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# Skewed X-Chromosome Inactivation Is Associated with Trisomy in Women Ascertained on the Basis of Recurrent Spontaneous Abortion or Chromosomally Abnormal Pregnancies

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**An increase in extremely skewed X-chromosome inactivation (XCI) (≥90%) among women who experienced recurrent spontaneous abortion (RSA) has been previously reported. To further delineate the etiology of this association, we have evaluated XCI status in 207 women who experience RSA. A significant excess of trisomic losses was observed among the women who had RSA with skewed XCI versus those without skewed XCI ($P$ = .02). There was also a significant excess of boys among live births in this group ($P$ = .04), which is contrary to expectations if the cause of skewed XCI was only that these women carried X-linked lethal mutations. To confirm the association between skewed XCI and the risk of trisomy, an independent group of 53 women, ascertained on the basis of a prenatal diagnosis of trisomy mosaicism, were investigated. Only cases for which the trisomy was shown to be of maternal meiotic origin were included. The results show a significantly higher level of extreme skewing (≥90%) in women whose pregnancies involved placental trisomy mosaicism (17%) than in either of two separate control populations ($n$ = 102 and 99) ($P$ = .02 compared with total control subjects). An additional 11 cases were ascertained on the basis of one or more trisomic-pregnancy losses. When all women in the present study with a trisomic pregnancy ($n$ = 103) were considered together, skewed XCI was identified in 18%, as compared with 7% in all controls ($n$ = 201) ($P$ = .005). This difference was more pronounced when a cutoff of extreme skewing of 95% was used (10% vs. 1.5% skewed; $P$ = .002). Maternal age was not associated with skewing in either the patient or control populations and therefore cannot account for the association with trisomy. Previous studies have shown that a reduced ovarian reserve is associated with increased risk of trisomic pregnancies. We hypothesize that the association between skewed XCI and trisomic pregnancies is produced by a common mechanism that underlies both and that involves a reduction of the size of the follicular pool.**

## Introduction

Recurrent spontaneous abortion (RSA), defined as loss of three or more consecutive pregnancies prior to 20 wk of gestation, is a devastating problem for couples trying to have children. Despite the extensive medical testing and experimental treatments that many of these couples undergo, a cause for their pregnancy loss often remains unclear (Stephenson 2001). Indeed "chance" may explain many of these losses, because 15%–20% of all clinically recognized pregnancies in the general population end in spontaneous abortion (SA) (Hassold et al. 1996). Nonetheless, the frequency of RSA (1%–2%) is greater than expected by chance, which suggests that, in at least some couples, a predisposing factor is present. Thus, the finding that skewed X-chromosome inactivation (XCI), defined as the preferential inactivation of one of two X chromosomes in female cells, is increased in women with RSA (Sangha et al. 1999; Lanasa et al. 2001; Uehara et al. 2001) has led to the exciting prospect that a cause for these pregnancy losses can be identified in a subset of women with RSA.

Because there are many potential causes of skewed XCI (see Brown and Robinson [2000] for a review), the reason for an association between RSA and skewed XCI is not clear and may be heterogeneous. Several authors have suggested that X-linked mutations underlie this association (Lanasa et al. 2001; Uehara et al. 2001). This hypothesis proposes that male lethal X-linked mutations cause skewed XCI in female carriers and would thus result in an increase in lost male conceptuses and a relative increase in female live births for

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female carriers. X-linked mutations have been clearly associated with skewed XCI and SA in some families (Pegoraro et al. 1997), and one study reported a slight excess of female live births in women with skewed X CI and RSA (Lanasa et al. 2001). However, without karyotype information on the SAs to demonstrate an excess of chromosomally normal males, it is difficult to determine how often X-linked mutations may explain these findings.

