

***XIST* expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage**

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In the somatic cells of female mammals, either the maternally or paternally derived X chromosome (X^M or X^P) is randomly inactivated to achieve dosage compensation for X-linked genes. In early mouse development, however, selective inactivation of X^P occurs first in extraembryonic lineages at the blastocyst stage around the time of implantation before later random inactivation in the embryonic ectoderm from which the fetus is derived. *Xist*, a gene mapping to the X-inactivation centre (Xic), is exclusively expressed from the inactive X-chromosome and is thought to be involved in the initiation of X-inactivation. Consistent with this, *Xist* is first expressed at the 4- to 8-cell stages, prior to functional inactivation at the blastocyst stage, exclusively from X^P in female embryos. This also suggests that genomic imprinting may influence the earliest expression of *Xist* resulting in selective inactivation of X^P and a candidate methylation site in the promoter region has recently been described. Here we report the expression of the human homologue, *XIST*, in human preimplantation embryos from the 5- to 10-cell stage onwards consistent with its role in the initiation of inactivation. In contrast to the mouse, however, transcripts were detected in both male and female embryos demonstrating *XIST* expression from the X^M in male embryos (X^MY).

INTRODUCTION

In the somatic cells of female mammals, dosage compensation for X-linked genes between XX females and XY males is achieved by transcriptional inactivation of genes on one of the two X chromosomes (1). Random inactivation of either the maternally or paternally derived X chromosome (X^M or X^P) occurs early in development and is subsequently stably inherited. Maintenance of inactivation involves heterochromatization characterised by delayed replication late in S phase (2–4), histone H4

deacetylation and methylation of CpG islands (5–7). Transcripts for the *Xist* gene in the mouse and the human homologue, *XIST*, are exclusively expressed from the inactive X chromosome (8,9) and the genes map to the X-inactivation centre (Xic), a region known to mediate X inactivation (10). Furthermore, *Xist* expression in the mouse has been demonstrated to be necessary for X inactivation to occur (11).

In the mouse, X-inactivation is first detected at the late morula/blastocyst stages of preimplantation development (12,13) restricted to the extraembryonic lineages in which X^P is selectively inactivated (14,15). Random inactivation of the X^M or X^P occurs later in the embryonic ectoderm lineage, and this pattern is maintained in adult somatic tissues. Consistent with a role in the initiation of inactivation, *Xist* transcripts are first detected in mouse embryos before functional X-inactivation at the 4-cell stage and exclusively from X^P suggesting that imprinting may be involved in repressing the initial expression of X^M . We demonstrate here that expression of *XIST* is also detectable in human preimplantation embryos from the 5- to 10-cell stage onwards. In contrast to the mouse, however, transcripts were detected in both male and female embryos indicating expression from X^M in the males.

RESULTS

Individual or pooled human oocytes, which had failed to fertilize following *in vitro* fertilization (IVF), and normally fertilized embryos at various stages of preimplantation development *in vitro* were lysed and reverse transcription PCR (RT-PCR) used to detect specific transcripts. The cDNA from each sample was split and analysed separately for *XIST*, *ZFX/ZFY* and *HPRT* expression. *ZFX* and *ZFY* transcripts from the respective genes on the X and Y chromosomes were amplified with a single set of primers and distinguished by restriction digestion to identify the sex of individual embryos (17). *HPRT* transcripts and omission of reverse transcriptase were used as positive and negative controls, respectively. In each case nested PCR was used to improve the sensitivity and primers designed to span introns to eliminate spurious results from genomic DNA. The *XIST* primers spanned exons 5, 6i and 6 (Fig. 1).

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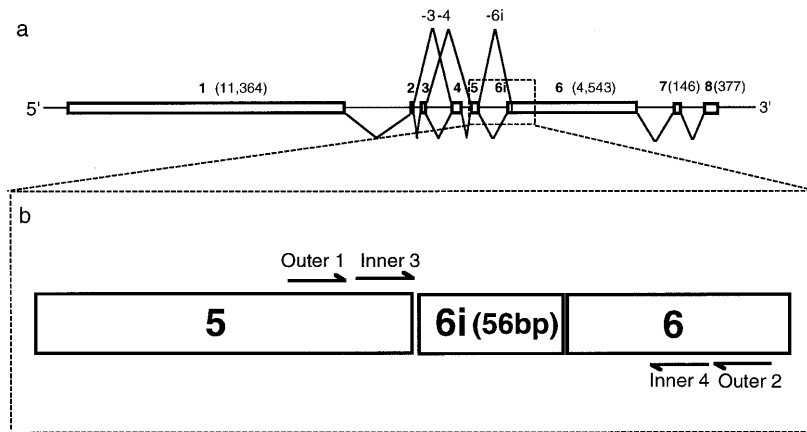


Figure 1. (a) Structure of the *XIST* cDNA and differential splicing patterns as described in ref. 32. (b) Details of the amplified region and location of the nested primers.

