Differential Pattern of Xist RNA Accumulation in Single Blastomeres Isolated From 8-Cell Stage Mouse Embryos Following Laser Zona Drilling

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ABSTRACT  Xist gene expression begins at the late 2-cell stage in female mouse embryos and by the third division results in the accumulation of an average 100 copies of Xist RNA per cell, as measured by real-time reverse transcription-polymerase chain reaction (RT-PCR). In the blastocyst, the trophectoderm maintains the paternally imprinted pattern of Xist expression present during early development, while either the maternal or the paternal X chromosome can express Xist among cells of the inner mass. Fluorescent in situ hybridization (FISH) has previously established that Xist transcripts are localized on the silenced X chromosome, forming aggregates of variable dimensions in blastomeres of 8-cell embryos. This observation and the fact that Xist RNA accumulation per cell sharply decreases after morula stage raise the possibility that cells of cleaving embryos contain different levels of Xist RNA, perhaps linked to their subsequent developmental fates. We show here that Xist RNA is efficiently recovered from single blastomeres isolated from 8-cell embryos following laser zona drilling. Sexing of the samples and simultaneous quantification of Xist RNA in individual cells is achieved with a multiplex Xist/Sry real-time RT-PCR assay sensitive to the single-copy level. This analysis reveals that Xist RNA is indeed accumulated to substantially different levels in individual blastomeres of the same 8-cell embryo and that two blastomeres contain most of the molecules per embryo. These results support the conclusion that cells of the early mammalian embryo are not all functionally equivalent. Differential Xist gene expression could arise from differences in DNA methylation, or the order in which cells divide. Mol. Reprod. Dev. 64: 41–51, 2003.
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Key Words: Xist expression; Sry amplification; quantitative RT-PCR; molecular beacons; single-cell gene expression; single-copy gene amplification

INTRODUCTION
In mammalian embryos differentiation of distinct cell types is first morphologically apparent when trophectoderm (TE) and inner cell mass (ICM) are formed at the blastocyst stage. However, an increasing number of studies point to a much earlier determination of embryonic cell fate (reviewed by Gardner, 2001; Zernicka-Goetz, 2002). In the mouse, a number of genes are known to be preferentially expressed either in the TE (endo A and β-actin genes, Duprey et al., 1985; Brison and Schultz, 1996; DSC2, Collins et al., 1995; the genes involved in the production of leptin and STAT3 proteins, Antczak and Van Blerkom, 1997; Fgrf2, Haffner-Krausz et al., 1999; Bex1/Rex3, Williams et al., 2002) or in the ICM (oct-4, Okamoto et al., 1990; Palmieri et al., 1994; fgf-4, Nisson et al., 1989; Brison and Schultz, 1996), and some of these genes have been hypothesized to actually control subsequent cellular differentiation because they are expressed in very early embryos. The mouse Xist gene is responsible for transcriptional silencing of one of the two X chromosomes in female cells, leading to dosage compensation of X-linked genes (Lyon, 1961). This gene maps to the X inactivation center (Xic) localized on X chromosomes (Borsani et al., 1991; Brockdorff et al., 1991) and, therefore, female cells carry two copies of Xist while male cells contain one. High accumulation levels of Xist transcripts, however, are only required in female cells and occur in cis along the X chromosome selected for inactivation through a mechanism of counting and choice (reviewed by Avner and Heard, 2001). A protein product is never translated from Xist RNA, while the strict association of the transcript itself with the inactive X chromosome has been documented by fluorescent in situ hybridization (FISH) (Sheardown et al., 1997; Panning and Jaenisch, 1998). In preimplantation embryos the paternally inherited X chromosome is targeted for Xist gene expression and chromosomal silencing due to imprinting during gametogenesis (Takagi and Sasaki, 1975) or, according to a more recent view, is transmitted to the zygote in a preactivated state (Huynh and Lee, 2001), and this pattern is maintained in the blastocyst’s TE that gives rise to the placenta. In contrast, the pluripotent ICM from which the embryo proper develops exhibits very low levels of Xist. By the time the epiblast is formed, X inactivation takes place randomly on either
the maternally or the paternally inherited chromosome (reviewed by Boumil and Lee, 2001). A change in both the pattern and level of Xist gene expression is therefore necessary for lineage differentiation in female embryos and may predict cellular destiny at an earlier stage.

