Spindle abnormalities in normally developing and arrested human preimplantation embryos in vitro identified by confocal laser scanning microscopy

Katerina Chatzimeletiou1,4,5, Ewan E.Morrison2, Nikos Prapas3, Yannis Prapas3 and Alan H.Handyside1,4

1The London Bridge Fertility, Gynaecology and Genetics Centre, One St Thomas Street, London SE1 9RY, 2CRUK Clinical Centre at Leeds, Division of Cancer Medicine Research, St James’ University Hospital, Leeds LS9 7TF, UK, 3Iakentro Advanced Medical Centre, Thessaloniki, 542 50, Greece and 4School of Biology, University of Leeds, Leeds LS2 9JT, UK

BACKGROUND: Despite recent technical improvements, many human preimplantation embryos fail to develop to the blastocyst stage or implant after transfer to the uterus. A possible cause for this developmental arrest is the high incidence of nuclear and postzygotic chromosomal abnormalities observed during cleavage, including chaotic chromosome complements, suggestive of defects in mitotic chromosomal segregation. The underlying mechanisms are largely unknown, but similarities with chromosome instability in human cancers led to the proposal that cell cycle checkpoints may not operate at these early stages. METHODS: To investigate this and to examine whether spindle abnormalities contribute to chromosome malsegregation, we have used fluorescence and confocal laser scanning microscopy, following immunolabelling with antibodies specific for α-tubulin, γ-tubulin, or acetylated tubulin, combined with a DNA fluorochrome to visualize nuclei, spindle and chromosome configurations in normal and arrested human embryos, from cleavage to blastocyst stages. RESULTS: In addition to frequent interphase nuclear abnormalities, we identify for the first time various spindle abnormalities including abnormal shape and chromosome loss and multipolar spindles at cleavage and blastocyst stages. CONCLUSIONS: We propose that a major pathway leading to postzygotic chromosomal abnormalities is the formation of binucleate blastomeres with two centrosomes which result either in a bipolar spindle and division to two tetraploid blastomeres, or in a multipolar spindle, chromosome malsegregation and chromosomal chaos.

Key words: cell cycle checkpoints/confocal laser scanning microscopy/human preimplantation embryo/mitotic spindle/nuclear abnormalities

Introduction

The development of improved culture media, in particular, sequential media tailored to the changing metabolic needs of human embryos at different stages, has significantly increased pregnancy rates following extended culture and transfer at the blastocyst stage (Gardner et al., 1998a,b; Gardner and Lane, 2003). However, a significant proportion of embryos do not reach the blastocyst stage or implant after transfer. One possible cause of early developmental arrest is the high incidence of nuclear and chromosomal abnormalities observed in embryos at these stages. The most common nuclear abnormality observed at cleavage stages is that of binucleate blastomeres caused by failure of cytokinesis (Hardy et al., 1993a). Other abnormalities include multinucleate and anucleate blastomeres (Winston et al., 1991). Multinucleate blastomeres in general (including binucleate blastomeres) are more frequent in embryos with poor morphology (Kligman et al., 1996) and are associated with lower pregnancy rates following transfer (Jackson et al., 1998).

