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XIST Expression in Human Oocytes and Preimplantation Embryos

R. Daniels,[1] M. Zuccotti,[1] T. Kinis,[2] P. Serhal,[2] and M. Monk[1]

[1]Molecular Embryology Unit, Institute of Child Health, and [2]Assisted Conception Unit, Department of Obstetrics and Gynaecology, London

Summary

During mouse preimplantation development, the exclusive expression of the *Xist* gene from the paternally inherited allele is thought to play a role in the inactivation of the paternally inherited X chromosome in the extraembryonic cell lineages of the developing female embryo. Recently, inactivation of the paternally inherited X chromosome has also been shown to occur in the extraembryonic cell lineages of the human female conceptus. In this paper, we determine whether the pattern of *XIST* expression in human preimplantation embryos is similarly correlated with paternal X inactivation. We developed procedures sensitive to the single cell, for the simultaneous analysis of *XIST* and *HPRT* expression and of sexing, initially using human fibroblast cells. Application of these procedures to human cleavage-stage embryos derived by in vitro fertilization revealed a pattern of *XIST* expression different from that in the mouse. Transcripts of the *XIST* gene were detected as early as the 1-cell zygote and, with increasing efficiency, through to the 8-cell stage of preimplantation development. In addition, transcripts of *XIST* were detected in both male (hence from the maternally inherited allele) and female preimplantation embryos. This pattern of expression is not consistent with a role for the early expression of the *XIST* gene in the choice of paternal X inactivation in the extraembryonic cell lineages of the developing human embryo.

Introduction

In female mammals, one of the two X chromosomes is inactivated to compensate for the extra dosage of X chromosome–linked genes present in females, compared with that in males (Lyon 1961). During development, the choice of X chromosome to be inactivated is random (maternal or paternal) in somatic cells, whereas the paternally inherited X chromosome is preferentially inactivated in the extraembryonic cell lineages (Takagi and Sasaki 1975; West et al. 1977; Monk and Harper 1979; Harper et al. 1982; Harrison 1989; Goto et al. 1997). In marsupials, preferential inactivation of the paternally inherited X chromosome occurs in all tissues (Richardson et al. 1971; Sharman 1971).

The inactivation of an X chromosome is regulated by the gene *XIST* (X inactive–specific transcript; *Xist* in mouse, *XIST* in human), which is located at the X-inactivation center (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991). Penny et al. (1996) have introduced a targeted deletion into a single *Xist* allele in a mouse XX embryonic stem-cell line and have shown that the *Xist* gene is required for X inactivation to take place. The mechanism of action of *XIST* is unknown, although cytogenetic studies have shown that the spliced *XIST* RNA binds along the length of the inactive X chromosome (Clemson et al. 1996).

Xist has also been implicated in the preferential paternal X inactivation in the extraembryonic lineages in the mouse. In preimplantation embryos of the mouse, expression of the paternally inherited *Xist* allele is first detected at the 4-cell stage and prior to the inactivation of the paternal X chromosome (Kay et al. 1993, 1994). Expression of the maternally derived *Xist* allele can be detected later in development (by 6.5 d postcoitum; Kay et al. 1994), when X inactivation is random in the embryonic lineages.

In human preimplantation development, the profile of *XIST* expression is not known. However, recent experiments have shown that preferential paternal X inactivation has occurred in first-trimester human trophoblastic cells (Goto et al. 1997), a situation that persists until birth when preferential paternal X inactivation has been demonstrated in full-term placentas (Harrison 1989). We have investigated *XIST* expression in human oocytes and preimplantation embryos, to determine whether *XIST* expression occurs during early development of the human and whether the paternal allele is favored, as in the mouse. The procedures for sensitive (single cell) detection of *XIST* were first developed on readily available human fibroblast cells before analysis of the human gametes and embryos. As a control, *HPRT* (hypoxanthine phosphoribosyl transferase) transcription was also measured, and the embryos were sexed as female or male by amplification of X- and Y-specific *AMG* (amelogenin) sequences. The expression of *XIST* (and *HPRT*) can be detected already in the fertilized 1-cell zygote and, with increasing efficiency, in 2-cell, 4-

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Address for correspondence and reprints: Prof. Marilyn Monk, Molecular Embryology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, United Kingdom. E-mail: mmonk@ich.ucl.ac.uk

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cell, and 8-cell embryos. Unexpectedly, and in contrast to the mouse, we found that expression of the human *XIST* gene occurs in both female and male embryos at the 8-cell stage and thus from both the paternally and the maternally inherited X chromosomes.

