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Differential X Reactivation in Human Placental Cells: Implications for Reversal of X Inactivation

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X inactivation—the mammalian method of X chromosome dosage compensation—is extremely stable in human somatic cells; only fetal germ cells have a developmental program to reverse the process. The human placenta, at term, differs from other somatic tissues, since it has the ability to reverse the X-inactivation program. To determine whether reversal can be induced at other stages of placental development, we examined earlier placental specimens using a cell-hybridization assay. We found that global X reactivation is also inducible in villi cells from first-trimester spontaneous abortions but not from first-trimester elective terminations. These differences in inducibility are not associated with detectable variation in histone H4 acetylation, DNA methylation, or *XIST* expression—hallmarks of the inactivation process—so other factors must have a role. One notable feature is that the permissive cells, unlike nonpermissive ones, have ceased to proliferate in vivo and are either beginning or in the process of programmed cell death. Cessation of mitotic proliferation also characterizes oocytes at the stage at which they undergo X reactivation. We suggest that, along with undermethylation, the apoptotic changes accompanying cessation of cell proliferation contribute to the reversal of inactivation, not only in placental cells, but also in oocytes entering meiosis.

Introduction

X inactivation—the developmental program that mammals use to compensate for the sex difference in numbers of X chromosomes (Lyon 1961)—ensures that only a single X is transcribed in cells of both sexes (reviewed by Migeon [2002]). The other X chromosome in females—or, for that matter, any X chromosome in excess of one in either sex—becomes inactive. Inactivation is mediated by the *cis*-acting RNA molecules, encoded by the X inactive–specific transcript gene (*XIST* in humans, *Xist* in other mammalian species) (Brockdorff et al. 1991; Brown et al. 1991*a*, 1991*b*). Located within the X-inactivation center, *Xist* is expressed uniquely from inactive X chromosomes, is repressed on active X chromosomes, and is both necessary and sufficient to initiate *cis* X inactivation. The abundant *Xist* transcripts bind to the X chromosome from which they are transcribed (Beletskii et al. 2001) and induce the chromatin modifications along the chromosome, which in turn silence most genes on the chromosome.

The process responsible for X inactivation occurs only during embryonic development (Wutz and Jaenisch 2000) or in totipotent cells of embryonic origin (Martin et al. 1978). Adding extra X chromosomes to a cell—past the stage when inactivation is initiated—has no effect (Migeon et al. 1996; authors' unpublished observations). Once initiated, the inactive state is maintained consistently from one cell to its daughters during cell division, so that all cells in a clonal population have the same inactive X (Davidson et al. 1963). The long-term silencing responsible for clonality is mediated by the methylation of cytosine residues in clustered CpG dinucleotides on the inactive X.

Piecemeal reactivation—which affects some genes but not others—occurs in marsupials (Kaslow and Migeon 1987) and in chorionic villi (CV) of humans (Migeon et al. 1985) and mice (Mann et al. 2004) and is associated with demethylation of the re-expressed gene (Migeon et al. 1985; Kaslow and Migeon 1987). Such localized reactivation is not associated with global changes in the chromosome; the hallmarks of an inactive X chromosome—late replication and chromosome condensation—are not affected.

On the other hand, global reversal of X inactivation is programmed to occur in female germ cells during their differentiation into mature gametes (Chapman 1986; Khalil et al. 2004). Nothing is known about the under-

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lying mechanisms, and most of what is known comes from studies of mice. As they migrate from hindgut to the germinal ridge, the primordial germ cells (PGCs) with two X chromosomes inactivate one of them randomly (McMahon et al. 1981). However, according to McLaren (2003), the silent X is reactivated about the time the germ cells enter the genital ridge, coincident with the end of mitosis (the final 2–3 rounds) and the beginning of the premeiotic stage. Although an exact time is unknown, reactivation occurs before the PGCs enter meiosis. Like XX germ cells in the female, the XXY cells in males undergo reactivation in the primitive gonad, but the male cells do not enter meiosis (Mroz et al. 1999). It is conceivable that reactivation occurs in response to some signal from neighboring somatic cells of the gonad.

Somewhat analogous to X inactivation in female germ cells is the transient inactivation of both X and Y during spermatogenesis (Khalil et al. 2004). Although inactivation of the XY body is associated with *Xist* transcription, the *Xist* transcripts are not needed; even when the *Xist* locus has been ablated, the XY body forms normally in primary spermatocytes, and normal spermatogenesis ensues (McCarrey et al. 2002). It is not merely coincidental that reversible inactivation in germ cells of both sexes is associated with a lack of methylated CpG islands on the transiently inactive X at the time that reversal occurs (Driscoll and Migeon 1990; Ariel et al. 1994). The absence of methylation to lock in the repression of genes on the silent X chromosome greatly facilitates reversal of the inactive state.

