

## Rapid visualization of metaphase chromosomes in single human blastomeres after fusion with in-vitro matured bovine eggs

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**The present study was aimed to facilitate karyotyping of human blastomeres using the metaphase-inducing factors present in unfertilized eggs. A rapid technique for karyotyping would have wide application in the field of preimplantation genetic diagnosis. When cryopreserved in-vitro matured bovine oocytes were fused with human blastomeres, the transferred human nuclei were forced into metaphase within a few hours. Eighty-seven human blastomeres from abnormal or arrested embryos were fused with bovine oocytes in a preclinical study. Fusion efficiency was 100%. In 21 of the hybrid cells, no trace of human chromatin was found. Of the remaining 66, 64 (97%) yielded chromosomes suitable for analysis. The method was used to karyotype embryos from two patients with maternal translocations. One embryo which was judged to be karyotypically normal was replaced in the first patient, resulting in one pregnancy with a normal fetus. None of the second patient's embryos was diagnosed as normal, and hence none was transferred. The results of the present study demonstrated that the ooplasmic factors which induce and maintain metaphase in bovine oocytes can force transferred human blastomere nuclei into premature metaphase, providing the basis for a rapid method of karyotyping blastomeres from preimplantation embryos and, by implication, cells from other sources.**

*Key words:* electrofusion/FISH/nuclear transplantation/spectral karyotyping/translocation

### Introduction

The karyotyping of blastomeres isolated from cleaving embryos prior to embryo replacement offers a good example of a situation where the problems of conventional methods become acute (Munné and Cohen, 1998). In the best circumstances, only a relatively small proportion of the cells yield analysable metaphases (Santaló *et al.*, 1995). This can be frustrating when the cells are in short supply and time is limited, as in cases of preimplantation genetic diagnosis (PGD) involving

numerical and/or structural chromosomal abnormalities. In human precompaction embryos, the cells are in interphase for most of their approximately 16-hour cell cycle. A relatively small proportion of blastomeres, isolated at random from such embryos, can be expected to enter metaphase during the short period available for analysis. Even after overnight culture of isolated blastomeres in the presence of colcemid, only one-third of the cells produce metaphases suitable for karyotyping, but a lower efficiency is more common (Santaló *et al.*, 1995).

Cytoplasmic factors have immediate control over the cell cycle and thus govern the behaviour of the nucleus (Masui, 1996). Nuclear transplantation involving unfertilized sheep and cow eggs bears striking testimony to this. Such experiments were first undertaken in the 1980s with the aim of cloning embryos as a means of breeding livestock (Willadsen, 1986, 1989). It was found that viable embryos could be produced by transfer of a nucleus from a cleaving embryo to an enucleated metaphase II (M-II) oocyte, which was collected an estimated 10–12 h after completion of the first meiotic division. However, when freshly matured oocytes (collected 2–6 h after completion of the first meiotic division) were similarly tried, it was found that hardly any of the resulting nuclear transplant 'embryos' cleaved during culture. Instead, the transferred nucleus was arrested in a configuration that, at least superficially, looked very similar to an M-II. The explanation was thought to be that while the ooplasm of older oocytes was easily activated, making the transferred nucleus behave like that of a 1-cell embryo, the ooplasm of freshly matured oocytes was not activated by the procedure. In the latter instance, therefore, the composite cell produced by the nuclear transfer behaved, not as a 1-cell embryo, but as an unfertilized egg and, in accordance with this, the transferred nucleus assumed a metaphase configuration. The ability of such nuclear transplant eggs to emit a 'second polar body' upon subsequent activation lent support to this explanation (Willadsen, 1992).

These observations were confirmed with in-vitro matured cattle oocytes, where the inherent precise timing of meiotic events is more easily controlled and exploited (Willadsen, 1973, 1992). As expected, similar results were obtained when donor nuclei other than those of cleaving embryos were used (Willadsen, 1992). Similar events were described recently after fusion of mouse oocytes with spermatogenic cells (Sasagawa *et al.*, 1998). A primary spermatocyte nucleus injected into an oocyte undergoing the first meiotic division will arrange itself into an M-I spindle and emit a 'first polar body' and form a (male) M-II at the same time as the oocyte emits its first polar body and reaches M-II. If the oocyte is subsequently activated, a male pronucleus is formed from the spermatocyte-derived M-II after emission of a 'second polar body'.