Alternatively, we have suggested that skewed XCI may be indicative of an increased risk of trisomy due to a reduction in size of the pool of embryonic precursors during early development of the affected woman (Sangha et al. 1999; Robinson et al. 2001*a*). Any event that reduces the number of embryonic cells present at the time of XCI increases the chance of skewed XCI. For example, a diploid fetus that arises from "trisomic zygote rescue" is often associated with skewed XCI (Lau et al. 1997). In this situation, a trisomic conceptus loses one extra chromosome in early development, producing a mosaic placenta and/or fetus. Reduced size of the pool of embryonic precursors may be associated with RSA by at least two mechanisms: (1) residual trisomic cells in the oocytes (in females whose own development was associated with trisomy mosaicism) and (2) poor in utero growth (of the female when she herself was an embryo), leading to a reduced total number of primordial ovarian follicles. Since reduced ovarian reserve has been associated with an increased risk of trisomy (as a result of an unknown mechanism) (Freeman et al. 2000; Kline et al. 2000), an excess of trisomic SAs would be expected if the size of the follicular pool was reduced in the women with skewed XCI who experience RSA (Robinson et al. 2001).

To investigate this potential cause of skewed XCI and RSA, the karyotypes of conceptuses lost by women with skewed XCI and without skewed XCI were analyzed and compared. Women with RSA and skewed XCI were found to have significantly more trisomic losses than women with RSA without skewed XCI. To further confirm that skewed XCI is associated with an increased risk of trisomy, we then investigated skewed XCI in mothers who were ascertained only on the basis of having a trisomic pregnancy and for whom the trisomy was confirmed to be of maternal meiotic origin.

## Patients, Material, and Methods

### Patients

*Patients ascertained on the basis of RSA.*—Patients ($n = 230$) were ascertained through the Recurrent Pregnancy Loss Clinic at the British Columbia's Women's Hospital and Health Centre by one of the authors (M.D.S.), between September 1997 and February 2001. Each pregnancy was documented by a positive result of a serum or urine test for human chorionic gonadotropin, by ultrasound, or by results of a pathologic examination. Ethics approval was obtained from the University of British Columbia clinical research ethics board. Peripheral blood (7 ml) was obtained after informed consent. XCI results from 76 of these patients have been reported elsewhere (Sangha et al. 1999). Women were included in the study regardless of the outcome of their clinical evaluation, except for nine women in whom—or in whose partner—a structural chromosomal abnormality was found. A total of 207 samples were informative for our XCI assay (194 for the androgen receptor [*AR*] locus and 13 for the fragile-X mental retardation 1 [*FMR-1*] locus). The mean age was 34.0 years (range 19–45 years) at the time blood was sampled in the informative women with RSA. The mean number of live births and SAs in these patients was 0.9 and 4.4, respectively.

*Patients ascertained through trisomy.*—Women ($n = 53$) were ascertained through prenatal diagnosis of trisomy mosaicism by tests of chorionic villus samples (CVS) or amniotic fluid (AF), as part of a separate study on the origin of trisomy mosaicism (Robinson et al. 1997). This group included 1 case each of trisomy 7, 10, 12, and 13; 2 cases each of trisomy 2, 9, 20, and 21; 3 cases of trisomy 14; 36 cases of trisomy 16; and 5 cases of trisomy 22. The trisomy was determined to be of maternal meiotic origin by molecular analysis, as reported elsewhere (Robinson et al. 1997; Peñaherrera et al. 2000), except in nine cases of trisomy 16 or 22 mosaicism, in which a maternal meiotic origin was assumed because virtually all cases of trisomy mosaicism involving these two chromosomes are consequences of maternal meiotic errors (Robinson et al. 1997). Age was known for 29 of the 53 patients informative for either *AR* ($n = 47$), *DXS6673E* ($n = 4$), or *FMR-1* ($n = 2$) XCI assays, and the mean age of the group was 35.6 years (range 25–51 years). However, because all blood samples were obtained during or shortly after pregnancy, these were all women of "child-bearing age."

In our study of trisomy, we also included four women with a trisomic SA who were ascertained through the Recurrent Pregnancy Loss Clinic but were not included in our study of RSA because they did not have three consecutive losses. Also, after our RSA study, seven women, from various centers, were ascertained solely on the basis of having had a trisomic loss. The mean age of these women was 36.5 years (range 25–42 years). Of the 11 women, 10 were informative for the *AR* locus, and 1 was informative for the *DXS6673E* locus.