Most attempts to reverse inactivation in somatic cells by experimental manipulation have been futile. Even removal of its X inactivation center does not affect the silence of the inactive X (Brown and Willard 1994). Yet piecemeal reactivation of one or several genes on the inactive X can be induced in cultured somatic cells or hybrids by demethylating agents (Mohandas et al. 1981) that demethylate the CpG islands within or near the repressed genes (Wolf et al. 1984).

To date, the placenta is the only human somatic tissue known to be capable of global X reactivation. This organ differs from all others in several features; two relevant differences are instability of X inactivation and undermethylated DNA. Some X-linked genes—subject to inactivation in other tissues—are partially expressed in CV, and this expression is associated with undermethylation of the relevant CpG islands (Migeon et al. 1985, 1986).

Because of the undermethylated status of its X-linked genes (Driscoll and Migeon 1990; Luo et al. 1993), it is not surprising that placental cells are susceptible to reversal of inactivation. In fact, reactivation is inducible in human CV. Unlike the inactive X in other somatic tissues, the inactive X in cells from term (newborn) placentas can be globally reactivated when these CV cells are hybridized with mouse A9 cells (Migeon et al. 1986). The reactivated chromosomes are indeed active; they replicate synchronously with human autosomes and fully express genes that are usually silent on inactive X chromosomes, such as *G6PD, HPRT, PGK,* and *TIMP.* Also, reactivation is associated with repression of the *XIST* gene that had been expressed previously (Luo et al. 1995).

To further explore the mechanisms involved in reversing X inactivation, we have extended our studies to first-trimester placental tissues, using reactivation of the inactive X in mouse-human cell hybrids as our assay for reversibility. Our results show that the ability to reverse X inactivation differs in different gestational environments and provide some insights into conditions that promote reversal of inactivation.

Material and Methods

Cells and Clones

Placental tissue from term pregnancies (NB1, NB2, and NB3) and from spontaneous (SP9, SP26, and SP77) and surgical (SA14, SA52, and SA58) abortions were obtained for previous studies, in accordance with a Johns Hopkins University internal review board (IRB)–approved protocol. In all cases, surgical procedures were performed in the absence of chemicals and for reasons unrelated to our studies; in no case did the investigators participate in the medical care of the subjects. The full-term placentas (38–40-wk gestational age) and surgical placental specimens (8–13-wk gestational age) were treated alike; taken immediately from the delivery room, the CV were dissected away from the maternal decidual tissues and were minced and explanted to plastic tissue culture dishes. In the case of specimens that had been spontaneously aborted during the first trimester, the product of conception was obtained close to the time of expulsion; villi were minced and explanted to culture. In the case of SP9 specimens, the small extraembryonic membrane was used instead of villi. Cell cultures established from these specimens were maintained in liquid nitrogen, and cultures were re-established just prior to the present study.

All the placental cells were heterozygous for the common AB electrophoretic variants (Davidson et al. 1963) of the enzyme glucose-6-phosphate dehydrogenase (G6PD [MIM 305900]). Clones derived from single cells, obtained by cell dilution, were assayed for these G6PD isozymes, to identify those clones suitable for hybridization (see section below). All clonal specimens had two normal X chromosomes. The other cells were human fetal fibroblasts derived from discarded products of conception at various gestational stages (with IRB permission), adult female skin fibroblasts, SM × TSA1,