The cytoplasmic factors and mechanisms governing nuclear behaviour appear to be similar in all mammals and, indeed, in all eukaryotes (Kishimoto and Okumura, 1997). In at least some instances, they have been shown to operate across species boundaries (Pavlath *et al.*, 1989; Willadsen, 1989; Mendonca *et al.*, 1991). The use of in-vitro matured bovine oocytes isolated from slaughterhouse material has become a routine procedure. With the above in mind, the present study was undertaken to examine the possibility of using freshly matured cattle oocytes to force transferred human nuclei into metaphase, thereby opening the way for rapid karyotyping of embryo-derived cells for preimplantation diagnosis of structural and numerical chromosome abnormalities. The study was aimed primarily at karyotyping of blastomeres in cases where alternative diagnostic techniques do not exist, are inadequate or have failed.

## Materials and methods

### *In-vitro maturation of bovine oocytes*

The bovine oocytes used in the present study were ordered from a commercial supplier (Biomed Inc., Madison, WI, USA) and shipped to the laboratory during maturation *in vitro*. The methods used for collection and culture of oocytes are described by the supplier. After 22 to 24 h of culture, the oocyte cumulus complexes were placed in PB1 (Whittingham, 1971) containing 5 mg hyaluronidase (Type VIII bovine testes; Sigma, St Louis, MO, USA) for 3 min and the cumulus cells removed mechanically. Morphologically normal oocytes with a polar body were frozen and stored at  $-192^{\circ}\text{C}$  until needed. Only oocytes that were judged to be morphologically normal after thawing and removal of the cryoprotectant were selected for the experiments.

In a preclinical study, blastomeres isolated from day 3 and day 4 embryos arrested at the 2- to uneven 8-cell stage were used. The zonae pellucidae were dissolved by brief exposure to  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free M2 containing 3 mg protease (*Streptomyces griseus*; Sigma). The blastomeres were separated after brief incubation at  $37^{\circ}\text{C}$  in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free M16 medium, using a fine-bore pipette. The media compositions were described by Hogan *et al.* (1994).

### *Micromanipulation*

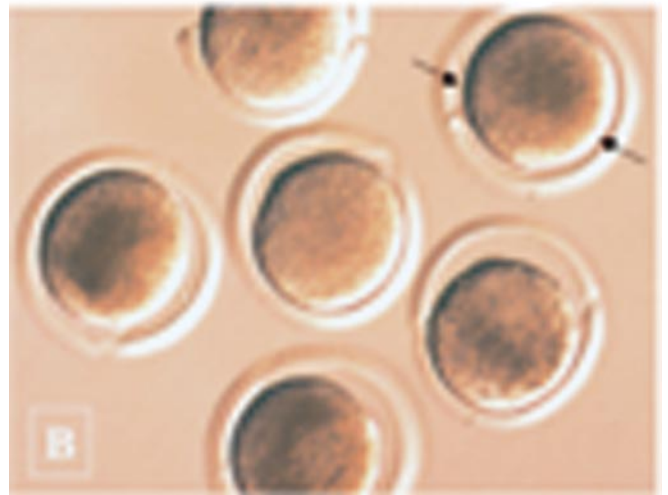
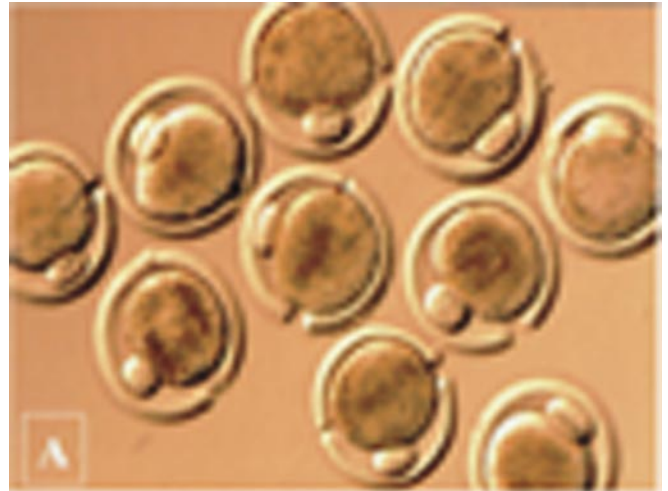
With a thin glass needle, a  $270^{\circ}$  equatorial slit was made in the zona pellucida of the bovine oocyte, centring over the first polar body. The polar body was removed with a fine-bore pipette, and a human blastomere was inserted into the perivitelline space, well clear of the slit in the zona (Figure 1).

