Original Article

Cytogenet Genome Res 105:29–35 (2004)
DOI: 10.1159/000078006

Cytogenetic and Genome Research

The use of a cell-cycle phase-marker may decrease the percentage of errors when using FISH in PGD

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Abstract. Fluorescent DNA probes are used to characterise the chromosome constitution of preimplantation embryos. FISH is used to select normal or balanced embryos in carriers of balanced chromosomal rearrangements, for embryo sexing or for aneuploidy screening in women of advanced age, who have had recurrent abortions or IVF failures. In most cases, FISH is performed on interphase blastomeres which are asynchronously dividing cells, that can be in G1, S or G2. However, a correct interpretation of a double FISH signal, which may correspond to a split signal, to a replicated chromosome region or to the presence of an extra chromosome is essential to establish an accurate diagnosis. To determine if the cell stage could influence the interpretation of FISH results, we compared the signal characteristics of one locus-specific probe, two different subtelomere region probes, and a centromere region probe in non-dividing Sertoli cells and in proliferating lymphocytes. Most cells had two signals per chromosome pair (i.e., a situation corresponding to G0 in Sertoli cells and to G1 or to a prereplication stage in lymphocytes). Nevertheless, in proliferating cells the percentage of nuclei with a number of signals different from the expected (two unreplicated chromosomes per pair) was different from that found in non-dividing cells ($P < 0.05$). It was estimated that 10.8% of double dots in dividing cells resulted from DNA replication. The sequence of replication was first the locus-specific region, second a telomere region, and third the centromere. In conclusion, the DNA replication process could result in errors of interpretation (misdiagnosis) in 7% of proliferating cells. Thus, the use of a cell cycle phase-specific marker could avoid errors by indicating the cell stage in which the nucleus analysed is found.

Preimplantation Genetic Diagnosis (PGD) using fluorescence in situ hybridisation (FISH) is extensively used to detect structural (Conn et al., 1998; Van Assche et al., 1999; Coonen et al., 2000; Iwarsson et al., 2000; Magli et al., 2001; Durban et al., 2001; Kuliev, 2002) and numerical (Preimplantation Genetic Screening: PGS) (Munné et al., 1998; Gianaroli et al., 1999; Egozcue et al., 2002; Kuliev et al., 2003) chromosome alterations and has represented an important advance in the detection of these abnormalities (Kahraman et al., 2000; Braude et al., 2002; ESHRE PGD Consortium Steering Committee, 2002). The aim of PGD is to avoid the birth of chromosomally abnormal children and to improve implantation and pregnancy rates. When performing PGD, one or two blastomeres of a 6–8-cell embryo are biopsied and analysed. Blastomeres are dividing cells and although they are frequently in interphase, it is not possible to distinguish between G1, early- or late-S (chromatin replication is bimodal and occurs gradually) or G2. When hybridising to a blastomere interphase nucleus, probes which produce a discrete signal are preferred (centromeric, locus-specific and telomeric) because whole-chromosome painting probes produce diffuse, overlapping signals.

Supported by Ministerio de Sanidad (FIS PI-020168), DURSI (2001 SGR-00104) and Fundació Catalana Síndrome de Down/Marató de TV3 (1994-98). This work has also been supported by Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (Aïda Pujol has a Beca Predoctoral de Formació de Personal Investigador; FI/FIAP).

Received 29 August 2003; manuscript accepted 5 December 2003.

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0301–0171/04/1051–0029$21.00/0

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When performing a PGD by FISH, one of the most serious difficulties is to identify if double FISH signals are simply splits signals (double dots related to the probes used), result from the replication of the region hybridised, or correspond to an extra chromosome. This difficulty is likely to be a frequent reason for embryo misdiagnosis. For example, three dots could either correspond to a trisomy or to a disomy with splitting or replication of one of the signals; the interpretation of a double dot could either be an euploidy or, if the dots are very close, a monosomy with a split or a replicated dot.

To correctly count the number of chromosomes obtained in a hybridised cell, and to avoid misdiagnoses, some standards are applied depending on the size of the nucleus and the characteristics of the chromatin. For instance, in blastomeres, most groups practising clinical PGD consider that two chromosomes are present when the FISH signals are separated by more than the distance which would allow for two additional signals (Dailey et al., 1996).