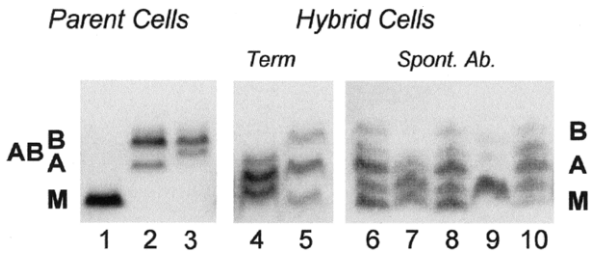

Figure 1 Cellulose acetate gel electrophoresis showing the G6PD phenotypes of placental cells and hybrids. *Left,* Newborn placental and mouse parent cells: A9 mouse cells expressing G6PD M (*lane 1*); human NB1 clone 1 expressing human G6PD B and the AB heterodimer (*lane 3*); and marker, mix of A plus B isozymes (*lane 2*). *Center,* Hybrids from term placenta, NB2. In these hybrids, G6PD B marks the active X, and G6PD A marks the reactivated X: hybrid 1, with reactivated X expressing G6PD A, mouse G6PD M, and the AM heterodimer (*lane 4*); hybrid 2, with active X, expressing B, mouse, and the BM heterodimers (*lane 5*). Note that the BM heterodimer migrates close to human G6PD A. *Right,* Hybrids from spontaneous abortus SP9. In these hybrids, G6PD A marks the active X, and G6PD B marks the reactivated X: hybrids 1 and 2, with both active and reactivated X, expressing all six isozymes (*lanes 6, 8,* and *10*); hybrid 3, with active X expressing A, mouse, and the AM heterodimers (*lane 7*); markers, mix of cells with A, B, and M (mouse) isozymes (*lane 9*). Note that G6PD A overlaps the BM heterodimer.

a human-mouse hybrid with an inactive X chromosome (Migeon et al. 1995), and mouse A9 cells.

G6PD Analysis

Parental cells, clones, and derivative hybrids were analyzed by cellulose acetate electrophoresis to determine which G6PD isozyme(s) was expressed (Migeon et al. 1985). Human G6PD B, the slowest to migrate through the electrophoretic field, is easily distinguished from the faster-migrating G6PD A, and both move more slowly than the mouse G6PD isozyme (G6PD M) (fig. 1, *lanes 1* and *2*). In hybrids, the presence of heterodimers—seen as bands migrating in between those for A, B, and M isozymes—means that two or more X chromosomes (i.e., mouse plus one or two human X chromosomes) are being expressed in the same cell (fig. 1, *lanes 4–6*).

Acetylation of Histone H4

To determine the histone H4–acetylation status of the X chromosomes, unfixed metaphase cells were labeled by indirect immunofluorescence with rabbit anti-serum R5/12, specific for histone H4 acetylated at lysine 12 (Jeppesen and Turner 1993; Migeon et al. 1999). To inhibit deacetylation of H4 occurring during metaphase arrest, and hence to ensure that even weakly acetylated H4 domains were immunolabeled, metaphase cells from some cultures were obtained in the presence of 4 mM sodium butyrate. Human X chromosomes were identified by subsequent FISH by use of an X-specific alphoid probe, as described elsewhere (Migeon et al. 1996).

XIST *and* TSIX *Expression*

RT-PCR.—Total RNA was isolated with TRIZOL (Invitrogen), was treated with DNaseI, and was analyzed, as described elsewhere (Migeon et al. 2002).

RNA FISH.—Interphase cells on slides were permeabilized, were fixed in paraformaldehyde, were dehydrated, and were hybridized without denaturation for 3 h, with labeled probes and with human COT-1 and salmon sperm DNA, as described elsewhere (Migeon et al. 2002). The FISH probes were PCR products cloned into the pCR2.1 TOPO vector and were either *TSIX* or *XIST* specific (Migeon et al. 2002). The *XIST* probe, labeled with digoxygenin-16-dUTP, was detected with anti–digoxygenin-rhodamine; the *TSIX* probe, labeled with biotin-11-dUTP, was detected with avidin/biotinylated anti-avidin; and 4,6-diamidino-2-phenylindole (DAPI) was used as counterstain. For DNA FISH, the slides were denatured prior to hybridization.

Results

Reversibility Assay

Figure 2 shows our strategy for detecting reversal of X inactivation in human-mouse somatic cell hybrids. The human parent cells were clonal populations (see the "Material and Methods" section) derived from single CV cells, heterozygous for the common G6PD AB elec-