### *Electrofusion*

The micromanipulated human–bovine cell pairs were placed in a solution of 0.3 mM mannitol, 0.1 mM  $\text{CaCl}_2$  and 0.1 mM  $\text{MgCl}_2$  and 5 mg bovine serum albumin/ml in water for 5 min. They were then transferred in the same medium to an electrofusion chamber with parallel electrodes (electrode distance 200  $\mu\text{m}$ ) and exposed to the following fusion conditions: 8 V AC/1000 kHz for 8 s followed by a single 24 V DC pulse lasting 99  $\mu\text{s}$ .

### *Cell culture and fixation*

After electrofusion treatment, the micromanipulated eggs were incubated in IVF-50 medium (Scandinavian IVF Science, Gothenburg, Sweden), and an hour later transferred to IVF-50 medium containing 0.02  $\mu\text{g}/\text{ml}$  colcemid (demecolcine; Sigma) and incubated for another 3 to 4 h. Demarcation lines were not always visible (Figure 1).



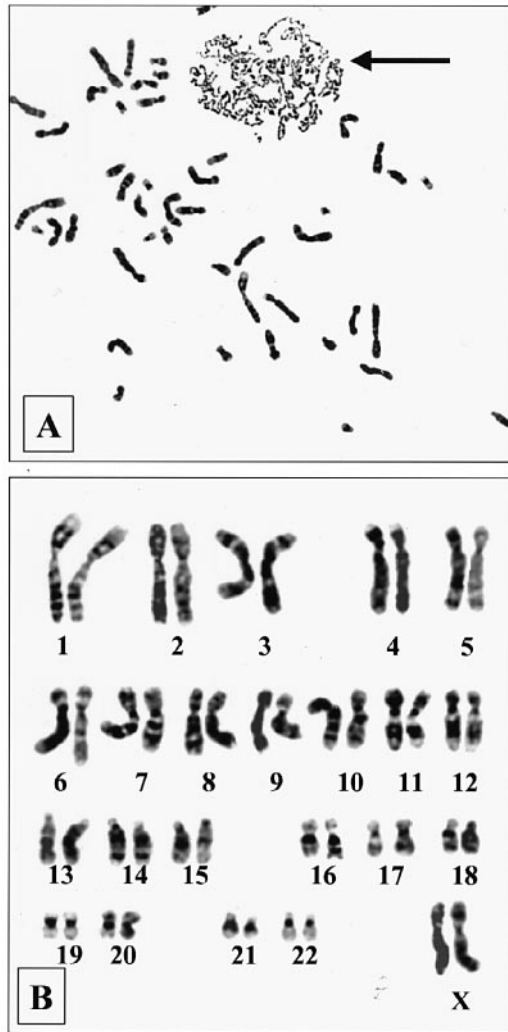
**Figure 1.** (A) Human blastomeres inserted under the micromanipulated zonae of thawed in-vitro matured bovine oocytes shortly before electrofusion. (B) Human blastomere–bovine oocyte hybrids 1 h after electrofusion. The arrows indicate a demarcation line still visible in one hybrid. Original magnification: A =  $\times 100$ ; B =  $\times 200$ .

The human–bovine hybrid cells were fixed on slides according to Tarkowski's method (1966) except that 0.02  $\mu\text{l}/\text{ml}$  colcemid was added to the hypotonic solution. A typical karyotype of a human blastomere after this procedure is depicted in Figure 2.

### *Clinical cases*

Two women carriers of reciprocal translocations underwent first polar body biopsy on their oocytes to determine which were chromosomally normal. Since first polar bodies are usually in metaphase at or shortly after oocyte retrieval, fluorescence in-situ hybridization (FISH) with chromosome painting probes can be used to analyse chromosome sets. However, a number of technical problems may occur when polar bodies are fixed (Munné *et al.*, 1998a,b). For instance, metaphase spreads may sometimes be suboptimal, hindering visualization of chromosomes; in other cells, there may be ooplasmic bridges, resulting in removal of metaphase chromosomes after polar body biopsy. For the second polar body or cleaving embryos, an alternative test may sometimes be required.