We have previously employed real-time PCR to quantify levels of Xist RNA in whole preimplantation embryos (Hartshorn et al., 2002). In agreement with other studies (Zuccotti et al., 2002), that analysis demonstrated that developing female embryos contain increasing amounts of Xist RNA from the two-cell stage onward, while male embryos, identified by the presence of the Y chromosome-specific Sry gene, do not. Total Xist RNA per embryo increases rapidly and peaks in compacted morulas, but a sharp change in the rate of Xist RNA accumulation takes place when the blastocoel begins to form. As a consequence, the average Xist RNA content per cell decreases during the next cell division cycles, although the development of the ICM cannot account for the magnitude of this decrease. We hypothesized that Xist is overexpressed in cleavage embryos at the earlier stages, possibly contributing an unbound fraction of Xist RNA in excess of the number of molecules needed to silence one X chromosome. Asymmetrical distribution of this free Xist RNA during subsequent cell divisions could provide a means of generating the two patterns of Xist expression found in the cells of blastocysts.

In order to investigate this possibility, we undertook the analysis of Xist RNA levels in single blastomeres isolated from embryos at the 8-cell stage, when cells first become polarized in preparation for the appearance of outer and inner blastomeres during the next cell cycle (Johnson et al., 1986). Blastomeres were readily separated after drilling the zona pellucida with the aid of a laser, a technique increasingly favored for genetic diagnosis of single cells ablated from human embryos (reviewed by De Vos and Van Steirteghem, 2001). We found this approach extremely convenient for removing not just one, but all blastomeres of an embryo in a sequential manner, while avoiding RNA degradation. A sensitive real-time PCR assay (Heid et al., 1996) involving the use of two molecular beacons conjugated to different fluorophores was devised in order to allow the identification of female and male samples and, at the same time, to measure their Xist RNA contents. The results demonstrate that most of the Xist RNA accumulated by female embryos at the 8-cell stage is contained in just two cells. This observation is consistent with other possible mechanisms of action that could determine Xist expression patterns without involving differential distribution of free transcripts. Future directions are discussed in light of these models.

MATERIALS AND METHODS
Embryo Culture and Single Blastomeres Isolation
Frozen late 2-cell stage embryos (B6C3F1 females bred with B6D2F1 males) were obtained from Embryotech Laboratories, Inc. (Wilmington, MA), and grown as previously described (Hartshorn et al., 2002) until the early 8-cell stage (about 20 hr, see Results for details). Single blastomeres were isolated with either of the following two methods. A 10–15 sec immersion in acidic Tyrode's solution (pH 2.5) containing 0.4% polyvinyl pyrrolidone (PVP) was used to remove the zona pellucida of the embryos in the first group, according to a well-known procedure (Nicolson et al., 1975). Samples were immediately transferred to Dulbecco's PBS (devoid of calcium and magnesium chloride) containing 0.4% PVP (both products from Sigma Chemical Company, St. Louis, MO). After one wash, blastomeres were disaggregated by pipetting up-and-down in glass capillaries of decreasing diameter and individually collected. Alternatively, the zona of each embryo was drilled using a ZILOS (zona infrared laser optical system; beam = 1480 nm) from Hamilton Thorne Research, Inc. (Beverly, MA). The software of this system visualizes the Isotherm Rings produced by laser pulses of different duration, allowing the operator to precisely burn a hole in the zona at the boundary of the 140°C isotherm (more details on this system can be found at http://www.hamiltonthorne.com/research/ivf). We used three pulses of 1 msec each (the power at target was set at 99%, equal to 140 mW) aimed at adjacent areas of the zona, in order to produce an opening slightly smaller than the diameter of a blastomere. Cells were extruded sequentially through this hole by using a glass capillary slightly larger than the embryo and gently pipetting the embryo up-and-down, without rupturing the rest of the zona. Blastomeres were collected one at a time, before release of the next cell outside the zona. For studies on whole embryos samples with intact zonae were collected directly in Dulbecco's PBS, after a couple of washes.

Nucleic Acid Isolation and Reverse Transcription
Single blastomeres or individual embryos were denatured immediately after harvesting using reagents from a Micro RNA Isolation Kit (Stratagene, La Jolla, CA). Nucleic acids (DNA and RNA) from each sample were purified using the phenol:chloroform:isoamyl alcohol procedure recommended by the manufacturer, but with a modified ratio (100 μl of phenol and 45 μl of chloroform/isoamyl alcohol solution per assay). Transfer RNA (10 μg/assay; Sigma Chemical Company) was added as a coprecipitant instead of glycogen. Reverse transcription was carried out in all the samples, using ThermoScript™ (Invitrogen, Life Technologies, Carlsbad, CA) as previously described (Hartshorn et al., 2002), in a 10 μl volume. At the end of the reaction, PCR reagents were added to each assay bringing the final volume to 100 μl.