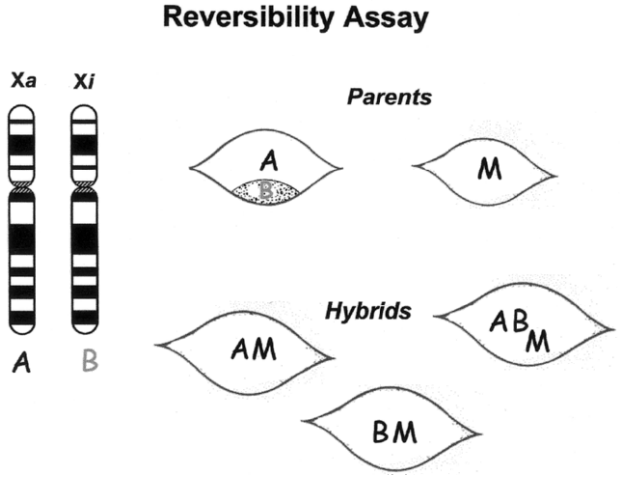

Figure 2 Scheme showing reversibility assay. A and B = human G6PD isozymes; M = mouse G6PD; Xa = active X; Xi = inactive X. G6PD B, encoded by Xi, is silent (*gray B*) in parent cell but expressed (*black B*) in hybrids. Parent clone expresses only G6PD A, since G6PD B is on the Xi. Hybrids express G6PD M plus either G6PD A (if they have the active X) or G6PD B (if they have a reactivated X).

trophoretic variants (Davidson et al. 1963). Each cell in the clone had the same active X, which could be identified by the G6PD variant it expressed. They also had an intact locus encoding the enzyme hypoxanthine phosphoribosyl transferase (*HPRT* [MIM 308000]). The mouse parent cells were *Hprt*-deficient A9. After hybridization, the cells were plated into hypoxanthine-amethopterin-thymidine (HAT) medium, to select for those expressing X-linked human *HPRT* from an active X. Well-isolated colonies were picked with cloning cylinders, were transferred into 35-mm petri dishes, were subcultured once, and were analyzed for their *G6PD* phenotype (Migeon et al. 1986). Therefore, the hybrids were analyzed in very early subculture.

Because X inactivation is not imprinted in human placental cells and because either X can be the active X (Migeon et al. 1985, 1986; Willemsen et al. 2002), the clones derived from a single cell can express either G6PD A or G6PD B (see table 1, clone SA14). The presence of heterodimers—seen as bands migrating in between those for A, B, and M isozymes—means that two or more X chromosomes are being expressed in the same cell (fig. 1, *lanes 4–6*).

Prior to hybridization, all the human parental cells expressed the G6PD variant coming from the active X (the single or stronger signal). In some clones, the AB heterodimer was minimally expressed because of leaky inactivation that characterizes some CV cells. In no clone—not even in the leaky ones—was there an electrophoretic signal for the G6PD isozyme encoded by the inactive X; any enzyme synthesized from the inactive X could be found only in the heterodimer, because such a small amount of this monomer is made. Of importance, at all gestational stages, heterodimers were seen only in placental tissues and never in the tissues derived from the embryo proper (Migeon et al. 1985).

Hybrid cells were identified by the presence of human-mouse heterodimers (shown in fig. 1, AM [*lane 4*] or BM [*lane 5*] or both [*lanes 6, 8,* and *10*]). The assay was considered positive for reversibility if the G6PD isozyme encoded by the previously inactive X was being expressed as a homodimer. It was considered negative if the only human G6PDs expressed were the ones expressed in the human parental cell.

Cell Hybrid Assay Shows X Inactivation Is Reversible in Placentas at Term and from First-Trimester Spontaneous Miscarriages

Table 1 shows the results of the reversibility assay. The enzyme expressed in the original human placental clone marked the allele on the active X. Of the 13 human parent clones hybridized with mouse A9 cells, 7 expressed G6PD A and 6 expressed G6PD B from their

Table 1

Human G6PD Variants Expressed in Hybrids as an Assay for Reversal of X Inactivation in Placental Cells

Stage, Tissue, and G6PD Variant Expressed	G6PD Expressed in Human Placental Clone			No. of Clones Analyzed	No. of Derived Hybrids with			Percentage of Hybrids with Reactivated X[a]
	A	B	AB Dimer		A	B	AB Dimer	
Newborn:								
Villi:								
NB1[b]Cl1	−	+	+	19	8	7	4	***63***
NB2 Cl1	−	+	−	13	7	4	2	***79***
NB3 Cl1	+	−	+	6	3	1	2	***50***
NB3 Cl2	+	−	+	6	4	2	0	***33***
Spontaneous abortion, 1st trimester:								
Villi:								
SP77 Cl1	+	−	−	20	11	7	2	***45***
SP77 Cl2	+	−	+	18	8	6	4	***55***
SP26 Cl1	−	+	−	6	0	6	0	***0***
Membrane:								
SP9 Cl1	+	−	−	8	1	5	2	***88***
SP9 Cl2	+	−	+	8	4	1	3	***50***
Surgical abortion, 1st trimester:								
Villi:								
SA14 Cl1	+	−	−	15	15	0	0	***0***
SA14 Cl2	−	+	−	17	0	17	0	***0***
SA58 Cl1	−	+	−	2	0	2	0	***0***
SA52 Cl1	−	+	−	11	0	11	0	***0***

Note.—A = only G6PD A expressed; B = only G6PD B expressed; AB Dimer = AB heterodimer expressed in the parent clone (Cl) or derivative hybrids. The hybrids with the AB dimer were karyotyped and had two human X chromosomes.