In two clinical cases, both involving a female carrier of reciprocal translocations, single blastomeres were biopsied from 8-cell stage



**Figure 2.** (A) Metaphase spread of a control blastomere obtained after fusion with a bovine egg. The human chromosomes are clearly condensed, whereas the bovine chromosomes are still in pro-metaphase (arrow). (B) Normal karyotype of human blastomere chromosomes depicted in panel A.

embryos resulting from fertilized eggs with dubious or no results after polar body analysis. The first case (A) concerned a 37-year-old woman carrier of a 46,XX,t(9;11)(p24;q12) translocation, from whom five mature eggs were obtained and biopsied. One embryo that had yielded unclear FISH signals on first polar body analysis, presumably because the cell was in advanced degeneration, was biopsied again on day 3, and the blastomere was processed using the fusion method described above.

The second case (B) concerned a 31-year-old woman carrier of 46,XX,t(11;16)(q21;q22). Eleven first polar bodies were biopsied. Eight of these either showed that the corresponding oocyte was unbalanced or did not fertilize. Three embryos were biopsied, and the extracted blastomeres were fused with frozen-thawed bovine oocytes. The fusion products were fixed as described above.

#### Fluorescence in-situ hybridization (FISH)

FISH was performed on the human metaphases by using whole chromosome painting probes. For case A, painting probes for chromosomes 9 (labelled in Spectrum Green; Vysis; Chicago, Illinois, USA) and 11 (labelled in Spectrum Orange; Vysis) were used in combination with a telomeric probe for 11q (biotin-labelled; Ning *et al.*, 1996)

and a centromeric probe for chromosome 9 (labelled in Spectrum Aqua; Vysis), as described previously (Munné *et al.*, 1998a,b). Repeated analysis of the same metaphases was performed using spectral karyotyping (SKY), which allows visualization of all 24 chromosomes. SKY hybridization was performed using the SKY™ kit (Applied Spectral Imaging Inc., Carlsbad, CA, USA) with a conventional FISH regime similar to the one described above and elsewhere (Márquez *et al.*, 1998).

The metaphases were evaluated with an Olympus BX60 fluorescent microscope equipped with a high-pressure mercury lamp for the excitation; a triple-band pass filter to detect Spectrum Aqua/Spectrum Orange/Spectrum Green dyes, another for 4',6-diamidino-2-phenylindole (DAPI) observation, and a third one for fluorescein isothiocyanate/propidium iodide observation. This microscope was also equipped with a SpectraCube™ SD 200 (Applied Spectral Imaging, Migdal HaEmek, Israel), which combines spectroscopy with imaging and enables the measurement of the visible spectrum for each pixel on the image observed. These metaphases were analysed using the inverted image of the DAPI stain, 'display colours' and 'spectra-based classification colours' simultaneously. 'Display colours' allows all chromosomes to be readily visualized after spectral imaging; while 'spectra-based classification colours' describes a chromosome classification algorithm based on spectral measurements at each pixel, permitting resolution of 24 colours (Schröck *et al.*, 1996).

For the second case, painting probes for chromosomes 11 (labelled in Spectrum Orange) and 16 (labelled in Spectrum Green) in combination with a centromeric probe for chromosome 11 (labelled in Spectrum Aqua) were used as described previously (Munné *et al.*, 1998a,b).

## Results

### Preclinical study

Eighty-seven human blastomeres ranging from 1/2 to 1/8 were coupled with bovine oocytes in the preclinical study. In all 87 pairs, fusion of the plasma membranes was evident within half an hour of the electrofusion treatment. For about an hour after fusion was complete, a demarcation line was observable between the human cytoplasm and the more heavily granulated bovine cytoplasm in a proportion of the hybrids (Figure 2). The hybrid cells were cultured for 3–4 h in medium containing colcemid before fixation. In 21 of the hybrid cells, no trace of human chromatin was found after fixation. Of the remaining 66, 64 (97%) yielded chromosomes suitable for analysis.