Multiplexed Real-Time PCR With Molecular Beacons
Two sets of primers, with compatible thermodynamic characteristics, were designed for simultaneous amplification of Xist and Sry templates. Because Sry is an
introns of a gene and the chosen Xist sequence is located with exon 1, either set of primers could amplify the corresponding genomic DNA as well as cDNA sequence present in a sample.

The current Xist primers amplified a sequence contained within the one formerly selected for PCR with SYBR Green (Hartshorn et al., 2002). The smaller size of the amplicon proved to be critical for molecular beacon's binding. These new primers were localized at positions 6450/6522 of the GenBank sequence with accession # L04961 (5' TACAGCAAGGGTACTAAG 3', upstream primer; 5' AGTCTTGGAAATTTAAGAGT 3', downstream primer). The Sry primers were chosen at positions 8472/8598 (5' GAGGCAGAGATTGAA 3', upstream primer; 5' CCACCAGCAGATTGTA 3', downstream primer) of the GenBank sequence having accession # X67204. A modification was introduced in the lower primer at position 8611 (C was substituted for T), in order to decrease primer duplexes formation.

Molecular beacons specific for Xist and Sry were designed according to the method of Tyagi and Kramer (1996), as detailed on the internet site http://www.molecular-beacons.org. Theoretical folding of these molecules and of their target sequence was analyzed with the program accessible at the internet site http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi. Each beacon satisfied all the thermodynamic and conformational requirements needed for specific binding at the chosen annealing temperature, as previously described (Pierce et al., 2000). These two beacons were also designed with very similar thermodynamic characteristics in order to allow hybridization to their respective targets under the same annealing conditions, in a multiplexed reaction. The Xist-specific molecular beacon was conjugated to the fluorophore TET at the 5' end and to the quencher DABCYL at the 3' end. Its sequence was as follows: 5' CGGCTTTTTGCTCTCTCATCTCTGAGGC CGG 3'. The Sry-specific molecular beacon was covalently attached to the fluorophore FAM at the 5' end and to the quencher DABCYL at the 3' end. This beacon's sequence was 5' CCCCCACTCCTGTTGACACTTAAAGCCAG CGG 3'. Both beacons were synthesized by Research Genetics, Inc. (Huntsville, AL).

The composition of the PCR mixture was as following: 100 mM Tris, pH 8.3, 4 mM MgCl2, 0.3 μM each primer, 0.4 mM each dNTP, 0.3 μM each of the two beacons, and 1 μl of Taq polymerase (Promega; Madison, WI). The polymerase was incubated at room temperature with Platinum Taq antibody (Invitrogen) for 5 min before addition to the reaction mixture (hotstart PCR). The cycling profile was: 95°C for 5 min; 10 cycles consisting of the following three steps: 95°C (20 sec), 57°C (60 sec), and 72°C (30 sec); 45 cycles with the following three steps: 95°C (20 sec), 53°C (60 sec), and 72°C (30 sec). This cycling protocol had been optimized in order to allow maximum binding of the fluorescent probes in spite of the large volume of the PCR assays (100 μl). Real-time PCR was carried out in an ABI Prism 7700 Sequence Detector and fluorescence readings were taken at the annealing temperatures. A standard scale prepared from male mouse genomic DNA (Sigma Chemical Company) was used to convert the "threshold cycle" (C\textsubscript{T}) values at which each fluorescent signal was first detected above background into template copy numbers, as detailed by Hartshorn et al. (2002). A twofold increase in the number of templates present at the beginning of the reaction resulted in a one-cycle, left-to-right shift of the fluorescent signal during real-time PCR (smaller C\textsubscript{T} values thus signify higher template numbers). PCR conditions for this study were carefully selected in order to obtain amplification of the same numbers of Xist and Sry targets at very similar threshold cycles. (The same number of Xist and Sry sequences is normally found in male genomes, having one X and one Y chromosome.) The specificity of both molecular beacons was confirmed by using a standard scale of female mouse genomic DNA, which completely failed to generate Sry amplicons. The amplification products' sizes, established by electrophoresis of the samples through a 3% agarose gel, were as expected for both Xist and Sry sequences.

**Microscopy**

Blastomeres isolation was performed using an Olympus IX70 inverted microscope equipped with a 50× laser-grade lens (Hamilton Thorne Research; Beverly, MA) through which the laser pulses were delivered. Images were collected with a COHU solid state CCD camera (Cohu, Inc., Electronics Division, San Diego, CA).