Analysis of chromosomes in nuclei of single cells, biopsied from cleavage stage human embryos, by multicolour fluorescence in situ hybridization (FISH) for preimplantation genetic diagnosis (PGD), initially to identify the sex of embryos in couples at risk of X-linked disease (Griffin et al., 1993), and more recently, for aneuploidy screening (Lamb et al., 1997; Tesarik et al., 2000; Lewis et al., 2001; Gianaroli et al., 2002; Verlinsky et al., 2002; Munne et al., 2003), has provided a powerful tool for interphase molecular cytogenetics, albeit on a limited number of chromosomes determined by the availability of probes and different fluorochromes. Using this approach, numerous studies have confirmed the high incidence of aneuploidy in human gametes and embryos and the well-established increase associated with advanced maternal age (Munne et al., 1995a, 2002).
In addition, however, subsequent multicolour FISH analysis of all the nuclei in biopsied embryos either from fertile patients, which had been rejected for transfer following PGD for X-linked disease, or in surplus embryos from infertile patients, has revealed that about a third of embryos are chromosomally mosaic (Delhanty et al., 1997). In these mosaic embryos, some nuclei have the normal diploid number of the chromosomes analysed, indicating fertilization with eusomic gametes, but other nuclei can be haploid, polyploid (most commonly tetraploid) or aneuploid (Harper et al., 1995; Munné et al., 1995a,b, 2003). At the chromosomal level, therefore, human embryos can be classified as diploid, diploid/haploid/polyploid or diploid/aneuploid mosaics. Furthermore, some embryos from fertile patients appeared to have a random distribution of chromosomal abnormalities in a majority of their nuclei, with some evidence that this was patient specific, and these were classified as chaotic (Delhanty et al., 1997).

These postzygotic nuclear and chromosomal abnormalities arising during cleavage, often involving chromosomal malsegregation, closely resemble the genetic instability observed in tumour cells, suggesting that cell cycle checkpoints may not operate at these early stages (Delhanty and Handside, 1994). To investigate this, and to examine whether spindle abnormalities contribute to chromosome malsegregation, we have used laser confocal scanning fluorescence microscopy with combinations of a DNA fluorochrome to visualize nuclei and chromosomes and several antibodies specific for either spindle- or centrosome-associated proteins in human embryos either developing at the normal rate at cleavage to blastocyst stages or in arrested, mainly cleavage stage embryos. These antibodies included anti-α-tubulin to label spindle microtubules and anti-γ-tubulin and anti-acetylated tubulin to label spindle poles.

Materials and methods
Source of human preimplantation embryos
Surplus embryos were donated from consenting couples undergoing IVF treatment in the Assisted Conception Unit (ACU) of the Laken- tro IVF Clinic in Greece, where the embryos were fixed and labelled.

Ovarian stimulation
A standard protocol was used to induce ovulation and control timing of oocyte retrieval. Pituitary down-regulation was achieved by the administration of GnRH analogues (Cetrotide; Serono; or Orgalu- tran; Organon, for the first 10–14 days, followed by ovarian stimulation with pure FSH (Metrodin; Serono, UK) or FSH and LH (Altermon; IBSA) or recombinant FSH (Puregon; Organon or Gonal-F; Serono) for 12–14 days. The patients were monitored by ultrasound regularly starting on day 6 of gonadotrophin adminis-

Oocyte retrieval
Oocytes were retrieved by flushing ovarian follicles with IVF 20 (Vitrolife) incubated in 5% CO₂ in air at 37°C and subsequently fertilized by conventional IVF or ICSI and cultured until the day of transfer (day 3) in G1.2 medium (Vitrolife). Embryos of patients requesting blastocyst transfer were further cultured in CCM medium (Vitrolife) until day 5. Spare embryos from day 3 or day 5 transfers were processed for immunofluorescence analysis.

Human embryo fixation, immunostaining and confocal imaging
Embryos were rapidly transferred from culture to ice-cold methanol (BDH) and fixed for 10 min. Following fixation they were briefly washed in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS; Gibco BRL) containing 2% bovine serum albumin (BSA; Sigma) and transferred into 10 µl drops of the primary antibodies (1:1000 dilution in PBS/BSA) under mineral oil (Sigma) and incubated at 4°C for 1 h. Primary antibodies included: (i) a rat monoclonal antibody specific for α-tubulin (Serotec); (ii) and (iii) mouse mono-

Embryo classification
Embryos were divided into three groups depending on their develop-