Material and Methods

Human Samples

Sperm.—Sperm samples were obtained, with permission of donor, from the Assisted Conception Unit (ACU) (University College Hospital, London). Sperm samples were washed in PBS, and groups of ~20 sperm were isolated into individual droplets of PBS under oil, by use of finely pulled glass pipettes.

Oocytes.—Oocytes that had failed to be fertilized after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) were donated by patients attending the ACU. All oocytes either had been mixed with sperm, for IVF, or had single sperm injected by ICSI. Oocytes were scored as unfertilized if a male pronucleus had not formed 24 h after the IVF or ICSI procedures and if the oocytes did not cleave after further incubation. It should be noted therefore that all oocytes used in this study may contain a sperm genome that had failed to form a pronucleus.

Preimplantation embryos.—Embryos derived by IVF or ICSI and assessed as unsuitable for transfer or freezing were donated by patients attending the ACU (University College, London). The zona pellucida of each oocyte or embryo was removed as follows. By use of finely pulled glass pipettes, oocytes and embryos were placed in drops of acid tyrodes solution (pH 2.0–2.4; Sigma) under oil and were observed under a dissection microscope until the zona pellucida had just dissolved (time 20–40 s). Oocytes and embryos were then washed through drops of PBS or culture medium. Great care was taken to remove any contaminating sperm or cumulus cells.

Fibroblasts.—Cultured fibroblasts were obtained from John Rainer (Biochemistry Unit, Institute of Child Health). After trypsinization, fibroblasts were resuspended in Hai's medium (Imperial Laboratories) and were kept on ice. Fibroblasts were then isolated singly or in groups of two, in droplets of PBS under oil, by use of finely pulled glass pipettes.

Sample Preparation

Samples (0.5 μl) were added to 1.5 μl lysis buffer B (0.8% Igepal (Sigma), 1 U RNAsin (BRL)/μl, and 5 mM DTT (BRL), were centrifuged briefly at 12,000 *g,* and were overlaid with one drop of mineral oil (Sigma). Samples were then frozen in liquid nitrogen and were stored at −70°C. Prior to reverse transcription, samples were held at 80°C for 5 min, in order to denature the mRNA. Samples were then transferred immediately to ice, before the addition of reverse-transcription reagents.

Reverse Transcription

The reverse-transcription reagents were mixed on ice, and 3 μl were added to each cell lysate to produce a final volume of 5 μl, comprising the cell lysate, 1 × Reverse Transcription Buffer (BRL), 40 U Superscript reverse transcriptase (RT) (BRL), 0.9 mM each deoxynucleotide (Pharmacia), 5 mM DTT (BRL), 2.25 μg random hexamers (BRL), and 2 U RNAsin (Boerhinger). Negative controls, without the RT enzyme and without the added sample, were included in all experiments. Contamination was very rare, but, when it did occur, the results of that experiment were discounted. Reverse transcription was performed at 37°C for 1.5 h. Samples were then immediately returned to ice.