[a] Percentage of hybrids that express the G6PD variant from the previously inactive X.

[b] NB1 hybrids were reported elsewhere (Migeon et al. 1986).

active X chromosome. Of the 13 clones, 5 had a less intense G6PD AB heterodimer, which indicates leaky expression from the gene on the inactive X (fig. 1, *lane 3*).

After hybridization, all four full-term placental specimens gave rise to hybrids expressing the G6PD allele that was silent in the human parent clone. For example, full-term villus NB2 clone 2 expressed only G6PD B, but 7 of the 13 hybrids derived from this clone expressed only G6PD A. Two of the 13 hybrids derived from NB2 clone 2 expressed the AB heterodimer, which indicates that both human X chromosomes were present in the hybrid and both were being expressed (table 1). AB heterodimers were seen in six other hybrids, even when none was present in the parent human clone, so that reactivation occurred even in cells initially lacking a heterodimer (table 1, hybrid NB2). The presence of two X chromosomes in the hybrids with AB dimers was confirmed by karyotype analysis (data not shown).

Similar results were obtained in specimens from first-trimester spontaneous abortions, associated with fetal death in utero (table 1). Four of the five clones tested gave rise to hybrids expressing the *G6PD* gene from the X that was silent in the parent clone; 11 of 54 hybrids expressed the G6PD AB heterodimer (fig. 1, *lanes 6, 8,* and *10*). Only one clone (SP26 clone 1) did not yield many hybrids, and all of those hybrids were exactly like the parent clone. When this culture was established in 1980, lab notes referred to the specimen as "fresh," a term not used to describe any other specimen in this category. However, some clones from this specimen had heterodimers, but the clone used for hybridization did not. The low number of hybrids from this specimen may account for the absence of hybrids with a reactivated X.

Hybrid Cells with Reactivated X Have Characteristics of an Active X

Our previous studies of term placentas showed that the hybrid cells, which expressed the G6PD allele from the inactive X, also expressed other housekeeping genes on that chromosome (Migeon et al. 1986). In addition, the reactivated X replicated synchronously with the active one. Therefore, for this study, we could use the G6PD phenotype as an indicator of global reactivation. In support of the global reactivation of the chromosome in the present study was our finding that the *HPRT* locus on the previously silent X was also derepressed (enabling selection in HAT medium) and the presence of acetylated histone H4—a hallmark of active chromatin—on the reactivated X in hybrids (fig. 3*B* and discussed below).

Not All Placentas Can Reverse X Inactivation

We also derived 45 hybrids from four CV specimens from pregnancies terminated by surgical procedures, without the use of chemicals, in the first trimester (table 1, tissues SA14, SA58, and SA52). Unlike the other placental specimens, none of the clones derived from these specimens expressed the AB dimer. And although the conditions of specimen procurement and cell hybridization were the same as for term placentas, the results were not the same. In each case, the hybrids expressed both human and mouse G6PD enzymes and the human-mouse heterodimer, as expected for hybrid cells. However, the human G6PD variant encoded by the inactive X chromosome was not expressed in any of the 45 hybrids.

The Search for Factors Underlying Competence for Reversal

Because not all CV could reverse inactivation, we looked for features that might explain the difference between competent and incompetent cells. Our previous studies of DNA methylation patterns in CV of full-term placentas showed marked undermethylation both of the tissue as a whole (Driscoll and Migeon 1990) and of the CpG islands in every gene on the inactive X that we studied, including *HPRT, G6PD,* and *FMR1*. Methylation analysis of placental tissues from surgical terminations shows that 18 of the 20 *Hpa*II sites within the CpG island of the *FMR1* locus on the inactive X were completely unmethylated in all specimens of 6–14 wk gestational age (Luo et al. 1993), and the remaining 2 sites were methylated in only a minority of cells. Even in specimens that are unable to reverse inactivation, there is marked hypomethylation. Therefore, differences in reversibility cannot be attributed to global differences in DNA methylation alone.

