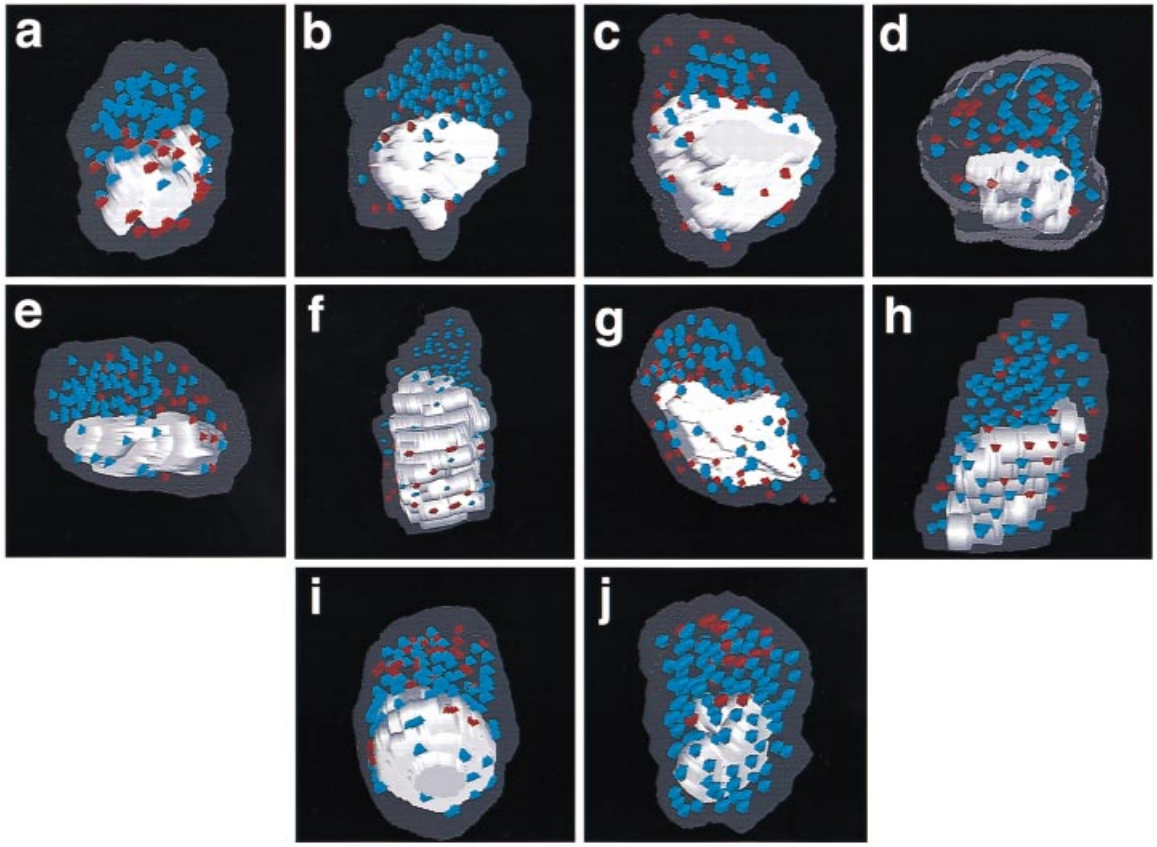

Fig. 2. 2-D views of the 3-D reconstructions (*a–e*), 4n↔2n blastocysts; (*f–j*) 2n↔2n blastocysts. Red dots, *Tg*-positive nuclei (4n in 4n↔2n chimaeras); blue dots, *Tg*-negative nuclei; white shading, blastocyst cavity; light shading, outline of the blastocyst.