PCR Amplification

After reverse transcription, 1/10 (0.5 μl) of the final reaction mix was amplified by use of primers for *HPRT* cDNA (Gibbs et al. 1989). The remaining 4.5 μl was then amplified by use of primers for *XIST* cDNA (fig. 1*b*) and *AMG* DNA (Nakahori et al. 1991; Ray et al. 1995) in duplex. PCR amplification was performed in a total volume of 25 μl comprising the cDNA, 1 × PCR buffer (Perkin Elmer), 25 pmol each primer (in the case of *AMG,* 5 pmol of each primer were used in the first round of PCR amplification), 200 mM each dNTP (Pharmacia), and 1.25 U Amplitaq DNA polymerase (Perkin Elmer). The PCR cycle parameters for *XIST, HPRT,* and *AMG* were as follows: 2 cycles of 93°C for 5 min, 60°C for 1 min, and 73°C for 2 min, followed by 30 cycles of 93°C for 1 min, 60°C for 1 min, and 73°C for 2 min, in a Techne PHC-II or GeneE thermal cycler. A second round of PCR amplification was performed for each gene sequence separately, by use of nested or hemi-nested primers, by diluting 1 μl of the first-round reaction into 25 μl of a new reaction mix prepared as described above. Optimum annealing temperatures for each primer set were determined empirically. In the case of *HPRT,* the sensitivity and specificity of the PCR reaction were increased by the addition of 1.25% formamide.

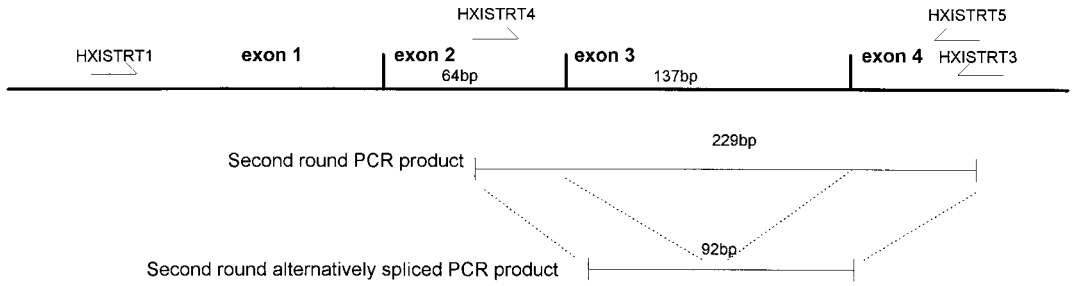
Gel Electrophoresis

Fourteen microliters PCR product were mixed with 2 μl loading buffer and were electrophoresed on a 3% agarose gel containing 2 μg ethidium bromide/ml, for 60 min at 120 V. The products were visualized and photographed under short-wavelength UV light.

Product Recovery and Sequencing

PCR products bands to be recovered and sequenced (to confirm identity) were cut out of the gel by use of a sterile scalpel blade and were isolated from the gel slices

a)



b)

XIST	1st round amplification primers	HXISTRT1 GTTTCTCGGAGAAGGATGTC HXISTRT3 TCCACCTAGGGATCGTCAAA
	2nd round amplification primers	HXISTRT4 GTAGTCATGTCTCCTTAGGC HXISTRT5 GGATCGTCAAAGGGAATGGA
HPRT	1st round amplification primers 244 and 246. 2nd round amplification primers 244 and 243.	Primer sequences taken from Gibbs *et al*, (1989).
AMG	1st round amplification primers AMG1 and AMG2. 2nd round amplification primers AMGa and AMGb.	Primer sequences taken from Nakahori *et al*, (1991). Primer sequences taken from Ray *et al*, (1995).

Figure 1 *a*, Diagram of the *XIST* cDNA region amplified by the RT-PCR procedure, showing exon/intron boundaries (indicated by vertical bars), primer positions, and PCR product sizes. Note that two PCR products are obtained after amplification of *XIST* cDNA: the product from the full-length transcript, 229 bp in size, and an alternatively spliced cDNA PCR product, lacking exon 3, 92 bp in size. The *HPRT* and *AMG* PCR procedures have been modified from those of Gibbs et al. (1989), Nakahori et al. (1991), and Ray et al. (1995). *b*, PCR primers.

by use of a Qiaquick gel extraction kit (Qiagen). Samples were sequenced by the Biotechnology Service Department, Charing Cross and Westminster Medical School, London.