However, even using this criterion, and knowing that the efficiency of the probes most frequently used in PGD has already been confirmed in other kinds of cells as well as in clinical diagnoses (Tepperberg et al., 2001), misdiagnosis could occur as a result of the different replicative stages in which the blastomeres analysed may be, and also because in the same cell, replication does not occur synchronously. Thus, a cell-cycle replication marker could facilitate this interpretation and reduce the number of misdiagnoses.

In order to evaluate the number of confusing signals which could lead to an error because of the replication characteristics mentioned above, the following delineating experiment has been carried out: FISH analysis was applied to two types of cells; non-dividing cells which were in the G0 stage of the cell cycle (Sertoli cells) and proliferating cells (stimulated lymphocytes) considered for this reason to be analogous to blastomeres.

The evaluation of the frequency of deviant FISH signal patterns (more or less than two signals for each chromosome pair in one cell) in dividing and non-dividing cells using centromere, locus-specific and subtelomere region probes was used to investigate if the replication process may be a cause of misdiagnosis.

Previous studies have shown the importance of the spread nucleus diameter in relation to FISH error (Munné et al., 1996; Velilla et al., 2002). For this reason, we chose, for the analysis, a cell type with a similar nuclear diameter: Lymphocytes and Sertoli cells (21.5 and 24.1 μm respectively). Other cell types, like oral epithelia, were discarded for this study due to their being half of the size of a lymphocyte's nucleus.

Chromatin is in a different conformation in undifferentiated cells (like blastomeres), in differentiated cells (like Sertoli cells) and in reprogrammed cells (like lymphoblasts), and fixed blastomeres usually have a larger nuclear size than the cells used for the study. For these reasons, the results of this study are not entirely analogous to what can happen in a blastomere, but since both blastomeres and lymphocytes are proliferating cells, the results could be used as an approximation to the diagnostic difficulties encountered when interpreting FISH signals in blastomeres.

Materials and methods

Lymphocytes were obtained from a non-synchronised standard culture from an 18-year-old blood donor. Cells were cultured and harvested using standard methods. Sertoli cells were obtained from a testicular biopsy from a different donor with a normal karyotype. The biopsy was made under informed consent and due to the study of an idiopathic sterility and was treated in order to obtain meiotic chromosomes (Chandley et al., 1994). The study was approved by the local ethics committee. Both types of cells were fixed with Carnoy (methanol:acetic acid, 3:1) and spread onto degreased slides.

To eliminate cytoplasmic debris, a 3–10 min enzymatic treatment using pepsin (Sigma, Spain) was applied (50 μg/ml in 10 mM HCl at 37 °C, rinsing at room temperature with purified water and allowed to air-dry) before adding a drop of freshly prepared Carnoy fixative.

A standard FISH protocol was applied to the slides using centromeric probe (CEP) 17 (band region 17p11.1→q11.1, locus D17Z1 at 17cen labelled with SpectrumOrange and SpectrumGreen), locus-specific (LSI) 13 (detected RB1 gene at 13q14, labelled with SpectrumGreen) and telomeric (Tel) probes that detected unique DNA sequences located in chromosome 14q (STS-X58399, SHGC-36156, STS-AA034492, telomeric IGHV segment) for lymphocytes or chromosome 19q (D19S238E) for Sertoli cells (both labelled with SpectrumOrange) (Vysis, Downers Grove, IL, USA). Prior to FISH, preparations were treated with 50 mM $MgCl_2$, PBS (4 min), post-fixed with 3% formaldehyde in 50 mM $MgCl_2$, PBS (8 min), cleansed in PBS (4 min) and dehydrated in 70, 80 and 95% ethanol (2 min each), air dried and then dried on a hotplate at 45 °C for 4 min. The hybridisation solution was applied (5 μl) to each slide and covered with a 20 × 20 mm coverslip. The slides were sealed with rubber cement and placed on a hotplate for 5 min at 72 °C to co-denature nuclear DNA with the probes; they were then allowed to hybridise overnight at 37 °C in a dark, moist chamber. The slides were then washed using the rapid-wash procedure recommended by the probe's manufacturer. Slides were mounted with 8 μl DAPI II (Vysis). Visualisation was made under an Olympus BX 60 microscope equipped with a high-sensitivity camera (Roger Scientific, Photometrics; Tucson, Arizona USA) and with a triple-band pass filter and connected to a Power Macintosh G3 computer with software for Smartcapture (Digital Scientific; Cambridge, UK), which helped during the analysis.