To explore the possibility that competence to reverse X inactivation might be reflected in the degree of histone acetylation, we examined histone H4 acetylation in placental cells and derivative hybrids. The extreme underacetylation of histone H4 is a marker of inactive chromatin and is one of the hallmarks of an inactive X (Jeppesen and Turner 1993). All fibroblasts cultured from CV, whether full term (NB1 and NB2) or elective terminations (SA14 and SA58), contained inactive X chromosomes indistinguishable by acetylated H4 labeling from normal inactive X chromosomes, even when metaphases were prepared in the presence of sodium butyrate to enhance any possible weak H4 acetylation. Figure 3*A* shows the typical unlabeled inactive X in NB2 cells. The studies also show that the X chromosomes in the two hybrid clones with reactivated chromosomes had active chromatin, on the basis of their histone H4 acetylation—compelling evidence of the global nature of the reactivation event. The pattern of histone acetylation of the X chromosome in the hybrids with the reactivated Xs (NB1 hybrids 1 and 2) was the same as that in the hybrid with the original active X chromosome: in each case,

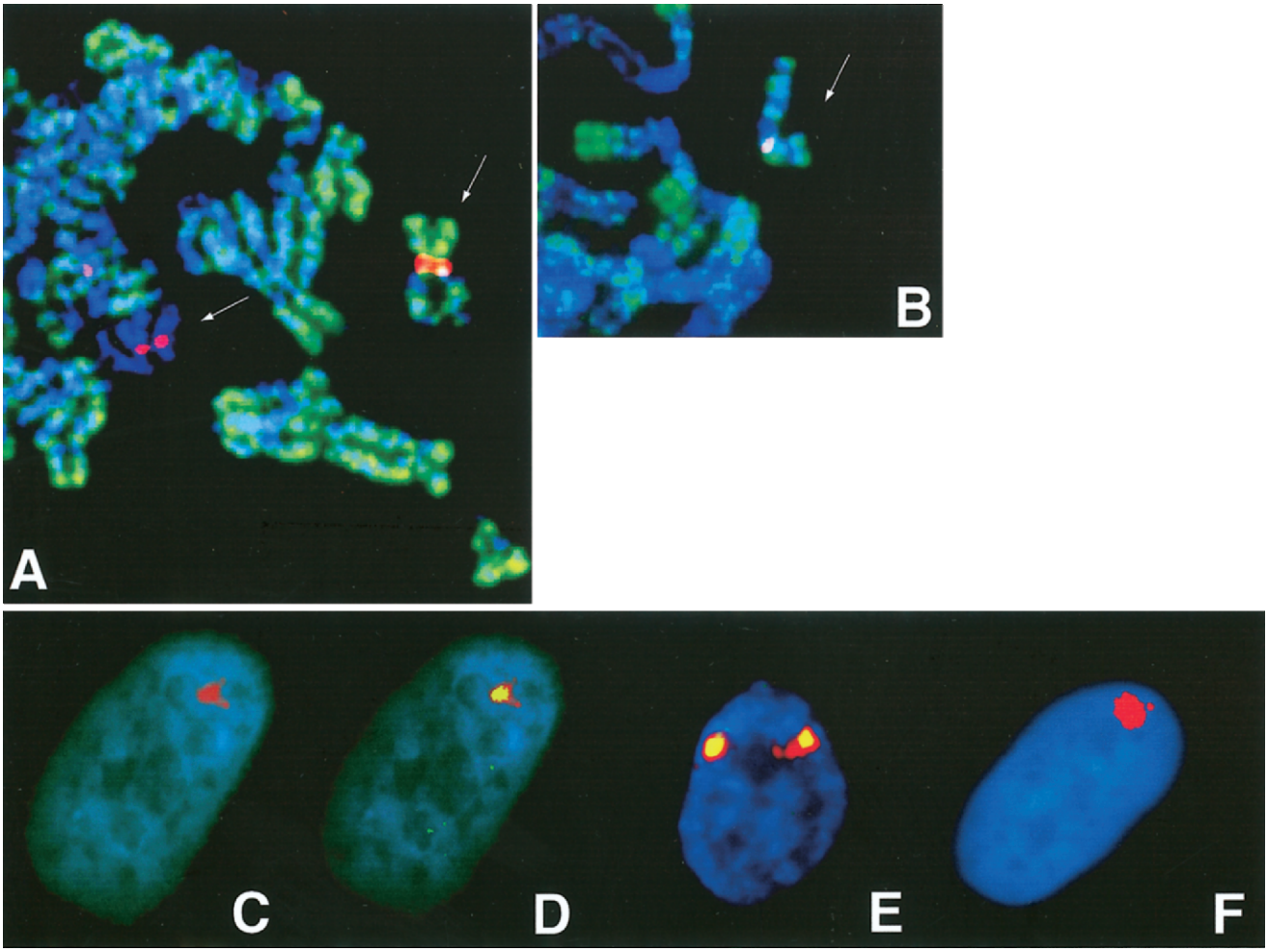

Figure 3 *A* and *B*, Immunolabeling of newborn placental (*A*) and hybrid (*B*) cells, with an antibody to acetylated histone H4, followed by DNA FISH. *A*, NB2 cells showing the active and inactive X. Both X chromosomes are labeled by the X centromere probe (*red*), whereas only the active X is labeled by the antibody to acetylated histone H4. *B*, NB1 × A9 hybrid 1, showing that the reactivated X chromosome (*centromere labeled red*) is well labeled by the antibody to acetylated histone H4, as expected for an active X. *C–F*, RNA FISH with *XIST* (*red*) and *TSIX* (*green*) probes, showing that both genes are well expressed in term-placental cells (*C–E*) and adult female skin cells (*F*). *C*, Term CV (NB5) cell showing normal red *XIST* signal; green signal not shown. *D*, Same as panel C, but merge of *XIST* (*red*) and *TSIX* (*green*) shows coexpression (*yellow overlap*). *E*, 47,XXX term villi cells with two inactive X chromosomes; merge shows that they both express XIST (*red*) and TSIX (*green*), as expected. *F*, 46,XX adult cell; merge shows expression of XIST (*red*) but no TSIX (*green*), as expected.