Classification of interphase nuclear and spindle abnormalities
All interphase and metaphase stage nuclei were carefully examined and counted in each of the embryos. Abnormalities of interphase nuclei were classified according to the criteria set out in Table I. In all cases, including the identification of anucleate blastomeres, the cell boundaries were clearly visible because of background cytoplasmic labelling with either the anti-tubulin antibodies or DAPI. At the blastocyst stage, cell boundaries in the mural trophoblast cells were also visible, allowing the identification of some abnormalities, particularly binucleate blastomeres.
The criteria for classifying spindle abnormalities are specified in Table II. A spindle with astral-shaped or fusiform poles and with chromosomes aligned at the equator was classified as normal. If there were one or more DAPI-labelled presumptive chromosomes not aligned with the other chromosomes on the spindle, this was considered abnormal as it would rarely be observed with normal somatic cells, and was classified as chromosome loss. Because of the variability of the morphology of spindles in these early stages, spindles were only classified as having an abnormal shape if the spindle poles were very poorly defined or apparently absent, generally with misaligned chromosomes, after careful examination of optical sections using the confocal microscope. Finally, spindles with more than two clearly defined astral poles and the characteristic ‘Y’- or ‘X’-shaped arrangement of DAPI-labelled chromosomes were classified as multipolar, after careful appraisal by confocal microscopy, and, in some cases, labelling of centrosomes at the spindle poles with γ-tubulin.

Statistical analysis
Statistical analysis of the results was carried out with the SPSS 9 (SPSS Inc., USA) statistical package for Windows. Fisher’s exact test was used to determine whether the incidence of spindle abnormalities differed in the normally developing and arrested cleavage to blastocyst stage embryos between days 3 and 7 post-fertilization.

Results

Human embryo morphology and development
A total of 182 normally fertilized human embryos, donated from 62 couples, were examined between the 2-cell and blastocyst stages on days 3–7 post insemination. Of these, 102 (56%) embryos were classified as developing normally based on cleavage rate (see Materials and methods), 66 (36%) were arrested, mainly at cleavage stages, and the other 14 embryos (8%) were degenerate and/or completely fragmented and not analysed further. Cell numbers in normally developing embryos, estimated by counting nuclei (taking into account cells with two or more nuclei, where these were identified), showed an approximate doubling every 24 h from day 3 to 7 (Table III). Arrested embryos, by definition, had significantly fewer cells ranging from $2.3 \pm 0.2$ to $10.1 \pm 1.0$ on days 3 to 5 respectively. The mitotic index in normally developing embryos, based on the identification of α-tubulin-labelled metaphase spindles, ranged from 1.8 to 3.9%. Interestingly, none of the embryos arrested at early cleavage stages on day 3 had metaphase spindles, whereas on days 4 and 5 the mitotic index was not significantly different from normally developing embryos on the same day ($P > 0.05$).
Nuclear abnormalities

The incidence of nuclear abnormalities among both normally developing and arrested embryos (as defined in Table I) was relatively high and included embryos with either at least one binucleate, anucleate or multinucleate blastomere as the only abnormality or a combination of both anucleate and binucleate blastomeres (Table IV). Binucleate blastomeres were observed in normally developing and arrested embryos at all stages including the blastocyst stage. Eighteen out of 76 (23.7%) normally developing cleavage to morula stage embryos had binucleate blastomeres, 11 (14.5%) had anucleate and five (6.6%) had a combination of binucleate and anucleate blastomeres. At the blastocyst stage although the incidence per cell was low (observations mainly in the mural trophectoderm), every blastocyst had at least one binucleate cell. Embryos with multinucleate blastomeres were more frequent in the arrested group (15/66, 22.7%) (Fig. 3a,g) compared with those developing at the normal rate (7/76, 10%). In addition to these abnormalities, micronuclei and apoptotic nuclei (Table I) were also observed in both normal and arrested embryos at all stages. Micronuclei were observed in 8/76 (10.5%) normally developing cleavage to morula stage embryos and in 8/66 (12%) arrested embryos and apoptotic nuclei in seven (9%) and eight (12%) embryos respectively.