### Clinical study

Clinical results are shown in Table I. In case A, the 37-year-old female partner of the couple concerned had a history of habitual abortion and had been diagnosed as having a 46,XX,t(9;11)(p24;q12) karyotype. Five of the six recovered oocytes were mature, but their general morphology was poor. The oocytes underwent first polar body biopsy and intracytoplasmic sperm injection. In two oocytes, the chromosomal material of the polar bodies was too condensed to allow analysis. One of these two was the only oocyte that became fertilized normally and developed properly, but its second polar body was degenerated and could not be analysed for the translocation. Blastomere biopsy was therefore carried out on day 3, when the embryo had reached the 8-cell stage. A single blastomere was removed and fused with a frozen-thawed bovine M-II oocyte as

**Table I.** Results of chromosome painting in two translocation patients after polar body biopsy and blastomere biopsy using the metaphase promoting signals from fused bovine eggs

	1st polar body painting	Painting unfertilized egg	Blastomere painting	Oocyte or embryo diagnosis (fertilization status)
<b>First case</b>				
	9 der9 11 der11	9 der9 11 der11	9 der9 11 der11	
1	Egg damaged	Not analysed	Unfertilized	No result (unfertilized)
2	0 (2) 0 (2)	Not analysed	Unfertilized	Normal (unfertilized)
3	PB damaged	Not analysed	Unfertilized	No result (unfertilized)
4	Unclear	Not analysed	2 0 2 0	Normal (transferred)
5	0 (2) 0 (2)	Fertilized	Not performed	Normal (abnormal fertilization)
<b>Second case</b>				
	11 der11 16 der16	11 der11 16 der16	11 d11 16 der16	
1	Lost PB	Fertilized	0 1 1 0	Haploid (fertilized)
2	(2) <sup>a</sup> 0 (2) 0	3PN	Not performed	Balanced (abnormal fertilization)
3	(11,der11)(16,der16) <sup>b</sup>	(11,der11)(16,der16)	Unfertilized	Balanced (unfertilized)
4	Broken PB	Fertilized	2 cells without nucleus	No result (fertilized)
5	0 (2) 0 (2)	(2) 0 lost 0	Unfertilized	Normal (unfertilized)
6	Broken PB	Fertilized	1 1 1 0	Unbalanced (fertilized)
7	(2) 0 (2) 0	Not analysed	Unfertilized	Balanced (unfertilized)
8	Incomplete PB	(2) 0 (2) 0	Unfertilized	Normal (unfertilized)
9	(2) 0 1 0	Fertilized	Not performed	Unbalanced (fertilized)
10	0 (2) (2) 0	Fertilized	Not performed	Unbalanced (fertilized)
11	(2) 0 (2) (2)	Fertilized	Not performed	Unbalanced (fertilized)

<sup>a</sup>(2) reflects monovalent chromosomes with two chromatids.

<sup>b</sup>A result of recombination between chromatids and not whole chromosomes. If fertilized, abnormalities could have still occurred during the second meiotic division.

PB = polar body; der = derivative.

described above. On the basis of the FISH analysis of the metaphase-transformed blastomere nucleus, the embryo was judged to have a normal karyotype (Figure 3). Repeat analysis with SKY, although performed after replacement, confirmed the result. The embryo was replaced on day 4, resulting in a XX pregnancy. Amniocentesis performed at 18 weeks confirmed the outcome of the PGD. The baby was delivered at 200 g but suffered from ventricular septal defect (VSD), a congenital anomaly that is not known to be linked with translocations. Repair of VSD at 5 months resulted in a variety of complications. The outcome of this was unknown at the time of writing.

In case B, the female partner was a carrier of a 46,XX,t(11;16)(q21;q22) with a history of recurrent miscarriage. She produced 11 mature M-II oocytes, of which first polar body analysis was successful in seven (Table I). Of the four undiagnosed eggs, three were fertilized, and one or two blastomeres of each resulting embryo were biopsied. The extracted blastomeres were fused with bovine oocytes and the resulting metaphases were analysed. The two cells biopsied from one embryo appeared to be anuclear. The metaphases of the two remaining embryos indicated that they were chromosomally unbalanced. Similarly, all the informative polar bodies were either unbalanced or, when balanced or normal, the eggs did not become fertilized. Embryos were therefore not replaced.