**RESULTS**

**Comparison of Methods for Blastomeres Isolation**

We initially isolated single blastomeres from 8-cell stage embryos after dissolving the zona pellucida with a brief exposure to acidic Tyrode's solution, according to a widely used technique (Garbutt et al., 1987; Hansis et al., 2001), see Material and Methods for details. Only cells deemed intact by morphological criteria were collected for nucleic acids extraction (Fig. 1, inset). Alternatively, the zona was perforated in a single area by using a laser beam to generate a hole whose diameter was slightly smaller than that of a blastomere. Figure 1 illustrates typical steps in this method. Right after a hole was drilled the blastomere in closest proximity herniated through it. This cell (panel A) and subsequent blastomeres (panels B–D) were sequentially released into the medium by gently pipetting the embryo up-and-down in a slightly constricted glass capillary. Only occasionally cells were released as doublets connected by cytoplasmic bridges (panel D); these were easily separated by suction in a thinner capillary.

Figure 2, panel A demonstrates that laser drilling resulted in a higher yield of intact blastomeres. In the course of three experiments, 18 embryos were treated with acidic Tyrode's solution and 87 blastomeres were recovered among 147 processed. Laser treatment was carried out on 12 embryos during two experiments, and 81 of 89 cells were harvested intact. The mean percentages of recovered cells for the two procedures
were 58 and 91%, respectively. These findings were not surprising in light of the fact that cells isolated following laser drilling are never exposed to acid pH and are subjected to far less mechanical shearing than blastomeres disaggregated from a cluster following complete removal of the zona.

To further compare the two isolation procedures we measured yields of Xist RNA in all recovered female blastomeres. Nucleic acids from each cell were purified, subjected to reverse transcription, and then amplified via real-time PCR, according to the protocol described in our initial studies on whole embryos (Hartshorn et al., 2002). Because DNA and RNA are recovered together, RT-PCR leads to the amplification of both genomic and cDNA sequences. Real-time PCR enables us to count the number of templates present in each sample (see Material and Methods for details on the conversion of PCR signals into copy numbers). In our previous work, a “No RT” control was routinely performed in order to establish the Xist genomic DNA copy number in each sample. The number of Xist transcripts was then deduced by subtracting this value from the “RT” copy number including DNA and RNA. In the present study, it was not possible to split each single cell sample. Instead we devised a multiplexed assay in which Xist and Sry amplicons, representing the sum of genomic and cDNA templates, were simultaneously measured with two differently labeled molecular beacons. Embryos were identified as female when none of the recovered blastomeres generated an Sry signal. The number of
Xist transcripts in each female blastomere was then estimated based on the knowledge that cells of this gender contain two copies of the Xist gene. Figure 2, panel B summarizes Xist expression data collected from single female blastomeres isolated following either exposure to acidic Tyrode's solution or laser drilling. A PCR signal showing the presence of more than 10 copies of total Xist DNA + cDNA in a single cell was considered positive proof of expression. Only 1 of 24 cells (4%) harvested from 7 acidic Tyrode's solution-treated embryos at the 8-cell stage contained Xist RNA (experiments carried out on embryos at different cleavage stages yielded similar results, not shown). Conversely, 21 of 28 (75%) of the blastomeres isolated from 4 embryos at the same stage contained Xist transcripts when the laser had been used to open the zona. (Unlike cDNA, genomic DNA was consistently recovered in blastomeres collected with both methods, not shown). In light of these findings, further analysis was limited to cells obtained from laser-treated embryos.

Xist/Sry DNA and RNA Detection in Single Blastomeres

Representative Xist and Sry real-time PCR plots obtained from one female and one male blastomere are reported in Figure 3. No significant amount of Xist RNA has been detected in male embryos at this stage (Hartshorn et al., 2002), thus the Xist signal reflects the presence of a single copy of the Xist gene on the single X chromosome in each male blastomere. The Sry signal arose with the same kinetics as the Xist signal. Because we have been unable to detect any Sry transcripts in early mouse embryos (J. Klein, J.E. Rice, and L.J. Wangh, unpublished results), these data are consistent with the fact that Sry is a single-copy gene present on the Y chromosome of each male cell. These Xist and Sry patterns were typical of the male samples, but 5 of 13 male blastomeres analyzed did not generate any amplicon, probably because the single Y and X chromosomes present in the cell were lost during nucleic acid purification. (In a larger group of samples, including 59 blastomeres isolated with either the acidic Tyrode's solution or the laser procedure, 40 cells, or 68%, produced at least one amplicon. Xist was detected in 33 of these cells, or 82%, and Sry in 23 samples, or 57%). Sry signals were routinely detected above background at a PCR cycle (Ct, see Materials and Methods) equal or very close to that of the Xist signals, and in each case corresponding to a single copy on a standard scale of genomic DNA (Fig. 3, inset). Two-copy Xist or Sry signals, arising one cycle earlier during PCR, were also found in some of the samples, indicating individual blastomeres in G2 or early M phase of the cell cycle.