### Table II. Classification scheme for metaphase spindles with microtubules labelled with anti-tubulin antibodies and chromosomes with DAPI and visualized by confocal laser scanning microscopy

<table>
<thead>
<tr>
<th>Spindle type</th>
<th>Definition</th>
<th>Diagrammatic representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Astral-shaped or fusiform poles with chromosomes aligned at the equator</td>
<td><img src="https://example.com/diagram1.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Normal with chromosome loss</td>
<td>As above with one or more chromosomes away from the equator</td>
<td><img src="https://example.com/diagram2.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Abnormal shape</td>
<td>Poorly defined/absent poles and misaligned chromosomes</td>
<td><img src="https://example.com/diagram3.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Tripolar</td>
<td>Three defined poles giving the characteristic ‘Y’ shape</td>
<td><img src="https://example.com/diagram4.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Tetrapolar</td>
<td>Four defined poles giving the characteristic cruciform or ‘X’ shape</td>
<td><img src="https://example.com/diagram5.png" alt="Diagram" /></td>
</tr>
</tbody>
</table>

### Table III. Mean cell numbers and mitotic index of normally developing and arrested human embryos between days 3 and 7 post insemination

<table>
<thead>
<tr>
<th>Day of analysis</th>
<th>No. of embryos analysed</th>
<th>Mean cell no. ± SEM</th>
<th>Total no. of spindles</th>
<th>Mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normally developing cleavage and morula stage embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>6.7 ± 0.3</td>
<td>8</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>20.2 ± 1.5</td>
<td>18</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>36.0 ± 3.1</td>
<td>8</td>
<td>2.8</td>
</tr>
<tr>
<td>Blastocysts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>81.6 ± 7.1</td>
<td>38</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>92.5 ± 11.8</td>
<td>21</td>
<td>3.2</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>198.5 ± 35.8</td>
<td>7</td>
<td>1.8</td>
</tr>
<tr>
<td>Arrested cleavage stage embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>2.3 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>4.9 ± 0.7</td>
<td>5</td>
<td>8.6</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>10.1 ± 1.0</td>
<td>7</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>168</td>
<td></td>
<td>112</td>
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</table>

**Nuclear abnormalities**

The incidence of nuclear abnormalities among both normally developing and arrested embryos (as defined in Table I) was relatively high and included embryos with either at least one binucleate, anucleate or multinucleate blastomere as the only abnormality or a combination of both anucleate and binucleate blastomeres (Table IV). Binucleate blastomeres were observed in normally developing and arrested embryos at all stages including the blastocyst stage. Eighteen out of 76 (23.7%) normally developing cleavage to morula stage embryos had binucleate blastomeres, 11 (14.5%) had anucleate and five (6.6%) had a combination of binucleate and anucleate blastomeres. At the blastocyst stage although the incidence per cell was low (observations mainly in the mural trophectoderm), every blastocyst had at least one binucleate cell. Embryos with multinucleate blastomeres were more frequent in the arrested group (15/66, 22.7%) (Fig. 3a,g) compared with those developing at the normal rate (7/76, 10%). In addition to these abnormalities, micronuclei and apoptotic nuclei (Table I) were also observed in both normal and arrested embryos at all stages. Micronuclei were observed in 8/76 (10.5%) normally developing cleavage to morula stage embryos and in 8/66 (12%) arrested embryos and apoptotic nuclei in seven (9%) and eight (12%) embryos respectively.
Spindle abnormalities