*Control Women.*—Two control groups were collected. Control group 1 ($n = 102$) consisted of anonymous healthy donors ($n = 25$) and unaffected spouses from families with Huntington disease ($n = 77$). Although pregnancy history was largely unknown among these

women, the group should be unbiased in terms of trisomic pregnancies or pregnancy losses. In control group 1, exact age was known in 90 women, with a mean age of 34.6 years (range 17–45 years). All were informative for the *AR* ($n = 94$) or *DXS6673E* ($n = 8$) XCI assay. In those women whose exact age was unknown, the women were known to be of child-bearing age (i.e., 20–45 years old). Control group 2 ($n = 99$) was collected for comparison with the mothers ascertained for prenatal diagnosis of trisomy, and it consisted of mothers ascertained through various studies of the origin of genetic abnormalities, including mothers of prenatally diagnosed mosaic aneuploidy of mitotic origin ($n = 10$), triploidy ($n = 44$), and deletions/duplications/translocations ($n = 45$). Exact age was known in 87 of 99 control group 2 women who were informative for either *AR* ($n = 92$) or *DXS6673E* ($n = 7$) XCI assays, with a mean age of 33.0 years (range 21–44 years).

### *XCI Assay*

DNA was extracted from peripheral blood by conventional methods. A DNA methylation-sensitive assay was used to distinguish the inactive X chromosome from the active one (Wolf et al. 1984; Yen et al. 1984; Tribioli et al. 1992). The presence of methylation at a particular site was determined by digestion with methylation-sensitive restriction enzymes, and the locus was amplified by PCR, with primers flanking the site of the restriction enzyme cut and a polymorphism. The *AR* locus was used, primarily, to assess skewing (Allen et al. 1992). If the *AR* locus was uninformative, skewing was assessed at either the *FMR-1* (Hecimovic et al. 1997) or *DXS6673E* (Beever et al., in press) locus. If a sample was found to have ⩾80% skewing, the assay was repeated, and an average of the tests was used.

Genomic DNA (300 ng) was digested with 5 U of *Hpa*II (for the *AR* and *FMR-1* assays)—or 5 U of *Hha*I (for the *DXS6673E* assay)—and 2 U of *Rsa*I in a total volume of 20 μl. For each sample, an undigested control was prepared with 300 ng of genomic DNA and 2 U of *Rsa*I. The digested and undigested samples were incubated at 37°C overnight. Complete digestion was assessed by amplification of the 5′ region of the *MIC2* gene, an X-linked gene which escapes XCI and is therefore always unmethylated at the 5′ end (Goodfellow et al. 1988; Anderson and Brown 2002). This assay was shown to detect incomplete digestion in a sample with 4.5% incomplete digestion (data not shown).

XCI skewing was estimated with the *AR* locus, according to the protocol of Allen et al. (1992), with the exception that digested and undigested samples (1.5 μl) were amplified in 500 nM of each primer (AR PCR1.1 and AR PCR1.2), 2 mM $MgCl_2$, 200 μM each dNTP, and 0.2 U of *Taq* (Rose Scientific), in a total volume of 15 μl. PCR conditions were 95°C for 5 min (initial denaturation); 95°C for 45 s, 60°C for 30 s, and 72°C for 30 s, for 30 cycles, with a final extension at 72°C for 7 min. PCR of the *FMR-1* locus was performed according to a modified protocol of Hecimovic et al. (1997), with primers designed by Fu et al. (1991). Digested and undigested samples (1 μl) were amplified in Expand long PCR buffer 1 (Roche), 10% dimethyl sulfoxide (DMSO), 350 μM dATP, 350 μM dCTP, 350 μM dTTP, 100 μM dGTP, 250 μM 7-deaza-GTP, 1 μM of each primer, and 0.4 U of Expand enzyme mix (Roche) in a total volume of 10 μl. Cycling conditions were 97°C for 30 seconds (initial denaturation), followed by 97°C for 30 s, 65°C for 45 s, 68°C for 4 min with an additional 20 s per cycle, for 25 cycles. For *DXS6673E*, digested and undigested samples (1 μl) were amplified in a total volume of 12.5 μl with 200 μM dNTP, 0.5 mM $MgCl_2$, 400 nM of each primer, and 0.1 U of Rose *Taq*. Cycling conditions were 95°C for 4 min (initial denaturation); 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min, for 35 cycles, followed by 72°C for 7 min (final extension). Primer sequences were as follows: forward 5′-ATGCTAAGGACCATCCAGGA-3′ and reverse 5′-GGAGTTTTCCTCCCTCACCA-3′ (Beever et al., in press).