Table 1. Stage specific *XIST* expression in pooled human embryos

Stage/day post insemination	No. of samples analysed	No. of embryos in each sample	No. of samples expressing <i>XIST</i>	No. of samples expressing <i>HPRT</i>
Failed fertilized	3	5–9	0	3
2	3	5–10	0	3
3	5	2–10	3	4
4	1	15	1	1
6	3	5–6	3	–

Table 2. *XIST* expression in single human embryos

Day post insemination	No. of embryos analysed	No. of positive samples ^a	No. of embryos expressing a specific transcript (no. of embryos analysed ^b)			
			<i>XIST</i>	<i>HPRT</i>	<i>ZFX</i> only	<i>ZFX/ZFY</i>
3	12	6	3 (6)	3 (6)	4 (6)	0 (6)
4	6	5	3 (5)	4 (5)	2 (4)	0 (4)
5–6	27	23	23 (23)	11 (12)	14 (21)	5 (21)
Total	45	34	29 (32)	18 (23)	20 (31)	5 (31)

^aSamples in which at least one of the four transcripts were detected.

^bOnly embryos in which at least one transcript was detected, confirming reverse transcription, are included.

XIST transcripts were not detectable in pooled failed fertilized oocytes or embryos 2 days post insemination (day 2) (Table 1; Fig. 2). On day 3, however, *XIST* transcripts were detected in three of four samples positive for reverse transcription and on subsequent days, up to the blastocyst stage on day 6, were detected in all four samples. This pattern of expression was similar in embryos analysed individually (Table 2). On day 3, PCR products for at least one of the transcripts were only detected in six of 12 samples. Three of these were positive for *XIST* and *ZFX* transcripts were detected in four. At the blastocyst stage on days 5–6, 23 of 27 samples were positive for at least one of the transcripts which included *XIST* in every case. Only five of these embryos showed *ZFY* expression indicating they were XY males but in each of these *XIST* was also expressed. The presence of alternatively spliced transcripts in which exon 6i was omitted

were observed in most of the samples (Fig. 2). No amplification products were detected in any of the 68 reverse transcription negative controls analysed. The use of nested PCR prevented any quantitative assessment of *XIST* expression in male and female embryos at different stages. The consistently strong amplification, however, suggests that it is unlikely that only low levels of transcripts, which may be of no functional significance, are being detected.

DISCUSSION

The expression of *XIST* as early as the 5–10-cell stage on day 3 is similar to the mouse in which expression of *Xist* was first detected at the 4-cell stage (16). In contrast to the mouse, however, *XIST* expression in a high proportion of embryos at all