Following the recommendations of the statistician, FISH signals of 200 interphase nuclei of each type of cell were scored.

Sertoli cells and lymphocytes were used to allow for the diagnosis of a high number of cells from the same individual. If blastomeres had been used, the study would have been impossible, and even if a good number of them could have been analysed, they would have come from different embryos from different donors and, consequently, the results would probably have shown a higher degree of variability.

The lymphocytes analysed came from a non-synchronised standard culture to obtain interphase nuclei in each of the three phases of the cell cycle (G1, S or G2) and, in addition, in different stages of the replicating phase (early- to late-S phase). The analysis of non-dividing cells allowed for the evaluation of the errors which may be related to factors other than replication, such as FISH artifacts which, on the other hand, were also expected to be present in proliferating cells.

Results and discussion

FISH signals were scored in 200 interphase nuclei of each type of cell. This number of cells is statistically sufficient, especially because the aim of the study was not diagnostic but rather designed to analyse a general phenomenon related to the stages of the cell cycle and the replication phase. The value of P is, in contrast to β error, independent of size of the sample. Thus, our main conclusion of differences, with $P < 0.05$, between proliferating or non-dividing cells, is independent of the number of cells in each group.

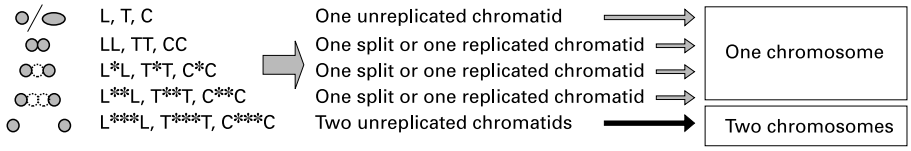

Fig. 1. Classification used when counting the FISH signals and conversion into number of chromosomes (L = LSI 13; T = Tel 14 and 19; C = CEP 17).

Table 1a. Distribution of LSI probe signals

Homologous region		Sertoli cells		Lymphocytes	
HR 1	HR 2	Frequency	Percentage	Frequency	Percentage
L	L	163	81.5	115	57.5
LL	L	9	4.5	39	19.5
LL	LL	4	2	11	5.5
L*L	-	2	1	1	0.5
L*L	L	14	7	12	6
L*L	LL	1	0.5	6	3
L*L	L*L	3	1.5	1	0.5
L***L	-	3	1.5	2	1
L***L	L***L	1	0.5	2	1
L	-			4	2
L	L***L			3	1.5
LL	-			1	0.5
L*L	L***L			2	1
L**L	L**L			1	0.5
Total		200	100	200	100

Table 1b. Distribution of Tel probe signals

Homologous region		Sertoli cells		Lymphocytes	
HR 1	HR 2	Frequency	Percentage	Frequency	Percentage
T	–	2	1	5	2.5
T	T	129	64.5	91	45
TT	–	1	0.5	4	2
TT	T	10	5	14	7
TT	TT	5	2.5	6	3
TT	T*T	2	1	7	3.5
T*T	–	6	3	4	2
T*T	T	18	9	18	9
T*T	T*T	5	2.5	10	5
T*T	T**T	1	0.5	1	0.5
T**T	–	3	1.5	9	4.5
T**T	T	3	1.5	4	2
T***T	–	10	5	5	2.5
T***T	T	3	1.5	5	2.5
T***T	T*T	1	0.5	2	1
T***T	T***T	1	0.5	3	1.5
–	–			3	1.5
TT	T***T			3	1.5
T**T	TT			1	0.5
T**T	T**T			4	2
T***T	TT			1	0.5
Total		200	100	200	100

Chromosomal regions and FISH signals

The FISH signals obtained by LSI and Tel probes were mostly small and well defined, while the ones for the CEP probes were larger and sometimes blurred. It has been described that chromosomes in interphase are very similar in length to metaphase chromosomes, and they are structured with a DNA-specific banding pattern characterised by the stretching of G^- bands but not of G^+ bands (Lemke et al., 2002). However, centromere signals are often larger than LSI or Tel signals.