MATERIALS AND METHODS

Production of chimaeric blastocysts

The protocol used for the production of 4n↔2n and 2n↔2n chimaeric blastocysts is as described by Everett & West (1996). Briefly, 2 genetically different strain combinations of 2-cell embryos were flushed from the reproductive systems of superovulated female mice. One of these embryo groups carried a reiterated β-globin transgenic sequence *Tg*N(Hbb-b1)83Clo (Lo, 1986; West et al. 1995) (*Tg*) that could be identified by DNA-DNA in situ hybridisation on histological sections (Lo et al. 1987; Katsumata & Lo, 1988; Thomson & Solter, 1988). Some 2-cell embryos of this strain combination were electrofused, forming 1-cell 4n embryos. All were placed in M16 medium under paraffin oil and cultured overnight. The following day these embryos were aggregated

with 8-cell embryos of the non-*Tg* strain which had also been obtained at the 2-cell stage by the method described above and cultured overnight. After aggregation, these chimaeric embryos were then cultured for a further 30–36 h.

The E3.5 chimaeric blastocysts were introduced into a freshly dissected *Tg*/*Tg* oviduct, fixed in acetic ethanol (3 ethanol : 1 acetic acid) and embedded in paraffin wax. Serial sections were cut at 7 μm and in situ hybridisation was carried out, using the digoxygenin-labelled probe pMβδ2 (Lo et al. 1987) and a diaminobenzidine endpoint (Keighren & West, 1993), to detect the β-globin sequences. In this way, the hemizygous diploid (*Tg*/-) or tetraploid cells (*Tg*/*Tg*/-/-), bearing 1 or 2 copies of the β-globin transgene respectively could be distinguished from cells from the other contributing embryo.

3-D digital reconstruction of chimaeric blastocysts

Serial sections through a total of 10 blastocysts were digitised using a Xillix Technologies Microimager-1400 12 bit CCD camera connected to a Sun Microsystems SparcStation Unix workstation and mounted on a Zeiss Axioplan microscope. Using a ×10 objective the digitised images had a pixel size of 0.68 μm. After digitisation the grey levels of each image was normalised and subsampled to 1.36 μm. The image capture and manipulation software is proprietary to the MRC and based on the woolz image processing system (Piper & Rutovitz, 1985).

To correct for sectioning distortions the 2-D images were restacked to create a 3-D voxel image which was warped using the Finite Element based method developed for this purpose (Guest & Baldock, 1995). Each reconstruction was then reviewed by reference to the original sections, viewed under the microscope at higher magnification, in order to distinguish the *Tg*-positive and *Tg*-negative nuclei. The tetraploid cells were expected to have 2 copies of the *Tg* and therefore 2 hybridisation signals per nucleus. However, part of the nucleus containing the *Tg* sequences may be excluded, due to the thickness of the sections, resulting in a false negative nucleus. Using 'Paint' (a program developed at the MRC for manual segmentation of structures within a 3-D voxel image), the 2 classes of nuclei (*Tg*-positive and *Tg*-negative) were delineated by different colour dots. The blastocyst cavity and blastocyst outline were also 'painted'.

This results in a set of data structures which can be read into the visualisation system AVS (Advanced Visual Systems, AVS/UNIRAS Ltd). In each case the cavity and outline have been displayed as transparent shells to aid orientation and the cell nuclei displayed as 'bubbles' which is a technique for visualising point data provided by AVS. The displayed colours and transparencies can be adjusted as well as the viewing position. On a Silicon Graphics Indigo 2 with the Extreme Graphics hardware the 3-D displays can be manipulated interactively and viewed using stereo glasses (Agar Scientific). To provide a clearer view of the data for publication, the AVS module to 'crop' a 3-D object to any coordinate plane has been utilised. More details of the image capture and processing systems will be published elsewhere and can be obtained from one of the authors (R.A.B.).