A total of 112 α-tubulin-labelled spindles were identified in the 168 embryos analysed (Table III). Twenty-nine out of 76 (38.2%) normally developing cleavage to morula stage embryos and all blastocysts had at least one spindle (range 1–6) (Table V; Figures 1–4). In contrast, only 8/66 (12.1%) arrested embryos had spindles. Five out of the eight arrested embryos (62%) had at least one abnormally shaped spindle, whereas 20/29 (69%) normally developing cleavage to morula stage embryos on days 3–5 had only normal spindles, five (17.2%) had abnormally shaped spindles and four (13.8%) had multipolar spindles (Table V). All 26 blastocysts examined had at least one normal spindle, but four (15%) had a tripolar spindle and two (8%) had tetrapolar spindles in addition to normal spindles (Figures 2 and 4a). The proportion of normal spindles increased significantly from 50% in the normally developing cleavage stage embryos on day 3 to 87% in the day 5 blastocysts (P < 0.05). The overall incidence of spindle abnormalities was significantly higher in the arrested embryos compared to both the normally developing cleavage and blastocyst stage embryos (P < 0.05). Chromosome loss, observed as one or more DNA-labelled chromosomes separate from the spindle, was evident in 5/29 (17.2%) normally developing embryos at cleavage and morula stages associated with either normal or abnormally shaped spindles (Figure 1a,b). Of the 12 spindles analysed in the arrested embryos, three (35%) were normal and nine (75%) abnormal including four associated with chromosome loss (Figure 3c).

Distribution of acetylated and γ-tubulin

γ-Tubulin was detectable at the spindle poles of mitotically dividing blastomeres and in several cases confirmed that spindles were multipolar (Figure 4a,b). Acetylated tubulin antibodies strongly labelled spindle midbodies during telophase and the spindle poles during both metaphase and anaphase (Figure 4c–e). Extra sperm bound on the zona of IVF embryos also displayed strong immunolabelling for acetylated tubulin along their tails (data not shown).
Detailed examination of interphase nuclei and spindles in normally developing and arrested human preimplantation embryos in vitro by fluorescence and confocal laser scanning microscopy, following labelling with anti-tubulin antibodies and a DNA-specific fluorochrome, has confirmed the well-documented incidence of nuclear abnormalities (Winston et al., 1991; Hardy et al., 1993b) and provided the first direct evidence of spindle abnormalities, notably multipolar spindles, and chromosome loss in cleavage to early blastocyst stages. Although this study is descriptive, if these observations are confirmed using antibodies to other centrosomal proteins such as pericentrin in addition to α-tubulin, for example, they have important implications for the interrelationship between nuclear and spindle abnormalities, chromosomal abnormalities and developmental arrest.

Nuclear abnormalities, including binucleate, anucleate and multinucleate blastomeres, were common at all stages, particularly in cleavage stage and arrested embryos (Figure 3; Table IV). In addition, micronuclei (possibly arising from chromosome loss) and/or apoptotic nuclei were observed in ~20–25% of normally developing and arrested embryos between days 3 and 5 (data not shown). However, labelling of nuclei for DNA strand breaks has demonstrated that apoptotic nuclei are only present following compaction in normally developing embryos (Hardy, 1999).

Binucleate blastomeres are thought to arise through failure of cytokinesis during cleavage (Hardy et al., 1993b). In one arrested 2-cell embryo with an anucleate fragment, both of the blastomeres were tetranucleate, suggesting that cytokinesis had failed in two successive cleavage divisions (Figure 3a,b). Interestingly, in another arrested 4-cell embryo, there

**Figure 1.** Spindle abnormalities in normally developing cleavage stage human embryos. (a1–3) A 6-cell embryo with a bipolar spindle and two unattached chromosomes (arrows; a3). (b) Confocal section of a 4-cell embryo with uneven-sized blastomeres, cytoplasmic fragmentation and a tetrapolar spindle with typical cruciform arrangement of chromosomes and spindle poles and an unattached chromosome (arrow). Note the prometaphase rosette in the adjacent blastomere (arrowhead). α-Tubulin (green) and DAPI (blue).