The Xist real-time PCR plot resulting from analysis of the single female blastomere in Figure 3 was in agreement with the expectation that female embryonic cells at this stage contain Xist RNA, as shown in our previous study of whole embryos. In fact, the Xist signal of this female cell was generated several cycles before the one of the male cell, indicating the presence of hundreds
of Xist templates. (The extent of Xist expression in different samples was found to be variable and a detailed quantification of Xist mRNA levels in individual female blastomeres is given below). None of the female cells produced a detectable Sry amplicon, confirming the specificity of the PCR primers and of the molecular beacon. Only 1 of the total 29 female blastomeres harvested after zona-drilling failed to generate a Xist signal. This result is likely due to the presence of multiple Xist RNA molecules in each female cell, since the apparent "PCR failure rate" for the single Xist and Sry genes in male cells was much higher (see above).

Quantification of Xist RNA in Single Female Blastomeres

Having established the reliability of our method at the single cell level, we investigated whether all blastomeres comprising an 8-cell stage female embryo contain similar amounts of Xist RNA. The kinetic plots of all blastomeres individually recovered from four female embryos in the course of two experiments are presented in Figure 4. It is immediately apparent that the number of Xist templates per reaction varied considerably. In Table 1, each C_T value has been converted to total Xist copy number per cell (inclusive of genomic Xist copies) and the order in which each blastomere was collected from the embryo is indicated in parentheses. Based on the knowledge that only two copies of the Xist gene are present in each diploid cell and that a maximum of four copies can be found in duplicating cells, it is clear that blastomeres from each set contained widely different Xist RNA levels, unrelated to the order in which the cells were isolated. Two cells in the same embryo often contained very similar numbers of Xist RNA molecules, but these two cells were not necessarily recovered sequentially. In at least one instance, two cells that were released from the embryo as a pair such as the one in Figure 1D, had very different levels of Xist RNA (Embryo 3, marked by asterisks). Although Xist expression in Embryos 1 and 2 was overall higher than in Embryos 3 and 4, suggesting day-to-day fluctuations, two blastomeres in every embryo consistently contained particularly high levels of Xist transcripts. Such elevated concentration of Xist RNA could not be accounted for by simply assuming that these specific blastomeres were tetraploid, because in most cases (embryos 2, 3, and 4) the Xist RNA present amounted to much more than a simple doubling relative to any other cell within the embryo. (A doubling of template copy number would appear as a right-to-left shift equivalent to a single C_T in the curve of Figure 4.)

Quantitative Comparison of Xist Templates in Single Blastomeres and Whole Embryos

To further investigate the accuracy and reliability of our method for measuring Xist DNA and RNA copies in isolated blastomeres, we compared results obtained from individual cells and whole embryos at the 8-cell stage. Specifically, the data in Table 1 were used to calculate total amounts of Xist RNA per embryo by summing RNA levels in single blastomeres, assuming diploidy in all cells and an average amount of Xist RNA
**TABLE 1. Xist DNA + RNA Copies Measured in Single Female Blastomeres**

<table>
<thead>
<tr>
<th>Experiment A</th>
<th>Experiment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo 1</td>
<td>Embryo 2</td>
</tr>
<tr>
<td>5 (6)</td>
<td>45 (4)</td>
</tr>
<tr>
<td>40 (2)</td>
<td>60 (2)</td>
</tr>
<tr>
<td>50 (5)</td>
<td>60 (6)</td>
</tr>
<tr>
<td>50 (7)</td>
<td>110 (5)</td>
</tr>
<tr>
<td>80 (4)</td>
<td>130 (1)</td>
</tr>
<tr>
<td>170 (1)</td>
<td>570 (7)</td>
</tr>
<tr>
<td>170 (3)</td>
<td>600 (3)</td>
</tr>
</tbody>
</table>

() Blastomeres collection order.
* Blastomeres deriving from a pair connected by a cytoplasmic bridge before collection.

**TABLE 2. Comparison of Xist RNA Levels Measured With Different Methods in 8-Cell Stage Female Embryos**

<table>
<thead>
<tr>
<th>Xist RNA copy numbers per embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green^a (Whole embryos)</td>
</tr>
<tr>
<td>363</td>
</tr>
<tr>
<td>489</td>
</tr>
<tr>
<td>600</td>
</tr>
<tr>
<td>726</td>
</tr>
<tr>
<td>834</td>
</tr>
<tr>
<td>942</td>
</tr>
<tr>
<td>954</td>
</tr>
</tbody>
</table>

*Xist RNA copy numbers calculated from total Xist copy numbers by subtracting "No RT" values (Hartshorn et al., 2002).