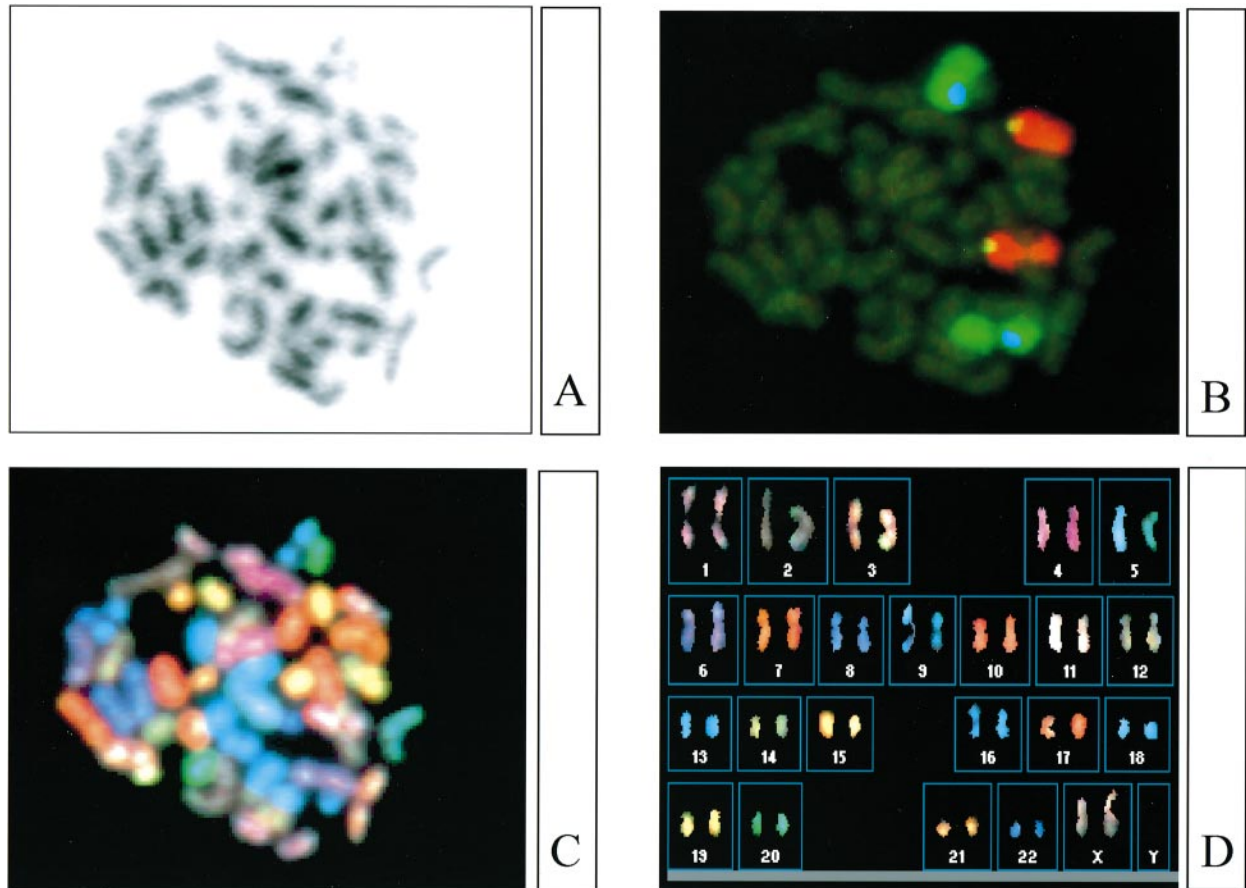
## Discussion

The results of the present study demonstrate that the ooplasmic factors, which induce and maintain metaphase in bovine oocytes, may indeed be used to force transferred human blastomere nuclei into premature metaphase, thereby providing the basis for a rapid method of karyotyping preimplantation

embryos. The vast majority of cumulus-cell-enclosed bovine oocytes isolated from ovarian follicles, with a diameter of more than 2 mm, will resume meiosis after being placed in culture and reach metaphase II within 20 to 22 h. Unless fertilized, nearly all such in-vitro matured oocytes will remain arrested in metaphase for at least another 24 h, provided their cumulus cells are not removed. Removal of the cumulus cells 32 h or later after the start of in-vitro culture tends to trigger activation of the oocyte. But prior to 32 h, an oocyte usually remains arrested, even if it is denuded of cumulus cells and also subjected to the electrofusion treatment employed in the present study (Willadsen, 1989, 1992). It is this time window of strong metaphase arrest that the present study aimed to exploit. However, preliminary experiments had shown that while fusion with bovine oocytes forced virtually all the blastomere nuclei into immediate mitosis, the arrest of such nuclei in metaphase was not entirely dependable. Hence the inclusion here of colcemid in the culture medium.

It is interesting to note that, although the ability of frozen-thawed bovine oocytes to develop to term after fertilization has been found to be low (Martino *et al.*, 1996; Izadyar *et al.*, 1998), such oocytes were quite adequate for the purposes of the present study, provided that they exhibited apparently normal morphology after thawing. This greatly simplifies the logistics of karyotyping by cell fusion.