PCR products were visualized, and XCI skewing was estimated by two methods: (1) densitometry of silver-stained polyacrylamide gels and (2) automated fluorescence analysis. Densitometry of silver-stained polyacrylamide gels has been described elsewhere (Lau et al. 1997). For automated fluorescence analysis, PCR was performed with a forward primer labeled with the ABI Prism Dyes HEX (for *AR* and *DXS6673E* forward primer) and 6-FAM (for forward FMR-1 primer). The amplification products were sized using capillary electrophoresis on an ABI Prism 310 genetic analyzer. Fluorescence was detected by ABI Prism data-collection software and analyzed by use of GeneScan software. The peak area for each allele was used to determine XCI skewing.

Degree of skewing was measured in relation to the most intense allele (the allele that was inactivated most often). To account for preferential amplification, the areas of the peaks of the digested sample were normalized in relation to measurements from the undigested sample. The degree of skewing was calculated as (d1/u1)/(d1/u1+d2/u2), where d1 and d2 represent the peak area of the more intense allele and the less intense allele, respectively, from the digested sample and where u1 and u2 are the corresponding bands from the undigested sample. At the *AR* locus, if alleles of a sample were 3 bp apart, shadow bands of the first allele overlap with bands of the second allele. In these cases, shadow bands were calculated to represent ~30% of the amplification from one allele, and peak area values were adjusted accordingly. In the case of RSA samples, skewed XCI was

**Table 1**

**Skewed XCI in Women with RSA or with a Trisomic Pregnancy**

| Ascertainment (*n*) | No. ≥90% Skewed XCI (%) | *P* | No. ≥95% Skewed XCI (%) | *P* |
|---|---|---|---|---|
| All RSA (207) | 25 (12) | .16,[a] .18,[b] .08[c] | 11 (5.3) | .13,[a] .06,[b] .03[c] |
| Women with trisomic pregnancy: | | | | |
| RSA (39) | 8 (21) | .02,[a] .03,[b] .007[c] | 5 (13) | .02,[a] .007,[b] .004[d] |
| Non-RSA (11) | 1 (9) | Not significant | 0 (0) | Not significant |
| Non-RSA, Mosaic (53) | 9 (17) | .05,[a,b] .02,[c] | 5 (9) | .05,[a] .02,[b] .01[d] |
| Total (103) | 18 (18) | .005[c] | 10 (10) | .002[d] |
| Control group: | | | | |
| 1 (102) | 7 (7) | | 2 (2) | |
| 2 (99) | 7 (7) | | 1 (1) | |
| Total (201) | 14 (7) | | 3 (1.5) | |

[a] Compared with control group 1, Fisher's test.
[b] Compared with control group 2, Fisher's test.
[c] Compared with both control groups, $\chi^2$ test.
[d] Compared with both control groups, Fisher's test.

initially quantified without knowledge of the karyotypes of conceptuses lost by each woman. Although this is not true for the women who had trisomic SAs, interpretation of data from ABI quantification should not be subject to human bias, because the results are quantified using the GeneScan software. Furthermore, to ensure accuracy, all borderline results were repeated several times.

It is important to note that the present protocol differs from the one used in our previous study (Sangha et al. 1999) in two ways: (1) the *MIC2* assay was not previously used to check for complete digestion and (2) approximately half of the samples of the present study were analyzed with the ABI 310, whereas all samples from the previous study were analyzed by the silver-staining method. A number of patient and control samples, particularly those with alleles of similar size that were reported previously, have been reanalyzed using the *MIC2* assay and the ABI 310, and, in some cases, skewing status changed. Three samples previously showing skewed XCI with the silver-staining method were no longer skewed when reanalyzed with the ABI 310, whereas two samples previously not skewed by silver-staining changed to skewed when analyzed with the ABI 310. All five of these samples had alleles that differed in size by only 3 bp, and such close alleles are difficult to separate and quantify with the silver-staining protocol. Analyzing these samples with the ABI 310 allows for more consistent and accurate quantification of alleles (authors' unpublished results). However, samples analyzed with both the silver-staining method and the ABI 310 show a high correlation in XCI skewing ($r = 0.839$, $n = 60$) (data not shown). Thus, samples previously analyzed by silver staining were retested on the ABI only if alleles were close or skewing was >80%.