**Figure 2.** Spindle abnormalities in normally developing human blastocysts on day 5 post-insemination. (a1) Human blastocyst on day 5 with a compact inner cell mass at top right and several spindles in the mural trophectoderm, including at higher magnification, (a2) a tripolar spindle, and (a3) two spindles, at prometaphase and telophase stages, and several midbodies (short and long arrows and arrowheads respectively). α-Tubulin (green) and DAPI (blue). (b1–2) Two confocal sections of a spindle with three distinct astral poles (arrow) and two normal anaphase stage spindles again in the mural trophectoderm of another human blastocyst on day 5. α-Tubulin (red) and DAPI (blue).
were two large binucleate blastomeres and two smaller anucleate blastomeres/cytoplasmic fragments, strongly suggesting that in this case, instead of failure of cytokinesis, the actin microfilaments of the contractile ring had been displaced to one end of the cell (Figure 3e,f). This may therefore represent one mechanism underlying the frequently observed phenomenon of cytoplasmic fragmentation, particularly if, in the absence of a nucleus, further cycles of aberrant cytokinesis continue, as occurs, for example, following enucleation of mouse zygotes (Petzoldt, 1990).

In the pig, actin microfilament polymerization has been shown to be important for oocyte maturation and early embryo development (Wang et al., 2000a) and is affected by the culture conditions (Wang et al., 2000b). Furthermore, in contrast to in vivo-derived embryos, those generated in vitro, by in vitro maturation of oocytes, fertilization and culture, were characterized by reduced perinuclear filamentous actin, binucleate and anucleate blastomeres and poor development to the blastocyst stage (Wang et al., 1999). The parallels with human preimplantation development in vitro following analysis by multicolour FISH with chromosome-specific probes. For example, sequential FISH analysis of nine chromosomes revealed multiple chromosome losses including several clones of cells missing one or more chromosomes separate from the spindle resulting presumably either from congression failure or anaphase lag (Figures 1a1–3, 3c,d). The latter could explain the frequent detection of chromosome loss in interphase nuclei from cleavage stage embryos following analysis by multicolour FISH with chromosome-specific probes. For example, sequential FISH analysis of nine chromosomes revealed multiple chromosome losses including several clones of cells missing one or more chromosomes of a particular pair in a small series of arrested cleavage stage embryos (Harrison et al., 2000). Of particular interest, however, is the observation of several tripolar (Figure 2a2, b1–2) and tetrapolar spindles (Figure 1b), with a characteristic ‘Y’- or cruciform ‘X’-shaped organization respectively, in some cases confirmed by γ-tubulin labelling of spindle poles (Figure 4a2–5), at cleavage and early blastocyst stages. Also, in a day 3 embryo, γ-tubulin labelling revealed three distinct foci at one pole and one at the other pole of a bipolar spindle, suggesting the presence of four centrosomes in an unbalanced arrangement (Figure 4b1–3). The localization of γ-tubulin at the spindle poles of mitotically dividing blastomeres and acetylated tubulin at midbodies during telophase and the spindle poles during both metaphase and anaphase are in agreement with previous reports on mouse and human oocytes (Schatten et al., 1988; George et al., 1996).

In humans, the centrosome of the zygote (the organizing centre of the mitotic spindle which is composed of two
Spindle and nuclear abnormalities in human embryos

Centrioles) is paternally inherited from the fertilizing sperm (Sathananthan et al., 1991). Following IVF and abnormal dispermic fertilization, three pronuclei are formed and the presence of two centrosomes most frequently results in a tripolar spindle, or less often in the formation of a bipolar spindle, when the supernumerary centrosome remains dormant (Plachot et al., 1989; Staessen and Van Steirteghem, 1997; Sathananthan et al., 1999). Segregation of the three sets of chromosomes on a bipolar spindle results in a uniformly triploid 2-cell embryo. However, tripolar spindle formation results in a mosaic embryo with three equally sized blastomeres at the first mitotic division, due to the random segregation of the sister chromatids of the three haploid sets of chromosomes to the three poles (Palermo et al., 1994; Staessen and Van Steirteghem, 1997; Sathananthan et al., 1999). By analogy, tripolar (or tetrapolar) spindles at cleavage and early blastocyst stages could also result in chromosomal malsegregation and explain the chromosomal chaos identified by multicolour FISH analysis in some cleavage stage embryos (Delhanty et al., 1997).