FISH signals were classified depending on the distance between them (Fig. 1). We found single signals for LSI, Tel and CEP probes (L, T and C); double signals close together (LL, TT and CC), double signals separated by a distance which would allow for one additional signal (L*L, T*T and C*C), double signals separated by a distance which would allow for two additional signals (L**L, T**T and C**C), and double signals separated by a distance which would allow for more than two additional signals (L***L, T***T and C***C).

The results obtained are represented in Tables 1a–c. In these tables it can be seen that lymphocytes had a higher variability of signals than Sertoli cells. We have assumed that this variability was probably related to the fact that lymphocytes were replicating, while Sertoli cells were in G0. Aneuploidy could be another reason for this variability, but since the blood came from a young donor with a normal karyotype and the probability of having aneuploidies previous to the culture was considered very low, the initial aneuploidy rate in his blood

Table 1c. Distribution of CEP probe signals

Homologous region		Sertoli cells		Lymphocytes	
HR 1	HR 2	Frequency	Percentage	Frequency	Percentage
C	–	1	0.5	3	1.5
C	C	82	41	115	57.5
CC	–	28	14	4	2
CC	C	23	11.5	38	19
CC	CC	8	4	7	3.5
CC	C*C	1	0.5	3	1.5
C*C	–	8	4	3	1.5
C*C	C	25	12.5	15	7.5
C*C	C*C	6	3	1	0.5
C**C	–	8	4	1	0.5
C**C	C	1	0.5	1	0.5
C***C	–	3	1.5	1	0.5
C***C	C	5	2.5	5	2.5
C***C	CC	1	0.5	1	0.5
CC	C**C			1	0.5
CC	C***C			1	0.5
Total		200	100	200	100

Table 2a. Number and percentage of cells with 0, 1, 2, 3 and 4 chromosomes when LSI probe was applied

LSI	Sertoli cells		Lymphocytes	
No. chromosomes	Frequency	Percentage	Frequency	Percentage
0				
1	2	1	6	3
2	197	98.5	187	93.5
3			5	2.5
4	1	0.5	2	1
Total	200	100	200	100

Table 2b. Number and percentage of cells with 0, 1, 2, 3 and 4 chromosomes when Tel probe was applied

Tel	Sertoli cells		Lymphocytes	
No. chromosomes	Frequency	Percentage	Frequency	Percentage
0			3	1.5
1	12	6	22	11
2	183	91.5	161	80.5
3	4	2	11	5.5
4	1	0.5	3	1.5
Total	200	100	200	100

Table 2c. Number and percentage of cells with 0, 1, 2, 3 and 4 chromosomes when CEP probe was applied

CEP	Sertoli cells		Lymphocytes	
No. chromosomes	Frequency	Percentage	Frequency	Percentage
0				
1	45	22.5	11	5.5
2	149	74.5	182	91
3	6	3	7	3.5
4				
Total	200	100	200	100

culture was probably minimal. Aneuploidy could originate as a consequence of the 72-hour blood culture performed; in three mitotic divisions there was enough time to introduce some mitotic errors.

Number of chromosomes

The number of chromosomes was counted under the criterion that two chromosomes were considered when the signals were separated by more than the distance which would allow for two additional signals. L, T, C, LL, TT, CC, L*L, T*T, C*C and L**L, T**T, C**C signals were counted as one chromosome; each L, T and C was considered as a single chromatid signal and each LL, TT, CC, L*L, T*T, C*C, L**L, T**T, C**C as single split signals or as corresponding to replicated chromatids. L***L, T***T and C***C signals were counted as two chromosomes, i.e., two unreplicated chromatids (Fig. 1).