### *Statistical Analysis*

Fisher's exact test or $\chi^2$ analysis was used to compare the proportion of skewed XCI in patient and control populations. Given our hypothesis that skewed XCI will be increased in our patient population, *P* values from a one-tailed Fisher's exact test were reported. Student's *t* test was used to compare the mean age of patient and control populations and to determine whether age was a confounding factor in the association of trisomic pregnancy and skewed XCI.

## Results

### *Skewed XCI in Women with RSA*

Among women with RSA, 25 of 207 (12%) were found to have extremely skewed XCI (defined, by convention, as ≥90% inactivation of one allele). In contrast, skewed XCI was observed in 7 of 102 (7%) women in group 1 and in 7 of 99 (7%) women in control group 2. Thus, women with RSA showed greater XCI skewing than did control women, although the difference was not statistically significant ($P = .16$ and $P = .18$, respectively, by $\chi^2$ test when women with RSA were compared with control groups 1 and 2 separately, and $P = .08$ if both control groups are considered together) (table 1). When a more stringent criterion for skewing (≥95%) was used, a greater-than-twofold increase in skewing was seen in women with RSA (5.3%), compared with control group 1 (2.0%) ($P = .13$, Fisher's exact test), and a fourfold increase was seen compared with control group 2 (1%) ($P = .058$, Fisher's exact test). In this case, the difference is statistically significant if the comparison is made with the sum of both control groups ($P = .03$, $\chi^2$ test). Using a more stringent cutoff

**Table 2**

**Karyotypes of Conceptuses Lost by Women with RSA**

| Karyotype | No. of SAs (%) among Women with Skewed XCI ($n$ = 16) | No. of SAs (%) among Women without Skewed XCI ($n$ = 85) |
|---|---|---|
| 46,XY | 6 (23.1) | 43 (29.7) |
| 46,XX | 4 (15.4) | 40 (27.6) |
| Trisomy | 14 (53.8)[a] | 43 (29.7) |
| Other abnormalities | 2 (7.7) | 19 (13.1) |
| Total | 26 | 145 |

[a] $P = .02$, by $\chi^2$ test, in comparison with all other losses.

for skewing may be more clinically relevant for the identification of women in whom RSA is associated with skewed XCI, because chance may play a smaller role in the cause of skewed XCI with this cutoff. The mean age of women with RSA was not significantly different from that of either control group.

### *Pregnancy History of Women with RSA and Skewed XCI*

To determine if male lethal X-linked mutations or reduced size of the pool of embryonic precursors is significantly contributing to pregnancy losses in the women with skewed XCI and RSA, we examined karyotypes of losses and sex of live births. No excess of male losses was seen in the women with ⩾90% skewed XCI in comparison with those without skewed XCI (table 2). In addition, an excess of female live births was not seen in women with skewed XCI (table 3). Unexpectedly, the opposite trend—an excess of male live births—was observed.

An increase in trisomic losses was observed in women with ⩾90% skewed XCI ($P = .02$, Fisher's exact test) (table 2). In some cases, more than one karyotyped loss from the same mother was included in the analysis; therefore, this test may introduce a bias, because clustering of chromosomally abnormal and normal losses can occur within family groups. For this reason, we also analyzed the data in terms of frequency of skewed XCI in mothers with at least one trisomic loss (21%, $n$ = 39) versus frequency of skewing in mothers with one or more karyotyped losses, none of which was trisomic (13%, $n$ = 63). Although this difference did not reach statistical significance, only the women with a confirmed trisomic loss showed a significant increase in skewing compared with controls (7%, $n$ = 201) ($P = .007$) (table 1). It would be expected that some of the women with a chromosomally normal loss also had trisomic losses, since most losses were not karyotyped, thus minimizing the difference between these two groups.