Results

Development of Single-Cell Sensitivity for Detection of XIST *and* HPRT *Expression, and Sexing as Male or Female*

Amplification of *XIST* and *HPRT* cDNA was performed by use of a nested-primer strategy (see Material and Methods and fig. 1). Amplification of the *HPRT* cDNA sequence served as a positive control for the reverse-transcription procedures. To distinguish individual male and female embryos, samples were sexed by use of PCR amplification of *AMG* sequences on the X and Y chromosomes (Nakahori et al. 1991; Ray et al. 1995).

We designed the *XIST* primers to produce a PCR product that spans the first, second, and third introns of the cDNA sequence (see fig. 1*a*). This sequence is subject to alternative splicing to produce two cDNAs with or without exon 3 (Brown et al. 1992). Therefore, we expect two amplified products from our PCR procedures to detect the *XIST* transcript—namely, 380 and 243 bp after the first-round PCR and 229 and 92 bp after the second-round PCR.

Using the human fibroblast cells, our procedures were refined to develop the sensitivity required to detect transcripts in single cells and, hence, in single oocytes and embryos. Figure 2*a* shows the results after RT-PCR of replicate female and male samples each containing a single fibroblast, together with control reactions omitting the RT enzyme and blank samples lacking added cDNA. Sequencing of the PCR products confirmed the amplified sequences as those of the *XIST* gene in the expected region (data not shown). Transcripts of the *XIST* gene were detected in single female fibroblast cells, with an efficiency of >90% (27/30), and in samples of two female fibroblasts, with an efficiency of 100% (29/29). Transcripts of the *XIST* gene were not detected in single male fibroblast cells (fig. 2*a*) and only very rarely (<10%), and with a PCR product of much lower intensity than the PCR product of females, in samples of two male fibroblasts (data not shown). Control reactions lacking RT or added sample showed that this low level of detection of *XIST* in male cells is not due to contamination. Low levels of *Xist* expression in mouse male somatic and undifferentiated embryonic stem cells have been detected by others (Borsani et al. 1991; Panning and Jaenisch 1996).

As a control for the reverse-transcription procedures, *HPRT* transcripts were analyzed. Amplification of *HPRT* cDNA was performed on 0.5 μl reverse-transcription reaction mix, by use of a heminested-primer set spanning seven intron/exon boundaries (Gibbs et al. 1989). Figure 2*b*