For LSI signals (Table 2a), nuclei with one, two and four chromosomes were found in both types of cells, while three chromosomes were only present in lymphocytes. Regarding Tel signals (Table 2b), one, two, three and four chromosomes were found in both types of cells, while the absence of both chromosomes was only observed in lymphocytes. For CEP signals (Table 2c), one, two and three chromosomes were found in both types of cells.

Most cells had two chromosomes of each pair. The frequencies for Sertoli cells and lymphocytes were, respectively: 98.5 and 93.5% for LSI (Table 2a), 91.5 and 80.5% for Tel (Table 2b) and 74.5 and 91% for CEP probes (Table 2c).

Comparing these results for each chromosome using Fisher's exact test, it was found that the percentage of cells with two, or with a number different from two chromosomes detected with LSI, Tel or CEP probes in Sertoli cells, was statistically different from the percentage obtained in lymphocytes ($P < 0.05$). The frequency of cells with two chromosomes of each pair was higher in Sertoli cells than in lymphocytes for LSI and Tel probes, but for the CEP probe it was lower, while there was a higher number of cells with only one chromosome.

It is worth pointing out that there were 45 Sertoli cells (22.5%) with only one large signal for the centromere (Table 2c). It has been described that in Sertoli cells, there is a tendency of homologous chromosomes to associate, mainly chromosomes 3, 7, 8, 13, 17 and 21 (Chandley et al., 1996). In our study, CEP 17 was used, and centromeres are mostly made of heterochromatin, which means that they are generally late replicating. Probably, most of the large single signals found corresponded to non-replicated, associated homologous chromosomes. If this is taken into account, 74.5% of Sertoli cells with two chromosomes (Table 2c) would increase to nearly 97%. Thus, as an additional and unexpected bonus, the use of CEP 17 (which would not be considered to be the best of choices in a cell type where chromosomes 17 tend to associate) showed that in these situations, a single, large dot can be reliably considered as a result of the association of two unreplicated centromeres.

Chromosome 13 was also hybridised, but using an LSI probe which binds a euchromatic region, and a single signal for chromosome 13 was almost never found; this is in agreement with the observation that these regions are early replicating.

See Figs. 3 and 4.

Possible errors related to the interpretation of FISH signals

In lymphocytes, FISH errors for each chromosome region were identified by adding the frequencies of signal patterns which were deviant from the presence of two chromosomes (Tables 2a–c).

It was deduced that in lymphocytes LSI and CEP produced a much lower number of FISH errors (6.5 and 9%, respectively) than Tel probes (19.5%). This suggests that Tel probes should be used carefully when FISH is applied to a proliferating cell.

The differences in the percentages of Sertoli cells and lymphocytes with two chromosomes of each pair were probably related to the proliferating state of lymphocytes; consequently to analyse a cell which is replicating can cause errors in the diagnosis.