The origins of seven trisomic losses from four women with skewed XCI were determined in a study of recurrent aneuploidy (Robinson et al. 2001*b*). Six of these seven trisomic SAs were found to involve a maternal meiotic nondisjunction event, whereas one case was due to a paternal nondisjunction event. The vast majority of sporadic trisomic SAs are also due to maternal meiotic errors. In women with trisomic losses, mean maternal age at the time of loss was slightly lower in women with skewed XCI than in those without skewed XCI. However, mean maternal age was increased in all trisomic losses compared with the mean maternal age for nontrisomic losses (table 4). Thus, the trisomic losses among the women with skewed XCI are also associated with advanced maternal age, as are trisomic losses among the general population (Hassold et al. 1996). Again, this analysis is confounded by the inclusion of more than one loss from the same woman, which may then be correlated, in terms of maternal age, with one another. A larger sample size, which then could allow correction for correlated samples, would be needed to determine whether, in fact, women with ⩾90% skewed XCI might be having trisomic SAs at a slightly younger age than women with <90% skewed XCI.

### *Skewed XCI in Women with a Trisomic Pregnancy*

To verify the association of skewed XCI and trisomy, we collected samples from 11 additional women who were ascertained on the basis of a trisomic SA and were not part of our original study, as well as from 53 women who were ascertained on the basis of a prenatal diagnosis of trisomy mosaicism for which the trisomy was previously shown to be of maternal meiotic origin (i.e., due to a nondisjunction event occurring during oogenesis). Skewed XCI (⩾90%) was observed in 18% of all women with a trisomic SA (including the original RSA group) ($n$ = 50) compared with 7% of women in control group 1 ($n$ = 102) and 7% of women in control group 2 ($n$ = 99); both comparisons were significant ($P = .04$, Fisher's exact test). Skewed XCI (⩾90%) was also observed in 17% of women with trisomic preg-

**Table 3**

**Sex of Live Births to Women with RSA**

| Sex | No. of Live Births to Women with ⩾90% Skewed XCI[a] ($n$ = 13) | No. of Live Births to Women without Skewed XCI ($n$ = 113) |
|---|---|---|
| Male | 15 | 91 |
| Female | 5 | 79 |
| Total | 20 | 170 |

[a] $P = .07$, compared with sex ratio among live births to women without skewed XCI; $P = .04$, compared with an expected male:female ratio of 1:1.

**Table 4**

**Maternal Age at Time of Pregnancy Loss among Women with RSA**

| Karyotype of Loss | Mean Age (no. of SAs) among Women with ≥90% Skewed XCI ($n$ = 25) | Mean Age (no. of SAs) among Women without Skewed XCI ($n$ = 182) |
|---|---|---|
| Trisomic | 37.4[a] (14) | 38.2[b] (42) |
| Not trisomic | 33.6 (12) | 33.5 (101) |

[a] $P = .001$.

[b] $P < .0001$, one-tailed $t$ test, compared with not trisomic, not skewed.

nancies ($n = 53$) compared with 7% of group 1 controls ($n = 102$) and 7% of group 2 controls ($n = 99$) ($P = .05$ for both comparisons, Fisher's exact test) (table 1). When a higher skewing cutoff (95%) was used, a fourfold and an eightfold increase, respectively, of skewed XCI was observed in mothers of mosaic trisomy (8%) compared with group 1 controls (2%) and group 2 controls (1%) ($P = .09$ and $P = .05$, respectively, Fisher's exact test). When all women with trisomic pregnancies were grouped together and compared with control groups 1 and 2 combined, the association of skewed XCI and trisomy was found to be highly significant ($P = .005$, Fisher's exact test) (table 1).

The mean age of all the mothers who had a trisomic SA was increased over control group 1 and control group 2 ($P = .003$ and $P < .0001$, respectively, one-tailed $t$ test). The mean age of the mothers of mosaic trisomy was increased over control group 2 ($P = .02$, one-tailed $t$ test) but not over control group 1 ($P = .25$, one-tailed $t$ test). Because skewed XCI has been reported to increase with very high maternal ages (>65 years) (Sharp et al. 2000), it was important to verify whether this could be a confounding factor in our study. However, there was no evidence for any correlation between skewing and maternal age in the total group of women who a had trisomic SA ($r = .002$; 95% CI = −22 to .22), nor was the correlation significant in the total control population ($r = .12$; 95% CI = −.03 to .26). Furthermore, there was no increase in maternal age when we compared the ≥90% skewing group with the <90% skewing group in either of our patient populations, nor in our control populations (table 5). In fact, the mean maternal age among women who had a trisomic pregnancy with ≥90% skewing was actually slightly lower than the mean age among those with <90% skewing. Of further note, the mean maternal age of 34.6 years among eight mothers who had trisomic pregnancy and >95% skewing and who were informative for maternal age was even lower than that for ≥90% skewing.