the observed R-like banding pattern is characteristic of active X chromosomes. Figure 3*B* shows NB1 × A9 hybrid 1.

XIST Expression in the CV Cells and Hybrids

There is considerable evidence that inactivation requires not only *XIST* expression but also a sufficient level of expression (Migeon et al. 2001). Therefore, using RT-PCR, we looked for differences in *XIST* expression between competent CV cells (term and first-trimester) and normal somatic cells (adult and fetal) that are not competent to reverse inactivation (data not shown). We also compared term villi cells with fetal and adult somatic cells, using FISH studies with an *XIST* probe hybridizing to *XIST* RNA (fig. 3*C*–3*F* and table 2). Neither assay revealed striking differences between *XIST* expression in reversal-competent and -noncompetent cells. Shown is the characteristic *XIST* RNA picture of the inactive X in clones from full-term villi with two (fig. 3*C* and 3*D*) or three (fig. 3*E*) X chromosomes. The *XIST* signal extensively overlaps the sex chromatin, as it does in adult female cells (fig. 3*F*). In both assays, the *XIST* signal in the villi cells from reversal-competent specimens was the same as in female somatic cells that cannot reverse inactivation. We also examined the expression of

Table 2

FISH Results of *XIST* and *TSIX* in Placental Cells, Hybrids, and Controls

Cell Type and Assay	No. of Cells with FISH Signals		
	XIST DNA	*XIST* RNA	*TSIX* RNA
Fetal lung[a] *(K-19)*	ND	37/50	26/50
Fetal liver[a] *(Fe-18)*	ND	41/50	18/50
Term placenta[b] *(NB5)*	ND	39/50	21/50
Term placenta with trisomy X[c] *(NBX3)*:			
Assay 1	ND	41/50*	32/50*
Assay 2	40/50*	29/50*	ND
Assay 3	41/50*	34/50*	ND
Assay 4	ND	41/50*	43/50*
Adult female skin cells 46XX:			
Assay 1	ND	40/50	0/50
Assay 2	ND	45/50	0/50
Hybrid with reactivated X[d] (*NB2 hybrid 1*):			
Assay 1	ND	7/50	1/50
Assay 2	ND	0/50	0/50
Assay 3	44/61	1/61	ND
Hybrid with reactivated X (*NB2 hybrid 2*):			
Assay 1	ND	4/50	0/50
Assay 2	ND	0/59	ND
Hybrid with inactive X[e]:			
Assay 1	ND	22/50	0/50
Assay 2	ND	23/61	0/61

Note.—An asterisk (*) indicates cells with two signals. ND = not done.

[a] Female fetal cells with inactive X.

[b] Villi cells from newborn female placenta.

[c] Villi cells from newborn female placenta with trisomy X.

[d] Villi hybrids with human reactivated X.