RESULTS AND DISCUSSION

Three-dimensional display of chimaeric blastocysts

4n↔2n (and 2n↔2n control) chimaeras were made by aggregating 2 embryos, one of which was *Tg*-positive. 7 μm sections of paraffin wax embedded blastocysts were cut and *Tg*-positive nuclei identified by in situ hybridisation. In total, the grey level voxel images of 10 blastocysts were reconstructed: 5 each of 4n↔2n and 2n↔2n chimaeras. These were viewed as 3-D objects using AVS by the usual 3-D visualisation techniques which allowed arbitrary rotation and shading of the nuclei. On the Silicon Graphics workstation, used for display, stereo glasses which synchronise with the display screen, permitted full-colour interactive stereo images to be viewed.

Two-dimensional display of chimaeric blastocysts

In addition to the 3-D visualisation techniques, the blastocysts can be represented by a variety of 2-D images. Figure 1 demonstrates how these images are interpreted in such a format. Several different display techniques are possible; these are shown in Figures 2 and 3. Figure 2 demonstrates the 'best view' of all the reconstructed blastocysts. These are arbitrary 2-D views of the 3D reconstructions. In this representation, some of the nuclei are obscured by the blastocyst cavity, but the overall distribution pattern can still be appreciated. Blastocysts *a–e* and *f–j* represent 4n↔2n and 2n↔2n chimaeras respectively. They are ranked according to the distribution of the positive nuclei, with the highest concentration of positive cells in chimaeras *a* and *f* occurring in the area surrounding the blastocyst cavity.

Figure 3*a* and *b* presents stereo pairs of 4n↔2n (blastocyst a in Fig. 2) and 2n↔2n (blastocyst j) chimaeras. These can be viewed using stereo glasses (Agar Scientific Cat no. O6001). In Figure 3*c* and *d*,