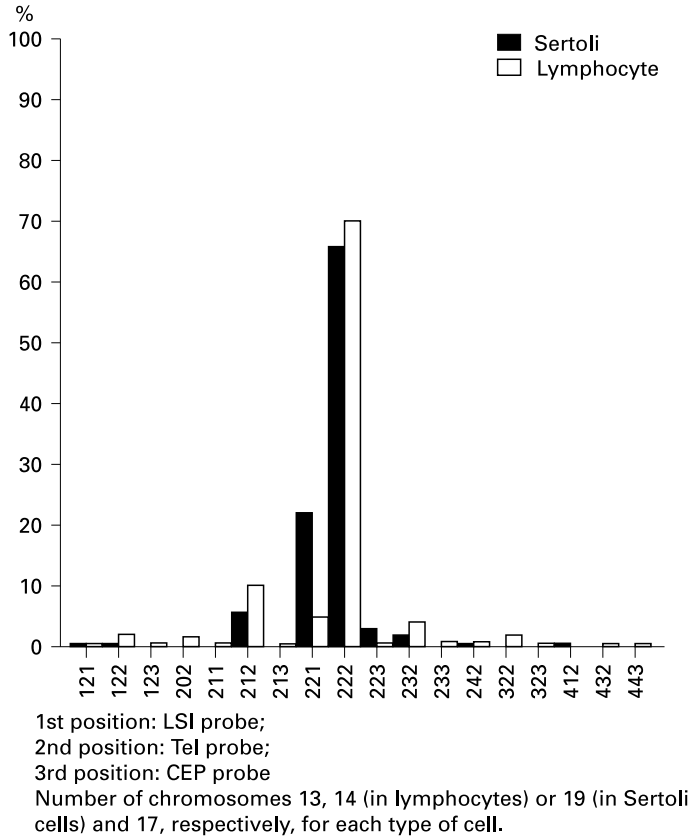

Fig. 2. Distribution of the number of chromosomes obtained in each cell type.

The distribution of the number of chromosomes in Sertoli cells and in lymphocytes (Fig. 2) was attained by combining the results obtained with the three types of probes in each cell. This distribution indicated that most cells have two chromosomes for each of the three chromosome pairs analysed, specifically 70% of lymphocytes and 65.5% of Sertoli cells. We considered that this 65.5% could change to 87.5% if 22% of the cells with two chromosomes for LSI and Tel probes, but only one CEP signal, is added to the total (as stated earlier, one large CEP signal was considered to be the same as two associated simple dots). The combinations of chromosome numbers are difficult to explain, i.e., those which included more than one monosomy, trisomy or a tetrasomy, were the ones least represented in our cultures, and they could be considered as artifacts. On the other hand, those results in which no signal for one chromosome was present could be considered as FISH failures. In Sertoli cells, these errors were 1.5% and, in lymphocytes, they were 7% (Fig. 2). This frequency (7%) corresponds exactly to the rate of error estimated by an experienced group when performing PGS using FISH (Munné et al., 2002). This error would result from the fact that the cells analysed are proliferating, and the cell stage cannot be identified.

3

4

Fig. 3. A lymphocyte considered normal with two single signals (C C) for CEP (yellow), two single signals (L L) for LSI probe (green) and two double signals close together (TT TT) for Tel probe (red).

Fig. 4. A Sertoli cell with two single signals (L L) for LSI (green), two single signals (T T) for Tel probe (red) and one large signal for CEP (yellow) considered to correspond to non-replicated associated homologous chromosomes.

Table 3. Combination of signals representing two chromosomes

LSI	Tel	CEP	Sertoli %	Lymphocyte %
* *	* *	* *	34.4	23.6
		* **	19.8	11.4
		** **	9.2	2.1
	* **	* *	7.6	11.4
		* **	5.3	4.3
		** **		1.4
	** **	* *	3.1	6.4
		* **	2.3	2.9
		** **	.8	
* **	* *	* *	5.3	10.0
		* **	4.6	2.9
		** **		1.4
	* **	* *	.8	2.1
		* **	1.5	1.4
		** **	.8	1.4
	** **	* *		5.0
		* **		2.9
		** **	.8	
** **	* *	* *	1.5	3.6
		* **		2.9
		** **		.7
	* **	* *	.8	.7
	** **	* *	1.5	1.4

* *: A simple signal for each homologous region.
* **: One homologous region with a single dot and the other with a double dot.
** **: Both homologous regions with a double dot.

Were all double signals replicated chromatin or were they split signals?

To distinguish between possible split signals (double dots from the probe itself due to the characteristics of the target DNA) and double dots which appear after replication, the number of dots found in those cells in which it was concluded that two chromosome pairs were present was analysed (Table 3).

Since Sertoli cells do not replicate, if a double dot was found, it was considered a split signal. Taking a similar splitting frequency in lymphocytes into account, we estimated that the excess frequency of double dots found in lymphocytes resulted from the replication process.

There were 34.4% of Sertoli cells with single dots for the three chromosome pairs analysed (Table 3); consequently, 65.6% had splits in some of the chromosomes studied. There were 23.6% of lymphocytes with single dots for the three chromosome pairs analysed; consequently, 76.4% of cells had one or two double dots for some of the chromosomes studied which could be either splits or the result of replication. Since 65.6% of double dots were considered splits in Sertoli cells, the extra 10.8% of double dots found in lymphocytes were probably the result of replication.