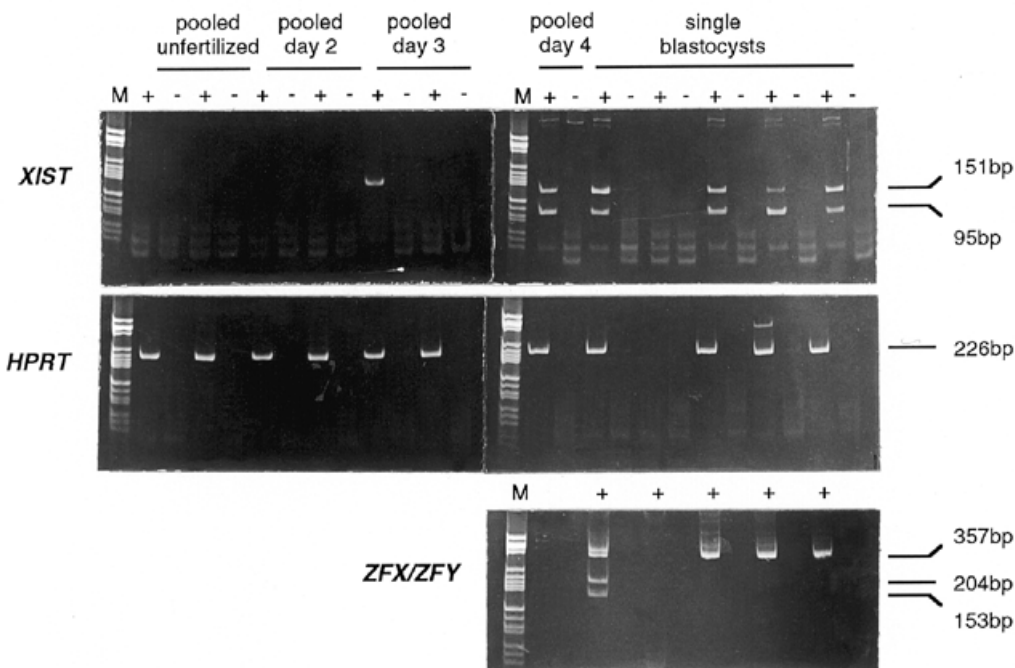


Figure 2. *XIST*, *HPRT* and *ZFX/ZFY* transcripts detected by RT-PCR of pooled and individual human oocytes and embryos. Top panel: *XIST* transcripts are not detectable in pooled failed fertilized and day 2 embryos (2–4-cell stage) but are present in one of the two pooled samples on day 3 (5–16-cell stage), in a pooled sample on day 4, and in four out of five single blastocysts. On day 3, only the full length product (151 bp) is detected, whereas on day 4 and with the single blastocysts both full length and truncated products (95 bp), predicted by the splicing out of exon 6i are present (Fig. 1). Middle panel: *HPRT* RT-PCR was used as positive control for reverse transcription. In all samples except one single blastocyst, *HPRT* transcripts (226 bp) were detected confirming successful reverse transcription. In the blastocyst which failed to amplify *HPRT*, amplification of *XIST* and *ZFX/ZFY* also failed indicating a failure of reverse transcription. Bottom panel: *ZFX/ZFY* transcripts were amplified to identify the sex of individual blastocysts. Following digestion with *Clal* the amplified fragment from *ZFX* remains undigested (357 bp) whereas the *ZFY* product is cut and yields fragments of 204 and 153 bp. The presence of *ZFY* transcripts is clearly demonstrated in one of the four single blastocysts expressing *XIST*, indicating that this embryo is male. Amplification products were only detected in reverse transcription positive samples (+), and never in the reverse transcription negative controls (-). M, molecular weight marker, pBR322 digested with *HhaI*.

stages and in five blastocysts positively identified as males by the simultaneous detection of *ZFY* transcripts indicates that expression can occur from X^M in males. The low proportion of embryos identified as males probably relates to the difficulty of detecting *ZFY* transcripts in individual embryos against a high background of *ZFX* transcripts inherited from the oocyte (17). However, all 23 individual blastocysts on days 5–6 in which the RT-PCR was successful showed strong expression and any unidentified males must therefore have also been expressing *XIST*. We were unable to distinguish expression from X^P or X^M in female embryos so we cannot conclude that expression is biallelic in these embryos. However, it seems unlikely that *XIST* would be exclusively expressed from X^M .

In the mouse, imprinting of *Xist* is thought to predetermine early expression and selective inactivation of X^P in extraembryonic lineages before the imprint is erased in the embryonic ectoderm prior to random inactivation (16). Methylation of specific sites in the paternal copy of *Xist* has been shown to persist even after the global demethylation that occurs in early cleavage and these sites are a candidate for the imprint in the mouse (18). In human, there has been conflicting evidence for selective inactivation of X^P in extraembryonic components of placental tissue (19–21). Recently RT-PCR analysis of trophoblast cells isolated from chorionic villus samples of female conceptuses demonstrated that only X^M is expressed (22). However, only two samples were analysed and it is possible that

inactivation was skewed or that the number of cells analysed was so small that they were of clonal origin. Also, the methylation status of *XIST* in sperm and eggs has not been investigated. Expression of *XIST* from X^M in male embryos at the blastocyst stage suggests that if it is imprinted in the human, it does not affect its expression at these stages.

At the blastocyst stage in the mouse, the imprint appears to be erased prior to random *Xist* expression and X-inactivation at the beginning of gastrulation in a tissue specific order (16,23,24). If *XIST* is not imprinted or the imprint does not affect expression in the human embryo during preimplantation development, this would explain expression in both male and female embryos and predict that both alleles are expressed in females. This would also explain differences in the effects of various abnormal combinations of *Xist/XIST* alleles in mouse and human early development. In the mouse, female embryos in which the functional *Xist* gene is deleted on X^P die soon after implantation as a consequence of failure of development of extraembryonic tissues whereas those inheriting the deletion from X^M are unaffected (25). A similar phenotype is observed with embryos with extra copies of X^M ($X^M X^M X^P$ and $X^M X^M Y$) presumably as a result of a failure to inactivate the extra X^M in the extraembryonic lineages due to imprinted repression of X^M *Xist* alleles (26). In contrast human XXX females and males with Klinefelter syndrome (XXY) develop relatively normally despite inheriting a supernumerary X^M in a majority of cases (27).