### *Association of Skewed XCI with Type of Trisomy*

The association with maternal age varies by type of trisomy (Hassold et al. 1996). Specifically, trisomy 16 increases linearly with maternal age, whereas most other trisomies increase exponentially with maternal age. We therefore examined skewed XCI separately, depending on whether the mother was ascertained on the basis of a trisomy 16 pregnancy. Interestingly, among the 33 cases ascertained through prenatal diagnosis of mosaic trisomy 16, only 2 women exhibited skewed XCI (6%), whereas, among the 20 women ascertained on the basis of other (non-16) mosaic trisomies, skewed XCI was found in 7 cases (35%). The frequency of skewing was thus more pronounced when trisomy 16 was excluded from the analysis, and the difference between these two subgroups was statistically significant ($P = .04$, Fisher's exact test). A similar trend was not noted among the women who had RSA that included one SA with trisomy 16; 4 of these 18 women (22%) showed skewed XCI. However, it is important to note that all of the cases in this group had other miscarriages and that most were confirmed to have other trisomic losses not involving trisomy 16.

## Discussion

In the present study, we report an increase in skewed XCI among women who experience RSA, although this difference is no longer as striking as the one in our earlier report (Sangha et al. 1999). The difference may be explained by changes in our methodology for estimating skewing, as well as by the addition of new case and control subjects. Nonetheless, because all studies reported to date (Kristiansen et al. 1999; Lanasa et al. 1999; Uehara et al. 2001; Sullivan et al. 2002) have shown an increase of skewed XCI in women who experience RSA, it appears likely that this effect is real, even if not statistically significant in all studies. Furthermore, the difference was greater when the more stringent cutoff of 95% skewing was used. The greater number of trisomic losses among women who had RSA with skewed XCI than among women who had RSA without skewed XCI is further evidence of a clinical difference between these two groups. We subsequently ver-

**Table 5**

**Maternal Age at Time of Blood Sampling**

| Population | Mean Age (No.) of Women with ≥90% Skewed XCI | Mean Age (No.) of Women without Skewed XCI | $t$ |
|---|---|---|---|
| Women with RSA | 34.2 (25) | 34.0 (182) | −.26 |
| Mothers of trisomy | 35.7 (14[a]) | 37.1 (65[b]) | −.95 |
| Control women | 35.4 (14) | 33.7 (164) | −.92 |

Note.—The skewed and nonskewed groups did not differ in mean maternal age in any of the three study populations.

[a] Includes 9 women from the RSA group.

[b] Includes 41 women from the RSA group.

ified the association between skewed XCI and risk of a trisomic conception in a separate population of women who were ascertained because of a trisomic pregnancy that resulted from a maternal meiotic error. These women were also found to have an increase in skewed XCI compared with both the normal control women and with a control group of women who had experienced a chromosomally abnormal pregnancy that was not due to an error of segregation at maternal meiosis. Although it would have been preferable to ascertain control subjects among women known to have no history of a trisomic pregnancy, there is no reason to think the control individuals should be biased in any way regarding XCI inactivation status. When all mothers of a trisomy are evaluated together, the increase in skewed XCI is highly significant ($P = .005$) (table 1). Thus, skewed XCI appears to be more strongly associated with a trisomic pregnancy than with RSA in general.

Little is known about the etiology of trisomy, except that maternal age is a strong contributing factor and that most errors leading to trisomy occur during maternal meiosis (Hassold and Hunt 2001). The present results provide additional evidence that certain women (e.g., those with skewed XCI) may be at increased risk of trisomy. Although some early epidemiological studies did not show evidence of increased recurrence risk after a trisomic SA when compared with control women (Warburton et al. 1987), age is such a strong risk factor for trisomy that the roles of other factors have been difficult to measure using an epidemiological approach (Morton et al. 1987). Several studies have suggested that it is follicular reserve—and thus how near a woman is to menopause, rather than chronological age—that is most important for aneuploidy risk. An increase in aneuploidy and a shortened reproductive lifespan were found in mice after surgical removal of an ovary (Brook et al. 1984). Similarly, it has been found that congenital absence or surgical removal of an ovary in humans significantly increased the risk of having a child with Down syndrome (odds ratio 9.6) (Freeman et al. 2000). A link between follicular reserve and aneuploidy is further suggested by the finding that median age at menopause was earlier among 111 women with trisomic SAs than among control women (Kline et al. 2000). Although women with premature ovarian failure (POF) have fewer live births than women with normal menopause, the former were more likely to have had three or more SAs (Chernyshov et al. 2001). Furthermore, miscarriage rates in women with diminished ovarian reserve—defined on the basis of levels of follicle stimulating hormone (FSH) on day 3 of the reproductive cycle—are significantly higher than those of age-matched controls (Hofmann et al. 2000; Levi et al. 2001) and are at a greater risk of fetal aneuploidy, most commonly trisomy (Nasseri et al. 1999).