By analysing the presence of a single dot or double dots with LSI, Tel or CEP probes (Table 4), the timing of replication for these different regions of the chromosome could be estimated. As mentioned, Sertoli cells do not replicate, and double dots found in those cells were considered split signals (17.5% for LSI, 25.2% for Tel and 22.5% for CEP). The same percentage of splits was expected in lymphocytes. The frequency of double dots (one or two) due to replication in lymphocytes was then obtained by subtracting the double dots considered as splits from the total number of double dots (36.5% for LSI, 41.4% for Tel and 35.8% for CEP). We obtained 19% of lymphocytes with double LSI dots due to replication, 16.2% for Tel and 13.3% for CEP. The region with a higher frequency of double dots due to replication would be the one that replicated earlier. This meant that, probably, the region labelled by the locus-specific probe replicated first, followed by telomeres, while centromeres were the last to replicate.

Table 4. Percentage of cells with single and double FISH signals for LSI, Tel and CEP probes

	LSI		Tel		CEP	
Dots	Single	Double	Single	Double	Single	Double
Lymphocyte	63.5	36.5	58.6	41.4	64.2	35.8
Sertoli	82.5	17.5	74.8	25.2	77.5	22.5
Signals due to replication		19		16.2		13.3

Was replication synchronous for the homologous regions of the two chromosomes of each pair?

In order to know whether replication occurred synchronously for each probe, cells with one double dot and those with two double dots were counted (Table 5). In general, there were more cells with only one homologous region replicated (one double dot) than with both (two double dots). This meant that, in the lymphocyte culture, replication occurred asynchronously. For

Table 5. Percentage of cells with one and two double FISH signals for LSI, Tel and CEP probes

	LSI		Tel		CEP	
Dots	1 double	2 doubles	1 double	2 doubles	1 double	2 doubles
Lymphocyte	27.2	9.3	22.8	18.6	30.9	4.9
Sertoli	13.7	3.8	6.8	18.4	10.9	11.6
Signals due to replication	13.5	5.5	11.2	0.2	20	- 6.7

the LSI probe, the difference was already observed in Sertoli cells, i.e., there were more splits (as indicated, double dots in Sertoli cells were considered splits) which affected only one homologous region rather than both. For Tel and CEP, although replication also occurred mostly asynchronously, there were more splits which affected both homologous regions rather than just one. This could be related to the conformation of the chromatin of telomeres and centromeres in differentiated cells such as Sertoli cells.

Conclusion

FISH is a successful technique and is widely used for PGD and PGS (ESHRE PGD Consortium Steering Committee, 2002). Misdiagnoses which are uncommon in PGD and PGS, at least as far as false negatives are concerned, may result from the observation of any of the different replication stages in proliferating cells. Moreover, the chromosomes of the different types of cells are not always organised in the nucleus as expected when a diagnosis is made using criteria established for other cell types. The cellular differentiation stage has also been related to FISH efficiency, indicating that a different structural organisation of some chromatin regions may be found in adult and prenatal material, decreasing the percentage of positive FISH results in foetal cells (Grao et al., 1993). Consequently, splits can be found, and their frequency should be evaluated for each cell type (although a similar percentage in both types of cells used here was assumed). The use of a proper criterion by an experienced person to correctly identify FISH signals is necessary to obtain an accurate diagnosis.

In the same way that two different probes for the same chromosome are used as a marker for this chromosome, usually, when Tel probes are applied, we would suggest that it would be very helpful to include markers for the beginning and the end of replication to be simultaneously used with other FISH probes in PGD, since they have been used in other cell types. For example, the CD3D gene and the muscle glycogen phosphorylase gene (PYGM) are early-replicating in the human lymphocyte Manca cell line; an IgH variable region probe showed a late-replicating pattern (Selig et al., 1992; Calza et al., 1984), and CFTR and β-globin replicate late in the S phase in fibroblasts (Ofir et al., 2002). The fact that some genes are known to replicate synchronously in disomic cells and asynchronously in aneuploid cells (Amiel et al., 1998, 1999) would support the usefulness of the application of a cell cycle phase marker. It should first be necessary to identify which marker would be the best for embryonic cells.

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