^a: Xist RNA copy numbers calculated from total Xist copy numbers assuming diploidy in all cells.

^A: A correction was introduced for blastomeres damaged during the isolation procedure and, therefore, not included in the experiments.
SYBR Green instead of molecular beacons (data extracted from Figure 5, panel A, in Hartshorn et al., 2002). All three methods of measurement revealed that the level of Xist RNA per female embryo varies widely at the 8-cell stage.

Comparative analysis of the three sets of data provided further information. First, the ranges of Xist RNA values found in whole embryos using SYBR Green or molecular beacons were similar. Second, calculation of Xist RNA levels by summation of single-cell measurements was fully consistent with results obtained by analysis of intact embryos. This was particularly true when comparing measurements performed with molecular beacons on the same day (indicated either by bold print, Experiment A, or by italics, Experiment B), suggesting that our procedure for Xist RNA isolation from single blastomeres was very reliable. Statistical analysis of the Xist copy number determinations collected in Tables 1 and 2 supported this conclusion. The average Xist RNA copy number per single blastomere (±SD) calculated from the 27 determinations in Table 1 (assuming diploidy in all cells, as before) was 103.2 (±155.2). As expected under ideal conditions, this amounted to about one eight of the average Xist RNA copy number per single 8-cell embryo (825.7 ± 637.6) obtained from the 14 whole-embryo measurements of Table 2.

The large standard deviations of the results above were not surprising, given the differences in Xist expression observed among samples. These included variations recorded in embryos that had reached the same developmental stage in different days. A more detailed analysis of the culture-times required by individual embryos to reach the 8-cell stage and their Xist RNA contents suggested some correlation between these two variables. Although wide fluctuations were present, faster growing embryos tended to have higher Xist RNA levels than slower embryos. Among the nine embryos analyzed in the present study using molecular beacons (Table 2; middle and right columns), the embryo with the highest Xist RNA level contained 2184 transcripts and reached the 8-cell stage in just 19 hr, while the two slowest-developing embryos were cultured for 25 hr and contained only 124 and 94 copies of Xist RNA. Values relative to all nine embryos are charted in Figure 5, which includes a trendline calculated by linear regression. (This trendline is only indicative, as its r-value is not significant. Analysis of many more samples will be needed to securely establish or exclude a correlation between the two variables in the graph.)

**DISCUSSION**

The use of an infrared 1.48 µm diode laser is increasingly favored for perforating the zonapellucida of human embryos when one or two blastomeres need to be biopsied for preimplantation genetic diagnosis (reviewed by De Vos and Van Steirteghem, 2001). This technique overcomes the risk of cytoplasmic acidification that occurs when a small stream of acidic Tyrode’s solution is expelled on a single spot of the zona pellucida (Depypere and Leybaert, 1994). Recent work on mouse also reports improved embryonic development following use of the laser approach (Phophong et al., 2001). Nevertheless, it is still current practice to dissolve the entire zona when multiple blastomeres of an embryo are to be analyzed (Collins et al., 1995; Szabó and Mann, 1995, 1996; Hansis et al., 2001). This procedure exposes all blastomeres to the possibility of damage due to a drop in intracellular pH.

The present study is the first analysis of multiple blastomeres harvested from the same embryo following laser zona-drilling. The levels of Xist RNA recovery clearly indicate that this method is advantageous in many respects besides the lack of exposure to low pH. For instance, damage due to mechanical shear is much less likely to occur in blastomeres collected after exiting the zona one at a time than in cells disaggregated by repeated pipetting of a denuded cluster. Also, for the same reason, the risk of contaminating the isolated cells with nucleic acids released from damaged samples is lower. The laser-assisted biopsy procedure is generally simpler and more reproducible than the chemical method. Finally, while blastomeres were collected at random for this study, the use of the laser also offers the potential of recovering cells from specific locations within the embryo.

Previous analyses of differential gene expression in individual embryonic cells are largely qualitative rather than quantitative (Collins et al., 1995; Hansis et al., 2001; Zuccotti et al., 2002), based on gel analysis of RT-PCR products or in situ hybridization. Although a great deal of valuable information can be drawn from these studies, RNA levels cannot be converted into copy numbers by application of those techniques. Relative quantification of transcripts present in groups of cells from the blastocyst’s TE and ICM (Brison and Schultz, 1996) or in single blastomeres at different stages (Kowalik et al., 1999) has been achieved with alternative RT-PCR-based methods. Gene expression levels in