With human cells in culture, binucleate cells formed by failure of cytokinesis, which inherit both centrosomes from the previous division, either form a single bipolar spindle at metaphase resulting in two tetraploid daughter cells following mitosis or, if both centrosomes divide synchronously or asynchronously, form abnormal tetrapolar and tripolar spindles which can result in malsegregation of chromosomes to two or more daughter cells (Cimini et al., 1999). As binucleate blastomeres are common at preimplantation stages, we propose that this could be a major pathway resulting in postzygotic chromosomal abnormalities in the human embryo in vitro. We hypothesize therefore that failed or asymmetric

Figure 4. Distribution of \( \gamma \)-tubulin and acetylated tubulin in human preimplantation embryos. (a1–a6) High power confocal sections of two normal (a1, a6) and one multipolar spindle (a2–5) from a human blastocyst on day 5. Note the localization of \( \gamma \)-tubulin labelling at the spindle poles in each case [\( \alpha \)-tubulin (red), \( \gamma \)-tubulin (green) and DAPI (blue)]. (b1–b3) High power fluorescence images of a spindle from a cleavage stage embryo showing three discrete areas of \( \gamma \)-tubulin labelling at one pole (arrows). (b1) \( \alpha \)-Tubulin (green)- and \( \gamma \)-tubulin (red)-labelled image only; (b2) the triple-labelled image [\( \alpha \)-tubulin (green), \( \gamma \)-tubulin (red), and DAPI (blue)]; (b3) \( \gamma \)-tubulin (red)- and DAPI (blue)-labelled image only. (c–e) Examples of confocal images of spindles from a cavitating morula and blastocyst labelled with \( \alpha \)-tubulin (red), acetylated tubulin (green) and DAPI (blue). Note the strong labelling with the acetylated tubulin-specific antibody (green) of (c) the centre of the prometaphase rosettes, (d) midbodies (arrow), and (e) spindle poles (arrow).
cytokinesis results in the formation of binucleate blastomeres which secondarily leads to tetraploidy or spindle pole abnormalities and chromosomal chaos. Subsequent abnormal mitoses in these cells could then lead to higher order polyplody and, combined with non-disjunction and chromosome loss of single or more chromosomes, this would provide an explanation for all of the different aneuploides observed at cleavage and blastocyst stages. This model links binucleate cells with the generation of tetraploid and higher order polyplody and chromosomal chaos and predicts that nuclear and chromosomal abnormalities are interrelated through abnormalities in cytokinesis and spindle formation.

The relatively high incidence of postzygotic chromosomal abnormalities detected by multicolour FISH analysis of cleavage stage embryos led to the hypothesis that, as with some invertebrate and lower vertebrate embryos, cell cycle checkpoints may not operate during cleavage before global activation of the embryonic genome, resulting in genetic instability similar to that observed with human tumour cells (Delhanty and Handyside, 1995). In human embryos, global activation of the embryonic genome occurs on day 3 at the 4–8-cell stage (Braude et al., 1988; Taylor et al., 1997). However, some maternal proteins inherited in the unfertilized oocyte appear to persist throughout preimplantation development (Leese et al., 1991; Taylor et al., 2001). Abnormal mitoses in the first three cleavage divisions would therefore exit mitosis and generate daughter cells with abnormal chromosomal complements whereas at the blastocyst stage, a functional spindle assembly checkpoint would minimize their deleterious effects by arresting mitosis until the defect is corrected or by eliminating mitotically arrested cells by apoptosis, essentially as has been suggested for somatic cells (Rieder and Palazzo, 1992; Musacchio and Hardwick, 2002). In vertebrate somatic cells and sea-urchin zygotes, mitosis is prolonged 2–3-fold when monopolar spindles assemble (Sluder and Begg, 1983; Wang et al., 1983). However, Sluder et al. (1997), who analysed the duration of mitosis in sea-urchin zygotes containing tripolar or tetrapolar spindles, reported that unlike monopolar spindles, the presence of supernumerary spindle poles did not delay anaphase, suggesting that the checkpoint control for metaphase–anaphase transition does not monitor excess spindle poles or bipolar spindle symmetry. The authors concluded that animal cells do not seem to have a checkpoint for the metaphase–anaphase transition independent of the checkpoint that monitors kinetochore attachment to the spindle, and they proposed that the spindle assembly checkpoint is in effect the kinetochore attachment checkpoint.