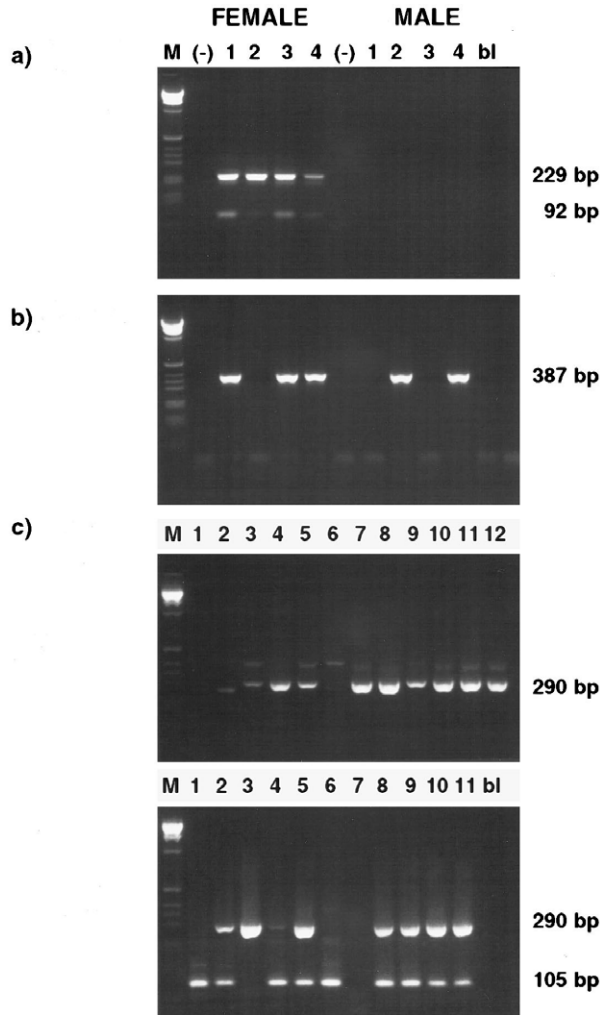


Figure 2 Expression of *XIST* and *HPRT*, and sexing, of single fibroblasts. *a*, Amplification of *XIST* cDNA in four single female and four single male fibroblasts, plus control samples omitting RT, (-), and PCR reaction blanks lacking cDNA (bl). *XIST* transcripts are readily detected in female samples only, showing both the full-length cDNA product of 229 bp and the alternatively spliced cDNA product of 92 bp. *b*, Amplification of *HPRT* in the same single fibroblasts analyzed in *a* for *XIST* expression. *c*, Amplification of *AMG* in female and male fibroblasts. The PCR products obtained from the X and Y chromosomes are 290 and 105 bp, respectively. Lanes 1–5 show results after amplification of single fibroblasts. The efficiency of the PCR in fibroblasts is greatly increased when two cells are used as template (Lanes 6–12 for female; lanes 6–11 for male).

shows amplification of *HPRT* cDNA in the same samples of female and male fibroblasts analyzed for *XIST* cDNA in figure 2*a*. *HPRT* transcripts were detected in single fibroblast cells, both female and male, with an efficiency of 75.9% (41/54), and in samples of two fibroblasts, with an efficiency of 83.6% (46/55).

Sexing of the fibroblasts was performed by use of a nested PCR amplification of a sequence of *AMG* present on both the X and Y chromosomes (Nakahori et al. 1991; Ray et al. 1995). Amplification of the X-chromosome sequence produced a PCR product 290 bp in size, whereas that of the Y-chromosome sequence produced a product 105 bp in size. Hence, both chromosomes can be detected with one nested PCR. Figure 2*c* shows sexing of single female and male fibroblasts (lanes 1–5) and sexing of two fibroblasts (lanes 6–12 for female and lanes 6–11 for male). The efficiency of sexing of single fibroblasts was ~50% but was greatly increased when two cells were used as template. For female cells, accurate sexing was achieved in 73.7% (42/57) of the 2-cell samples; for male cells, accurate sexing was achieved in 70.5% (43/61) of the 2-cell samples.

Analysis of XIST *Expression in Human Oocytes, and Preimplantation Embryos*

With techniques developed to the required sensitivity and efficiency, by use of the fibroblast cells, *XIST* and *HPRT* expression, as well as sex, were determined in individual human oocytes and preimplantation embryos. The results are summarized in table 1.

Of six "failed fertilization" oocytes (i.e., no male pronucleus was detected), analyzed individually, three were sexed as male and therefore must contain a sperm genome even though a male pronucleus was not detected. Only one of these showed *XIST* expression, whereas five of the six showed the presence of *HPRT* transcripts (table 1). Of a further six fertilized oocytes (i.e., a male pronucleus was present), two showed the presence of *XIST* transcripts and four were positive for *HPRT* transcripts (table 1). Figure 3*a* shows the results for three oocytes, two not expressing *XIST* and one expressing *XIST* and being sexed as male, which was originally assessed as belonging to the "failed fertilization" class.

Table 1

Sexing, and *XIST* and *HPRT* Expression, in Single Human Oocytes and Preimplantation Embryos

	No. Expressing	
Stage and Sex	*XIST*	*HPRT*
Oocyte:		
No male pronucleus (n = 6):		
3 Female		2
3 Male	1	3
Fertilized (n = 6):		
3 Female		2
3 Not sexed	2	2
2-Cell (n = 7):		
3 Female	1	2
1 Male	1	1
3 Not sexed	1	3
4-Cell to 8-cell (n = 17):		
7 Female	6	6
3 Male	2	2
7 Not sexed	5	7