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**Figure 5.** Correlation between Xist RNA content of individual embryos and culture-time needed to reach the 8-cell stage. Embryos were placed in culture at the 2-cell stage as described in Materials and Methods. The trendline was computed by linear regression.
single mouse blastomeres have also been rigorously measured with the RT-PCR single-nucleotide primer extension (SNuPE) assay (Szabó and Mann, 1995, 1996). This elaborate technique, however, involves the use of gel purification and radioactivity, requires the choice of a different internal standard, and is not suitable for fewer than several hundred molecules of transcript (Singer-Sam and Riggs, 1993). In contrast, real-time PCR allows the investigator to directly count templates as scarce as single copies in individual cells or embryos, and thereby provide a convenient means of validating overall procedures for handling blastomeres and isolating their nucleic acids. Based on our ability to recover single copies of DNA sequences, we conclude that the phenol/chloroform method of total nucleic acid isolation described here is overall quantitatively reliable and adequate for this kind of investigation. This confirms our previous results (Hartshorn et al., 2002) and establishes a basis for future evaluation of the efficiency and reliability of new commercially available kits designed for the isolation of DNA and RNA molecules from small numbers of cells. Finally, our comparative analysis of Xist RNA levels present in whole embryos and sets of blastomeres from single embryos also allows us to conclude that laser treatment does not cause a loss of RNA. To the contrary, in at least one case the blastomere closest to the laser beam was actually one of the two cells in that embryo that had the highest level of Xist RNA. It should be noted in this regard that the laser we selected offers two advantages over other available models. This kind of infrared diode laser delivers the light in a noncontact mode, reducing side effects (De Vos and Van Steirteghem, 2001). Moreover, its software enables the operator to visualize the peak isothermal rings produced by the laser beam at any given pulse duration (Douglas-Hamilton and Conia, 2001). We could therefore be certain to limit heating of the samples to a minimal area by using short, higher-power pulses rather than the longer, lower-power pulses favored in past studies by other investigators.

Real-time PCR results in quantitative amplification of Xist and Sry over a wide range of initial template molecules. Conversion of Xist and Sry PCR signals into copy numbers based on standards constructed from genomic DNA confirms that 8-cell male blastomeres usually contain one copy of both genes. Because cell cycles are not synchronized in cleavage stage embryos, however, a narrow range of variability for both Sry and Xist gene numbers is not surprising and in all likelihood is due to the occasional presence of tetraploid blastomeres. Corroborating our measurements of a single copy of Sry per male cell, Sry expression has never been found at this developmental stage (Zwingman et al., 1993; Cao et al., 1995), except for a single report (Boyer and Erickson, 1994). Xist RNA accumulation in male 8-cell embryos is equally negligible, although it reaches considerable levels in females (Hartshorn et al., 2002). Because the PCR primers used for this work are not strand-specific, transcripts of the antisense gene Tsix, known to block Xist RNA accumulation in embryonic stem cells (Lee et al., 1999a; Morey et al., 2001), could also be amplified during Xist PCR. However, Tsix expression in mouse embryos starts after the 8-cell stage (Sado et al., 2001).

Based on the above considerations we conclude that template copies can be efficiently recovered and accurately counted with our approach and that Xist RNA reaches significantly different levels in the blastomeres of female 8-cell embryos. Interestingly, previously published images of female mouse embryos at this stage analyzed via FISH (Sheardown et al., 1997) are consistent with our observation that individual cells of the same embryo contain greatly variable levels of Xist RNA (as shown by the greatly different dimensions of the Xist RNA FISH signals in the nuclei of cells comprising an 8-cell female embryo). We have demonstrated earlier that the average number of Xist transcripts per female cell increases quickly from the 2-cell to the morula stage and then steadily decreases during the later phases of preimplantation development, suggesting that an "excess" amount of Xist RNA is initially produced (Hartshorn et al., 2002). A role for these "extra" (possibly unbound) Xist RNA molecules is not known, but in light of our present results we speculate that they are differentially distributed among early embryonic cells. In female embryos, therefore, this difference may be predictive of the destinies of the cells themselves at the subsequent onset of lineage differentiation coincident with formation of a blastocoe. Once the TE and ICM lineages have been established, substantially lower levels of Xist RNA may be sufficient to guarantee that the chosen X chromosome remains silent.