As with the analogous genetic instability in human tumours, it is difficult to distinguish whether postzygotic nuclear and chromosomal abnormalities are the cause or consequence of developmental arrest. Furthermore, the identification of spindle abnormalities per se does not provide any information about the operation of cell cycle checkpoints, although most nuclei in arrested embryos were in interphase. However, the effects of these abnormalities on the development of human preimplantation embryos in vitro, whether primary or secondary, will depend on the stage at which they occur, the proportion of cells affected and the potential for further division. Clearly, if an abnormality arises in early cleavage then a large proportion of the embryo will be affected, whereas an isolated occurrence at the blastocyst stage may only have a marginal impact and chromosomally abnormal cells may be eliminated by apoptosis. For example, both blastomeres in the arrested 2-cell embryo shown in Figure 3a are tetranucleate. It is possible that failure of cytokinesis occurred in both blastomeres at the equivalent of both the second and third cleavage divisions and that tetrapolar spindles were formed as a result, producing the tetranucleate blastomeres. In this case, therefore, it seems highly unlikely that either blastomere would be viable but also that the nuclear abnormalities are secondary to the primary cause of cytokinetic failure.

Finally, the multipolar spindles observed at the blastocyst stage were in the mural trophectoderm (Figures 2a,b). In the mouse, tetraploid cells mainly contribute to the trophoderm at the blastocyst stage and are eliminated selectively from the inner cell mass lineage from which the fetus is derived (James et al., 1995). The latter has been proposed as a mechanism for confined placental mosaicism of aneuploid cells in human development (James and West, 1994). It is possible therefore that binucleate and/or tetraploid blastomeres normally contribute only to extraembryonic lineages and similarly a minority of aneuploid cells may simply be eliminated from the inner cell mass lineage. However, Evsikov and Verlinsky (1998) and Derhaag et al. (2003) reported the presence of aneuploid and tetraploid cells in both lineages in human blastocysts. Further research is therefore necessary to determine if this correlates with the presence of spindle abnormalities.

Multipolar spindles and chromosome loss inevitably lead to chromosomal malsegregation and may account for much of the observed postzygotic chromosomal mosaicism in human preimplantation embryos in vitro. Whether spindle abnormalities are limited to in vitro-produced embryos and are linked to parental infertility or culturing conditions, or whether they constitute part of normal development, warrants further investigation. Early identification of abnormal spindles may, however, allow better embryo selection in current IVF programmes. Polarizing microscopy has been employed for non-invasive visualization of the meiotic spindle in unfertilized oocytes to avoid damaging it during ICSI (Wang and Keefe, 2002a,b). It may therefore be possible to use a similar approach for non-invasive assessment of mitotic spindles in cleavage stage embryos. In combination with scoring for pronuclear morphology at the 1-cell stage (Gianaroli et al., 2003), this could provide an effective and non-invasive method for selecting viable embryos for transfer by identifying and rejecting those with extensive postzygotic chromosomal abnormalities.

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References


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