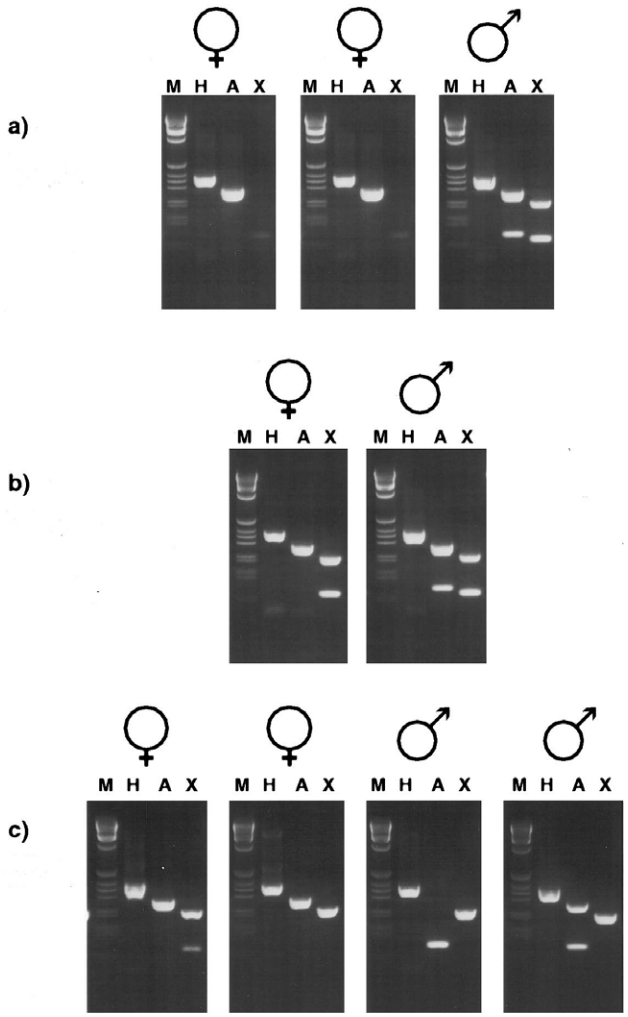


Figure 3 Sexing and analysis of *XIST* and *HPRT* expression in individual human oocytes and preimplantation embryos. Lanes M, 1-kb ladder. Lanes H, *HPRT* expression. Lanes A, Sexing by amplification of *AMG*. Lanes X, *XIST* expression. *a,* Representative results from three single oocytes. The first oocyte was scored as lacking a male pronucleus, was sexed as female, and was shown as expressing *HPRT* but not *XIST*. The second oocyte was assessed as fertilized by the presence of a male pronucleus; and it too was sexed as female and was shown as expressing *HPRT* but not *XIST*. The third oocyte was scored as lacking a pronucleus and was shown as expressing both *HPRT* and *XIST* but was sexed as male, indicating the presence of a Y-bearing sperm genome. *b,* Results from two single 2-cell embryos, both expressing *HPRT* and *XIST,* the first sexed as female and the second sexed as male. *c,* Results from four 8-cell embryos. The first two embryos were sexed as female, and the second two were sexed as male. The derivation of the fourth embryo in the figure, from an ICSI fertilization, removes the possibility that contaminating sperm produced a false male-sexing result. All four embryos show expression of *XIST* and *HPRT*. The absence of the alternatively spliced version of *XIST* cDNA at 92 bp was seen only in 3/13 embryos showing *XIST* expression at the 4-cell to 8-cell stage. All three were at the 8-cell stage and are shown here. The reason for the failure to amplify this band in these three 8-cell stage embryos is not known. The alternatively spliced transcript may be either absent or in lower abundance in these embryos.

No transcripts of *XIST* or *HPRT* were detected in samples containing ~50 sperm (data not shown) or in controls lacking either RT or a DNA sample. These controls and, in addition, controls with female (*XIST* expressed) and male (*XIST* not expressed) somatic cells were performed in each experiment. The somatic-cell controls confirm the *AMG* sexing procedure.