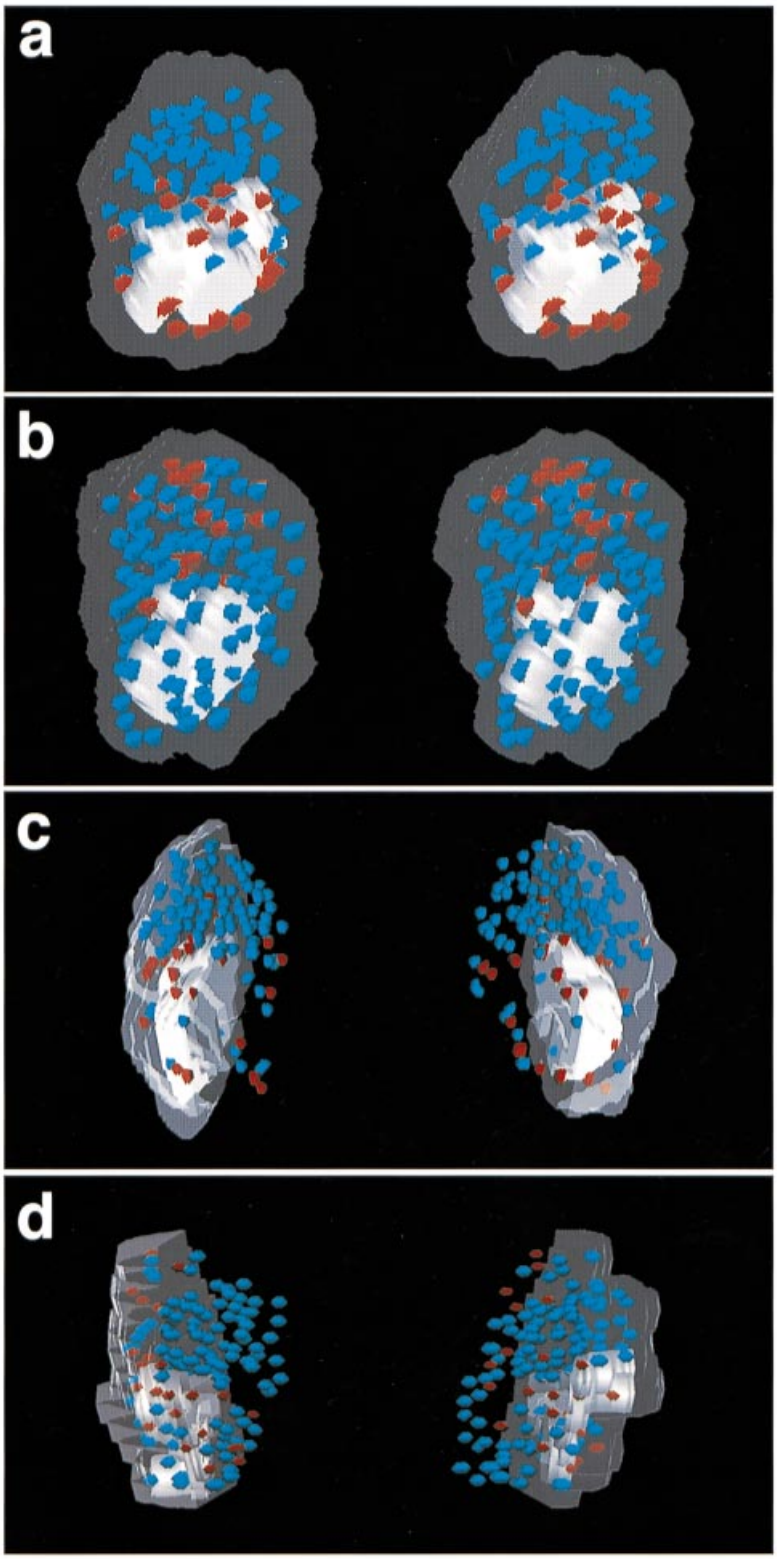

Fig. 3. Stereo pairs of (*a*) a 4n↔2n chimaera (blastocyst in Fig. 2*a*) and (*b*) a 2n↔2n chimaera (blastocyst 2*j*). Cropped and rotated images of (*c*) a 4n↔2n chimaera (blastocyst 2*b*) and (*d*) a 2n↔2n chimaera (blastocyst 2*h*) in which representations of the blastocyst cavity and blastocyst outline have been removed in half the chimaera, the remaining half being rotated through 20° to allow the nuclei behind the blastocyst cavity to be viewed. Red dots, *Tg*-positive nuclei (4n in 4n↔2n chimaeras); blue dots, *Tg*-negative nuclei; white shading, blastocyst cavity; light shading, outline of the blastocyst.

cropped and rotated images of blastocysts b and h are shown. Representations of the blastocyst cavity and blastocyst outline have been removed in half the chimaera, the remaining half has been rotated through

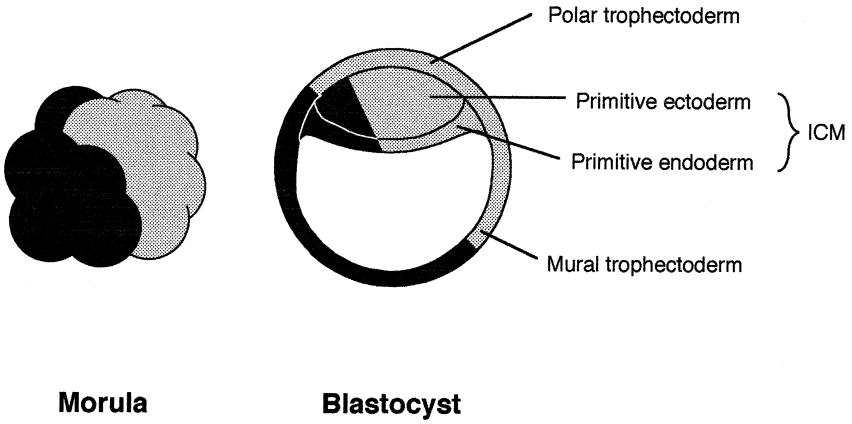

Fig. 4. Diagram showing the development of a chimaeric blastocyst from a morula (redrawn after West et al. 1984). The diagram assumes that there is little or no cell mixing at this early stage. The cells derived from the 2 8-cell embryos (shaded black and grey) occupy 2 hemispheres of the morula and these domains are retained in the blastocyst. If there was no cell mixing, each hemisphere would represent a single 'coherent clone' of cells.

