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INVITED EDITORIAL
The Timing of Twinning: More Insights from X Inactivation

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Since the formulation of the hypothesis, by Mary Lyon (1961), of X-chromosome inactivation in somatic cells of female mammals, studies of the biology and the consequences of this phenomenon have contributed to our understanding of many areas of genetics and development. Montiero et al. (1998 [in this issue]) have now extended the application of X-inactivation studies to a careful and quantitative dissection of the timing of MZ twinning, demonstrating that dichorionic MZ (DC-MZ) twinning, unlike monochorionic MZ (MC-MZ) twinning, occurs prior to the time of X inactivation in the embryo.

Recent discoveries have illuminated many details of the X-inactivation story, but the precise molecular events in the initiation and maintenance of X inactivation are not yet fully known. We now appreciate that expression levels of the great majority of X-encoded genes are equalized between XY males and XX females by permanent silencing of one or the other X chromosome in the cells of female somatic tissues. Normal X inactivation occurs in the early female embryo as a stochastic event—that is, a choice made independently in each cell, with an equal probability of the maternally derived versus paternally derived X chromosome becoming inactive. The X inactivation of individual embryonic cells is considered most likely to be initiated from the X-inactivation center by *XIST* (X inactivation–specific transcript), which, unlike most genes, does not encode a protein and, paradoxically, is expressed only from the inactive X in postinactivation somatic cells (Brown et al. 1992). At the time of X inactivation, *XIST* RNA functions in *cis* to spread an inactivating signal up and down the chromosome on which it resides (Willard 1995). X inactivation is then maintained throughout subsequent cellular proliferation and differentiation, by continued *XIST* expression and by methylation of DNA cytosine residues.

Because of X inactivation, tissues of females are normally a mosaic of two cell populations, each expressing gene alleles from either their paternal or their maternal X chromosome. Deviations from random X inactivation have been used to demonstrate clonal proliferations of malignant cells, positive or negative selection in carriers of adrenoleukodystrophy or X-linked immune disorders, and even primary disturbances of the X-inactivation process itself (reviewed in Belmont 1996; Puck and Willard 1998).

Measuring the distribution of X-inactivation skewing also has been exploited to estimate the number of embryonic progenitor cells giving rise to a particular tissue. If we assume that the maternal or paternal X chromosome is inactivated at random, analogous to tossing a coin and getting heads or tails, X-inactivation patterns would be expected to follow a binomial distribution from which the number of original inactivation events can be calculated. X-inactivation ratios for derivation of the best-fit binomial curve can be determined, provided that there are ways to (i) distinguish the two X chromosomes and (ii) indicate which X chromosome is active. This initially was done at the protein level, with samples from women whose X chromosomes were heterozygous for different glucose-6-phosphate dehydrogenase alleles (Fialkow 1973). Estimates suggested that X inactivation occurs when there are ~20 embryonic cells. Subsequently, fusion of human lymphocytes with rodent cells lacking the X-linked enzyme hypoxanthine phosphoribosyltransferase was used to select active human X chromosomes in hybrid cell lines, where they could be identified by polymorphic DNA markers (Puck et al. 1992). Quantitations from enumeration of active-X frequencies in somatic-cell hybrids yielded estimates consistent with the previous data, with ~10 cells giving rise to the population that forms bone-marrow stem cells. The proximity, in the X-linked androgen receptor (*AR*) gene, of a highly informative polymorphic $(CAG)_{17–26}$ repeat and methylation-sensitive restriction sites that are methylated on inactive X chromosomes recently has streamlined determination of X-inactivation

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ratios (Allen et al. 1994). Parallel DNA samples, one predigested with *Hpa*II and the other not, are subjected to PCR with primers flanking both *Hpa*II sites and the triplet repeat; only the methylated template strands from inactive X chromosomes remain intact to be amplified, separated by electrophoresis, and quantitated.

Monteiro et al. (1998) have used the latter method to evaluate X-chromosome inactivation in the context of another mysterious phenomenon of very early human gestation—the formation of MZ twins: X-inactivation patterns were determined in samples of both buccal-mucosal and blood DNA from a large series of 33 MZ-twin females and 21 singleton control females. The placental and fetal membranes of the twins were analyzed; 20 were MC-MZ, known to be the more common type and believed, on the basis of placental anatomy, to split later than DC-MZ twins (Derom et al. 1995). To validate their X chromosome–inactivation analysis, Monteiro et al. were careful first to establish that the *AR* methylation assay in their hands was quantitative and reproducible to <5% difference (Monteiro et al. 1998). Next, they determined norms for skewing of X inactivation in female singleton control blood and mucosal-cell DNA; as measured by *AR* methylation, there were overall similar degrees of skewing in the two tissues, with the single exception of highly skewed blood DNA in one subject (specific variation between tissues for each individual otherwise were not given, which is unfortunate in that such data are scarce in the published literature).

Monteiro et al.'s estimate of the number of cells at the time of X inactivation, 5–16, is in the range of previous reports, cited above, which used different methods. Although the overall mean and range of X-inactivation skewing of the twins was not different from that seen in the control singletons, there was a significant difference in X inactivation between co-twins. Not only was the blood DNA of MC-MZ twins matched in X-inactivation skewing (previously reported and possibly due to known mixing of blood in mingled placental circulations for this type of twin [Trejo et al. 1994]), but MC-MZ ectodermally derived buccal-cell DNA also showed highly similar X-inactivation skewing, <6% difference between one twin to the other. In contrast, the DC-MZ twins had significantly greater mean differences in buccal-cell and blood DNA skewing—14% and 23%, respectively. These results clearly indicate that DC-MZ twinning occurs before or at the time of X inactivation, whereas MC-MZ twinning occurs some three or so replication rounds later, after X-inactivation patterns have been established and the embryonic inner-cell mass contains on the order of 128–256 cells.

How, then, are we to interpret reports of MZ twins concordant or discordant for expression of genetic phenotypes—and, in particular, female MZ twins one of whom suffers from an X-linked recessive phenotype, such as Duchenne muscular dystrophy (Lupski et al. 1991)? Repeatedly, the female twin expressing such a phenotype has been shown to have extreme skewing of X inactivation; however, her status as monochorionic or dichorionic is generally not known. The report by Monteiro et al. emphasizes that, although such extreme skewing is not common in MZ twins, since it was not seen in their study cohort, the timing of twinning must be considered as an additional important variable in twin studies.

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