Similar results were obtained for the seven 2-cell embryos analyzed; three of the seven expressed *XIST,* whereas *HPRT* expression was detected in six of the seven (table 1). The *AMG* PCR showed that three of the seven were female and one was male and that, in three, the sexing was not conclusive. The results for the female and the male 2-cell embryos expressing *XIST* are shown in figure 3*b*. We consider it likely that the transcripts detected are due to new transcription from embryonic genes, rather than maternal transcripts inherited in the egg cytoplasm, since the proportion of embryos expressing *XIST* is increasing markedly through the 2- to 8-cell stage (see below). If new embryonic-gene transcription is assumed, *XIST* is being expressed from the maternal X chromosome in the male 2-cell embryo. However, since the *XIST*-expressing embryos were produced by IVF, it could be argued that the presence of an additional sperm genome containing the paternal Y chromosome results in female embryos being sexed as male.

From the 4-cell to 8-cell stage of development, 17 embryos were analyzed (table 1). The proportion of embryos showing expression of *XIST* at these stages has clearly increased; 13 of the 15 embryos analyzed showing *HPRT* expression also show *XIST* expression. The increased efficiency of *XIST* mRNA detection per single embryo, from 3/12 in oocytes to 13/17 in 4-cell to 8-cell embryos, is strongly indicative that expression of *XIST* mRNA is from the embryonic *XIST* genes and is not mRNA inherited from the egg cytoplasm. The presence of *HPRT* mRNA is detected with a high efficiency throughout these preimplantation stages, demonstrating that the RT-PCR procedures are reliable at all stages. The *AMG* sexing showed that, of the 17 embryos, 7 were female and 3 were male and, for the other 7 embryos, sexing was not conclusive. Figure 3*c* shows representative data obtained after sexing and analysis of *XIST* and *HPRT* expression in four 8-cell embryos, two female and two male. *XIST* is expressed in both the female (two X chromosomes, one maternal and one paternal) and the male (single maternally inherited X chromosome) embryos. One of the embryos expressing *XIST* and sexed as male was derived by an ICSI fertilization and therefore cannot have been wrongly sexed because of additional sperm. Note that the PCR fragment corresponding to the smaller *XIST* spliced mRNA is not present in three of these 8-cell embryos. This was observed only in these three embryos, and the reason is unknown.

Overall, of 23 embryos that were satisfactorily sexed, 16 were female and 7 were male. Of the 19 oocytes and embryos expressing *XIST,* 11 were sexed; and 7 were female and 4 were male. The preponderance of females is probably due to allele dropout and failure to amplify the Y chromosome, such that some male embryos might be sexed as female. However, this does not affect our conclusions, since the significant result is expression of *XIST* in male XY embryos. In addition, because of the low number of embryos sexed as male, we consider it highly unlikely that the male embryos were attributable to contaminating sperm, and we conclude that *XIST* expression occurs from the maternally inherited X chromosome in early human embryos.

Discussion

We have developed sensitive techniques to detect *XIST* expression, together with *HPRT* expression and sexing, in single human fibroblast cells. The techniques were refined to the level of the single cell prior to commencement of studies on single human preimplantation embryos. We have investigated *XIST* expression in individual human oocytes and preimplantation embryos.

In single oocytes (failed fertilization and fertilized), transcripts of the *XIST* gene were detected in one-third (3/9) of those positive for *HPRT* transcripts. In three of the six oocytes scored as lacking a male pronucleus after IVF, sexing revealed the presence of a sperm genome, indicating either the presence of contaminating sperm or that these oocytes were actually fertilized, although a male pronucleus was not seen. Hence results for ''unfertilized'' and fertilized oocytes cannot be viewed separately. Overall, the results from the 12 oocytes examined at this stage indicate that *XIST* transcripts are present in the 1-cell zygote; but the low frequency of detection (compared with *HPRT* expression) indicates a low level of *XIST* transcripts, given that our PCR techniques were shown to be 90% efficient at the 1-cell level. We cannot distinguish between maternal *XIST* transcripts present in the egg cytoplasm and new transcription at this stage, since we were unable to obtain fresh unfertilized oocytes that had not been either mixed with sperm or subjected to ICSI. In the mouse, *Xist* is not expressed during oogenesis, since both X chromosomes are active (McCarrey and Dilworth 1992). If the human is similar to the mouse in this respect, then *XIST* expression must commence in the human 1-cell zygote. Other instances of very early onset of transcription in the 1-cell human zygote have been shown by demonstration of the presence of paternal transcripts for *MPK* (Daniels et al. 1995), *SRY* (Ao et al. 1994), and *ZFY* (Fiddler et al. 1995). Studies on the expression of several housekeeping and tissue-specific genes suggest that nonmethylated CpG island–containing genes are expressed in human preimplantation development (R. Daniels et al., in press). It may be that the expression of *XIST* is part of this general picture.