20° to allow the nuclei behind the blastocyst cavity to be viewed.

Pattern of cell distribution in chimaeric blastocysts

The 3-D reconstructions have permitted the distribution pattern of cells from the 2 contributing embryos to be visualised. It is evident from Figures 2 and 3 that in the majority of both 4n↔2n and 2n↔2n chimaeric blastocysts, the *Tg*-positive (red) and *Tg*-negative (blue) nuclei remain as distinct clusters. This is particularly obvious when the labelled cells are clustered around the regions at the poles, with the boundary between cell types parallel to the roof of the blastocyst cavity. For example, 4n↔2n chimaeric blastocysts in Figure 2*a* and *b* and 2n↔2n blastocysts in Figure 2*f*. The clustering is less obvious, but still discernible, in those blastocysts in which the boundary between the 2 clusters of cells is oriented across the blastocyst cavity (e.g. Fig. 2*e*). This observation provides 3-D visual support for the previous analyses of chimaeric blastocysts by Garner & McLaren (1974) and Dvorak et al. (1995). Figure 4 illustrates this clustering diagramatically, with the 2 cell types represented by grey and black shading. This association would therefore suggest that the contribution of *Tg*-positive cells to the developmental lineages is essentially dependent on the spatial relationship between the orientation of the blastocyst and the boundary between the clusters of cells. This raises the question: is this spatial relationship biased, in 4n↔2n blastocysts?

In a previous study of 21 4n↔2n blastocysts, it was demonstrated that overall 4n cells showed a significantly higher contribution to the mural trophectoderm than to any other tissue of E3.5 chimaeras (Everett & West, 1996). It was suggested that if little cell mixing had occurred prior to the early blastocyst stage, this pattern may be the result of the preferential formation of the blastocyst cavity within the mass of 4n cells: a biased spatial relationship between the orientation of the blastocyst and the boundary between the clusters of cells. This suggestion was also consistent with observations in later stage chimaeras (E7.5 and E12.5), which showed a 4n cell contribution restricted to those tissues derived from the trophectoderm and primitive endoderm lineages (James et al. 1995). As we have already discussed, prior to E3.5 mixing between cells of the 2 contributing embryos was minimal. We have therefore addressed the possibility that the blastocyst cavity forms preferentially within the mass of 4n cells.

In Figure 2, the blastocysts have been ranked according to the proportions of *Tg*-positive nuclei showing an association with the blastocyst cavity. From these figures and the 3-D reconstructions it was evident that, overall for both 4n↔2n and 2n↔2n chimaeras, *Tg*-positive cells contributed to all lineages of the blastocyst (inner cell mass, mural trophectoderm and polar trophectoderm; see Fig. 4). Consequently the pattern of cell distribution in both types of chimaera was not strikingly different. It may be significant, however, that in all 4n↔2n blastocysts, the blastocyst cavity was associated with a large proportion of the labelled cells; this was not true of some of the 2n↔2n chimaeras (e.g. Figs 2*i* and 1*j*). If this trend was confirmed by analysis of a larger series, it would support the idea that the blastocyst cavity forms preferentially where 4n cells predominate (Everett & West, 1996).

The pattern of distribution of 4n cells was evidently unaffected by their relatively large size (i.e. they have

not been excluded from the inner cell mass for geometric reasons). This supports our previous study (Everett & West, 1996) which suggested that preferential allocation to the mural trophectoderm was due to ploidy per se.

CONCLUSIONS

3-D reconstructions allow the contribution of each aggregated embryo to the individual chimaera to be visualised. This has the advantage, over 2-D sections, that all dimensions can be viewed simultaneously on the computer screen. Additional information, regarding relative spatial relationships, can be obtained more readily using this technique.

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