A low level of *Xist* expression has been detected from X^M in undifferentiated male mouse embryo stem (ES) cells and from both active X chromosomes in female cells which reverted to the normal pattern of expression exclusively from the inactive X chromosome in female cells on differentiation *in vitro* (28). It will be important therefore to quantify expression in early human embryos to determine the level of expression at these early stages and indeed to compare the levels of expression in males and females. Because nested PCR is necessary to detect transcripts in the very small amount of total mRNA in individual oocytes and embryos, conventional semi-quantitative approaches are not possible. However, since pooled oocytes and embryos on day 2 showed no expression, we consider it unlikely that we are simply detecting 'leaky' transcription at the later stages. To examine the possibility that imprinting may not play a role in *XIST* expression and the initiation of X-inactivation in the human embryo, therefore, will require more direct approaches which could include analysis of allele-specific polymorphisms (29) or polymorphisms closely linked to specific methylation sites (30), or alternatively, RNA *in situ* hybridization (31,32).

MATERIALS AND METHODS

Human oocytes and embryos

Surplus oocytes and embryos were donated for research by couples undergoing IVF treatment with their informed consent. Oocytes were examined 19–20 h post-insemination (day 1) for pronuclei and only normally fertilized two pronucleate embryos were used in this study. Oocytes which failed to fertilize and did not cleave were used after 24 or 48 h. Embryos were cultured in Earles balanced salts solution (EBSS) supplemented with 10% heat inactivated maternal serum. This work was carried out under a Human Fertilisation and Embryology Authority license, with local approval from the Research Ethics Committee of the Royal Postgraduate Medical School.

Reverse transcription

After removing the zona pellucida with acidified Tyrode's solution (pH 2.2) and washing in phosphate buffered saline (PBS) with 4 mg/ml BSA, to remove possible contaminants, individual or pooled oocytes and embryos were lysed in 5 µl of lysis buffer (0.5% NP-40, 10 mM Tris pH 8.0, 10 mM NaCl and 3 mM MgCl₂) (33). The tubes were immediately plunged into liquid nitrogen prior to storage at -70°C. The lysate was then made up to 19 µl with reverse transcriptase buffer [50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DDT (Pharmacia, St Albans, Herts, UK), 300 pmol oligo (dT)₁₂₋₁₈ primer (Pharmacia), 0.5 mM dNTP (Pharmacia)]. The mixes were homogenized by pipetting, 5 µl was transferred into a fresh tube for the reverse transcriptase negative control (-) and 1 µl (100 U) of M-MLV reverse transcriptase (Gibco, Life Technologies Ltd, Paisley, UK) was added to the remaining 14 µl (+). Both tubes were incubated at 37°C for 2 h and the reaction terminated by heating at 95°C for 5 min.

Polymerase chain reaction

Following reverse transcription, nested PCR was used to detect specific transcripts in 2 µl (*HPRT* and *XIST*) and 4 µl (*ZFX/ZFY*) aliquots of cDNA and reverse transcriptase negative control. PCR

was carried out in a final volume of 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM of each dNTP, 2.5 U *Taq* polymerase (AmpliTaq, Perkin Elmer, Applied Biosystems, Warrington, Cheshire, UK) and 0.4 µM of each primer, covered with 50 µl of silicone oil and denatured at 95°C for 3 min before thermal cycling. For the second PCR with nested primers, 2 µl of product from the first reaction was transferred to fresh PCR buffer. The primers and cycling conditions for *HPRT* and *ZFX/ZFY* were as previously published (34,35). The outer and inner primers for *XIST*, annealing to sequences in exons 5 and 6 (Fig. 1), were as follows: outer, 5'-CCT ACA AGC AGT GCA GAG AGC-3' and 5'-GAG AAA ATG AGG CAA AGG CA-3' and inner, 5'-TCA GCC TTC CCA CCT GAA G-3' and 5'-AGC AAG AGA AAC ATG GAA ATG G-3'. Thermal cycling at 95°C for 45 s, 60°C for 60 s and 72°C for 60 s with a final extension at 72°C for 5 min was carried out using an Omnigene thermocycler (Hybaid, Teddington, Middlesex, UK) for 30 and 35 cycles for the first and second reactions, respectively.

ZFX/ZFY products were digested with *Cla*I at 37°C for 2 h. The amplified fragment from *ZFX* remained undigested (357 bp) whereas the *ZFY* product was cut and yielded fragments of 204 and 153 bp. RT-PCR products were analysed by gel electrophoresis on 10% polyacrylamide gels at 200 V for 45 min, stained with ethidium bromide and examined with a UV transilluminator (see Fig. 2).

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