It is likely that a range of nuclear phases, including developmental arrest, was represented among the blastomeres prior to cell fusion in the preclinical study. Yet at least two-thirds of the fused cells had metaphase sets which could be analysed even though it is possible that the efficiency was reduced due to the poor state of some of the donated material. In addition, the human nuclear material may have been lost during fixation. With more practice, the fixation efficiency should rise. It may



**Figure 3.** (A) Metaphase spread, under phase-contrast microscope of a blastomere from embryo 4 of case A [46,XX,t(9;11)(p24;q12)]. (B) The same metaphase after fluorescence in-situ hybridization with painting probes for chromosomes 9 (green) and 11 (orange), in combination with a telomeric probe for 11q (yellow) and a centromeric probe for chromosome 9 (blue). The results indicate that the cell was normal for these chromosomes. (C) The same metaphase after rehybridization with 24-colour painting probes and visualized with spectral karyotyping. (D) Whole karyotype of the same metaphase obtained with spectral imaging and showing that the cell was chromosomally normal for all 46 chromosomes.

also be that some of the cells were anucleate and represented type IV fragmentation (Warner *et al.*, 1998). In any event, it appears that the cell cycle status of the nucleus prior to fusion with the bovine oocyte is of little importance. Also, although blastomeres were the cells investigated in the present study, it is reasonable to expect that the method could be applied to other cell types.

In other preliminary experiments, enucleated freshly ovulated murine, rather than bovine oocytes, were used to provoke metaphase transformation. But both the frequency of metaphase transformation and the quality of the metaphases were very low. Somewhat better results were obtained by using two enucleated mouse oocytes for fusion with each blastomere, instead of one, which suggested that the relatively small ooplasmic volume of the enucleated mouse oocyte and its associated molecular signals were the main reasons for failure. If so, mouse oocytes should work well for karyotyping cells of substantially smaller volume than the blastomeres in the present study.

The method is well suited to determine any type of numerical and structural chromosome abnormality, and will be particularly useful for detecting translocations of paternal origin. Numerical chromosomal abnormalities in preimplantation

human embryos are very common (Munné *et al.*, 1995). The molecular techniques of polymerase chain reaction and FISH are powerful diagnostic tools and are widely used today in clinical human genetics and the study of human development (Munné and Cohen, 1998). However, such diagnostic methods have major drawbacks. Firstly, in interphase cells, only a few chromosomes can be visualized with FISH and, in randomly sampled cells, metaphase chromosomes are rarely available for analysis. Complete metaphase karyotypes can provide much more information and do not require a patient-specific approach. Secondly, only maternally derived translocations can be investigated using painting of metaphase-stage chromosomes of first polar bodies. Transformation of cells into metaphase, as proposed above, has the advantage that paternal as well as maternal translocations can be determined in blastomeres without the development of case-specific probes. Consequently, metaphase transformation by cell fusion would allow the differentiation between normal, balanced and unbalanced karyotypes (Cassel *et al.*, 1997; Conn *et al.*, 1998; Munné and Cohen, 1998). The present method for metaphase transformation of single cells by cell fusion can help to overcome these problems by allowing metaphases to be studied with conventional karyotyping.

With the advent of spectral imaging, it is now possible to detect all of the 24 different human chromosomes in polar bodies and blastomeres (Márquez *et al.*, 1998). Although the method could possibly be applied to interphase nuclei, the number of discernible pixels left for analysis would probably be too small to produce a complete chromosome count. Detection of structural chromosomal abnormalities using spectral imaging may only be possible with metaphase chromosomes. PGD involving full karyotyping, rather than analysis of a limited number of chromosomes, might enable more strict embryo selection. This might lead in turn to improvements in implantation and ongoing pregnancy rates. The present method of karyotyping will also be of value in the scientific study of embryonic development, and has a number of other potential fields of applications because of its modest time requirements and apparent effectiveness for analysis of non-dividing cells.

In conclusion, metaphase chromosomes of human preimplantation embryos can now be visualized just a few hours after blastomere biopsy. The method whereby this may be achieved relies on such general principles that its usefulness, in all likelihood, extends to cells other than blastomeres and, indeed, to other species than the human. It is interesting to note that this diagnostic tool is based on nuclear transplantation experiments aimed at the cloning of livestock.

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