At the 2-cell stage, *XIST* transcripts were detected in three of the six embryos that were positive for *HPRT* transcription, thus indicating that the *XIST* transcripts were of lower abundance than were the *HPRT* transcripts. One of the *XIST*-expressing 2-cell embryos was sexed as male. This is surprising, since, in the mouse, the maternally inherited *Xist* allele is not active early in development (Kay et al. 1993, 1994). We consider it likely that the *XIST* transcripts in human 2-cell embryos are derived by embryonic gene activity, on the basis of the marked increase in *XIST* expression from the 1-cell stage to the 4-cell to 8-cell stages. It could be argued that the conclusion that *XIST* expression occurs from the maternally inherited allele is erroneous, since these 2-cell embryos are derived by IVF and may contain an extra sperm genome. However, the sexing of IVF embryos is unlikely to be affected by contamination with Y-bearing sperm, since we do not see an excess of male embryos. In fact, of the 23 sexed embryos, 16 are sexed as female and 7 are sexed as male (table 1).

Embryos at the 4-cell to 8-cell stages clearly show increased *XIST* expression. Thirteen of 15 of the embryos positive for *HPRT* transcripts were also positive for *XIST* transcripts. It is clear that *XIST* expression occurs in both male and female embryos at these stages. Even if the sexing results are disregarded, it is highly unlikely that all 13 *XIST*-expressing embryos are female. In addition, the derivation of one of the male 8-cell embryos from an ICSI fertilization procedure eliminates the possibility that contaminating sperm produce a false sexing result in this case.

In conclusion, the expression of *XIST* in human preimplantation development from the 1-cell stage to the 8-cell stage and from both parental alleles contrasts with the situation in the mouse, in which the onset of *Xist* expression occurs from the 4-cell stage and then only from the paternal allele in female embryos (Kay et al. 1994). Thus, preferential inactivation of the paternal X chromosome in human extraembryonic tissues early in development (Goto et al. 1997) is not preceded by preferential *XIST* expression from only the paternal allele, since the expression of *XIST* in male embryos indicates expression of the maternally inherited allele. Probably both alleles are expressed, although we have no independent evidence for the expression of the paternal allele. A search for polymorphisms to distinguish maternal and paternal alleles in the 229 bp amplified by our single-cell RT-PCR procedure in 10 human individuals has been unsuccessful. A polymorphism is present 3′ to the poly(A) site in the *XIST* gene (Rupert et al. 1995). However, we could not use this polymorphism, since the analysis of unspliced mRNA would introduce problems involving the amplification of genomic DNA.

In the mouse, *Xist* expression in cleavage-stage embryos is not associated with X-chromosome inactivation, since both X chromosomes are active at this stage. It is possible that paternal *Xist* expression in cleavage-stage mouse embryos reflects its previously active state in spermatogenesis, rather than a future role in paternal X inactivation at the blastocyst stage. Given that there is no detectable difference in the expression of maternal and paternal *XIST* alleles in the human preimplantation embryos, it is likely that the early *XIST* expression per se has no role to play in the choice of X chromosome to be inactivated in the extraembryonic tissues of the blastocyst; rather, we must continue to search in both mouse and human for a differential molecular imprint carried on the parental *XIST* alleles themselves, through to the blastocyst stage, when the inactivation of the paternal X chromosome takes place.

Acknowlegments

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