An additional striking feature common to all female embryos analyzed in this study is that most of the Xist RNA produced by each of them is consistently recovered at equal levels within two cells. Because the embryos were at the third cleavage stage, the above finding could be explained by a model alternative to differential distribution during cell division but still leading to segregation of most Xist RNA within two cells. Epigenetic modifications of a number of mammalian genes occur during gametogenesis via methylation/demethylation of the cytosine residue in CpG islands. Further DNA modifications take place later on in the zygote, refining the mechanism by which parental imprinting of specific genes is achieved (reviewed by Latham, 1999). The mouse paternal genome is actively demethylated after fertilization (Oswald et al., 2000), a process that is completed within 4 hr and concomitant to pronucleus formation (Santos et al., 2002). Passive demethylation, leading to asymmetric methylation patterns in sister chromatids (Rougier et al., 1998), is then ongoing during the cleavage stages up to morula, after which de novo methylation is found in the blastocyst's ICM but not in the TE. By contrast, maternal genes remain methylated or become further methylated after fertilization (Oswald et al., 2000). Analysis of fluorescence patterns identifying the presence of 5-methylcytosine, however, indicates that, although maternal alleles
maintain their methylation levels, the frequency of the labeled chromatids decreases at each cell division. Thus, by the 4th metaphase (8-cell stage) methylated chromosomes only comprised 25% of those found at 1- and 2-cell stages and they all are formed by asymmetrically labeled chromatids (Rougier et al., 1998). It has been long known that Xist expression is paternally imprinted in the cleavage stage mouse embryo (Takagi and Sasaki, 1975). Methylation studies demonstrate that Xist is predominantly demethylated during spermatogenesis, although partial methylation persists on some sites of the Xist promoter, maybe through embryonic development, but is lost in differentiated (somatic) cells (Norris et al., 1994). It was also recently shown that some sequences of the male genome appear to resist active demethylation (Santos et al., 2002). Taken together, this body of information arises the possibility that a particular epigenetic modification, marking the paternal Xist allele or its promoter at the time fertilization, is transmitted to the daughter cells and confers them the capacity of synthesizing stable Xist RNA. This modification, likely due to CpG methylation, would not be replicated on the newly synthesized sister chromatids during the time when passive demethylation occur, and therefore would be only found in 25% of the cells comprising an 8-cell stage embryo. Thus, two cells in each embryo would have a higher or earlier capacity of transcribing the Xist gene, or would produce mostly stable rather than unstable Xist RNA (Panning et al., 1997). Generation of stable and unstable transcripts was initially thought to be dependent on activation of different promoters, although no specific sequences with this role have been identified to date (Lee et al., 1999b; Warshawsky et al., 1999).

Timing of cell division should also be considered, alternatively to epigenetic modifications or in combination with them, as a possible reason for the formation of different Xist RNA accumulation patterns among female embryonic cells. In fact, Xist expression in mouse embryos starts at the late 2-cell stage (Latham and Rambhatla, 1995; Hartshorn et al., 2002; Zuccotti et al., 2002). One of the first two embryonic cells, almost always the one encircling the sperm point of entry (Piotrowska and Zernicka-Goetz, 2001; Pluza et al., 2002), then divides before the other and recent evidence suggests that this “divisional advantage” eventually leads to the formation of the ICM, while the TE is derived from blastomeres with slower cell cycles (Piotrowska et al., 2001).

Based on all above information, it is tempting to speculate a connection between Xist RNA distribution in early embryonic cells and their later developmental fate, although this relation is not likely to be of a cause-and-effect type, because it can only happen in female embryos. (There are no reasons to assume that development to blastocyst should follow different pathways in male and female embryos, although recent studies point to different developmental rates in the two sexes, reviewed by Mittwoch, 2000.) Xist RNA patterns in cleavage-stage female embryos, however, could potentially be a very useful marker for studying embryonic cells fate.

In the next phase of our research we plan to quantify Xist RNA accumulation in embryos at earlier stages, in order to gain some insight into the mechanism responsible for differential Xist transcript distribution among blastomeres. In addition to PCR measurements, the recent successful introduction of RNA-specific fluorescent probes into living mammalian cells opens the possibility for this kind of investigation in developing embryos. Perlette and Tan (2001) were able to demonstrate with this method that considerably different β-actin mRNA concentrations are present in single rat kidney cells of the same population. A new, long-lasting type of probes appears to be suitable for fluorescence monitoring over several days (Molenaar et al., 2001).

Ideally, these probes could be injected in embryonic blastomeres at the 8-cell stage or earlier in order to mark high Xist-expressing cells during the following developmental steps and also to establish whether Xist transcripts are exclusively associated with the inactive X chromosome. Preliminary experiments in this direction are under way in our laboratory.

ACKNOWLEDGMENTS

We thank Dr. Ken Pierce for illustrating the blastomeres isolation procedure with acidic Tyrode’s solution. Hamilton-Thorne Biosciences, Inc., is acknowledged for a grant supporting this research and for providing the laser used in this study.

REFERENCES


Szabo PB, Mann JR. 1996. Maternal and paternal genomes function independently in mouse oocytes in establishing expression of the imprinted genes Surpr and Igf2r: No evidence for allelic trans-sensing and counting mechanisms. EMBO J 15:6918–6925.