The link between trisomy risk and follicular atresia leads us to suggest that skewed XCI in the women from the present study reflects a mechanism leading to fewer follicles at birth or to accelerated follicular atresia. It is possible that poor early growth could affect the oocyte pool. Earlier menopause has been associated with poor growth late in gestation (Cresswell et al. 1997). Furthermore, mice that survived treatment with a growth inhibitor during embryogenesis had a severe reduction in germ cell populations, accompanied by reduced fertility (Tam and Snow 1981). Any event that causes a reduction of size of the pool of embryonic precursors may not only affect the oocyte pool but would also be expected to lead to an increased risk of skewed XCI because of stochastic factors (Robinson et al. 2001*a*).

Increased follicular atresia can be caused by X-linked factors, as demonstrated by POF in women with X-autosome translocations, X-linked deletions, monosomy X, and certain mutations of X-linked genes (Simpson and Rajkovic 1999; Mumm et al. 2001). Although large rearrangements may lead to POF, it is possible that subtle changes may lead to less severe follicular depletion, which then manifests as a higher risk of trisomy at a slightly earlier age. X-linked deletions, translocations, and mutations are also frequent causes of skewed XCI. An increased rate of oocyte atresia has also been observed in female infants affected with monosomy X and trisomies 13, 18, or 21 (reviewed by Cunniff et al. [1991] and Ogata and Matsuo [1995]). Therefore, germline trisomy mosaicism in phenotypically normal individuals could also cause reduced ovarian reserve. Although germline trisomy mosaicism could contribute directly to trisomic pregnancies through the production of aneuploid oocytes, this has not been documented for trisomies other than 18 and 21 (Robinson et al. 2001*b*). We suggest that it is more likely that germline trisomy mosaicism leads to increased trisomy risk through reduced ovarian reserve. Germline trisomy mosaicism has been reported in association with mosaicism confined to the placenta (Stavropoulos et al. 1998), which, in turn, has been associated with skewed XCI (Lau et al. 1997; Peñaherrera et al. 2000).

There is no doubt that X-linked mutations can be associated with skewed XCI and an increased rate of SA in families (Pegoraro et al. 1997; Lanasa et al. 2001). Do male lethal X-linked mutations occur often enough to significantly contribute to the etiology of RSA? An increase in female live births in patients with skewed XCI, compared with the expected 1:1 ratio, has been observed, suggesting that male-lethal X-linked mutations could be a cause of loss of pregnancy among women with skewed XCI and RSA (Lanasa et al. 2001), although the losses were not karyotyped to corroborate this possibility. In contrast, the present study does not show an increase in SAs of male conceptuses or in fe-

male live births. Thus, male-lethal X-linked mutations are an unlikely explanation for the losses experienced by the majority of women with skewed XCI and RSA.

There are few proven causes of trisomy. The finding of increased occurrence of skewed XCI among women who have experienced a trisomic pregnancy thus provides a clue to its etiology and suggests that reduced size of the pool of embryonic precursors and reduced ovarian reserve could lead to increased susceptibility to chromosomally abnormal pregnancies. However, it will be important to conduct additional studies to confirm this possible association. In particular, there may be only a subset of trisomies to which these findings apply. Since the main underlying cause of the association between skewed XCI and trisomy is not yet known, skewed X CI is not currently recommended as a diagnostic tool. Nonetheless, with further research, skewed XCI could perhaps be used as an early indicator of an increased risk of trisomy and could allow women to plan their families with greater knowledge of their own specific age-related risk of aneuploidy or loss.

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