[e] Nonvilli hybrid (adult female) with human inactive X.

the *TSIX* gene that encodes transcripts antisense to *XIST*. In humans, *TSIX* is expressed only during gestation and is repressed gradually after birth, so that adult cells do not express it (Migeon et al. 2002). Table 2 summarizes the RNA FISH studies of relevant cells, showing the numbers of cells with *XIST* and *TSIX* hybridization signals. We found that *TSIX* was well expressed in CV cells at term but not in the hybrids derived from them (table 2 and fig. 3*D* and 3*E*). In the hybrids that showed the *XIST* locus by DNA FISH, the expression of both *XIST* and *TSIX* was repressed (table 2). As expected, the inactive X in hybrids from adult female human blood cells expressed *XIST* but not *TSIX* (table 2 and fig. 3*F*). From these studies, it seems that *XIST* and *TSIX* are well expressed from the inactive X in term CV cells but that both are repressed in their derived hybrids, as a consequence of the reversal of X inactivation.

Discussion

Even though its developmental program is relatively short-lived—9 mo instead of a lifetime—the placenta is among the most important human organs, since it directs the development of the fetus and is essential for its survival. One of the earliest tissues to differentiate, this trophoblast-derived tissue is closer to its totipotent cell progenitors than are most other tissues, and this may contribute to its competency to reverse X inactivation. In mouse embryonic stem cells, X reactivation is inducible for a short while—just after inactivation is initiated (Wutz and Jaenisch 2000), presumably because it is not yet locked in by DNA methylation.

What are the factors permitting reversal to occur in placental tissues but not in other somatic tissues? One common feature of the reversal-competent cells is that the inactive X is undermethylated. Clearly, hypomethylation has to be an enabler, since it eliminates the major mechanism serving to lock in inactivation. In noncompetent cells, a locus may be reactivated by a demethylating event that affects the maintenance of inactivation. However, global reversal of inactivation does not occur, even when these cells are hybridized with mouse A9 cells; the silent X retains its inactive state. The experimental induction of global reactivation requires a good deal of manipulation, which usually involves fusion with embryonal carcinoma stem cells (Takagi et al. 1983; Kazuhiko et al. 1986; Yoshida et al. 1997).

Although hypomethylation creates a permissive environment, it is not in itself sufficient to induce reversal of inactivation. Whereas all CV are remarkably under-

methylated, not all of them are competent (table 3). Villi from term placenta and from first-trimester spontaneous abortions are permissive, but those from first-trimester induced terminations are not. Of interest, Mohandas et al. (1989), studying CV obtained from elective terminations of pregnancy, did not observe heterodimers in cultured fibroblasts, heterozygous for the G6PD AB variants, or in two HAT-selected hybrids derived from those cells. Since the differences in competence of placental cells were not appreciated at that time, our present observations reconcile apparent discrepancies in the literature.

The difference in reversibility does not seem to reflect differences in the nature of the X-inactivation process. The patterns of *XIST* expression, histone acetylation, and CpG methylation in the term placentas did not differ from those characteristic of an inactive X in other somatic cells (table 3). Since *XIST* is well expressed in cells of term placentas, it is not surprising that the inactive X was underacetylated in those cells—consistent with the inactive state. There may be other modifications not yet examined that merit exploration, but such differences, if any, are unlikely to change our conclusions that X chromosomes, subject to reversal, have chromatin that is transcriptionally inactive before hybridization and transcriptionally active afterward.

Another factor to consider is that the milieu of the mouse A9 cells may promote reversibility. The *Xist* RNA seems to be less tightly packaged around the inactive X chromosome in some hybrids than it is in its own cell (Clemson et al. 1998). Yet the human inactive X from such hybrids (derived from A9 or other mouse somatic cells) usually remains inactive. In any case, A9 cells do not provide the same degree of competency for all placental cells. Because the assay conditions were the same for all specimens, the environment provided by A9 cells is not sufficient to induce reactivation in the absence of other permissive factors.

The most obvious difference between spontaneous and induced abortions is that, in the former, the fetus has expired—usually a number of days before the expulsion of the placenta. The death of the fetus usually induces changes in the placenta that lead to the cessation of growth and initiation of apoptosis. Conceivably, the difference between villi that are capable of reversing inactivation and those that cannot is related to senescence or aging of placental tissues at term or after spontaneous death of the fetus in utero.

The molecular mechanisms that control trophoblast proliferation are not known, but, clearly, placental development involves the simultaneous activity of two processes—cell proliferation and degradation through programmed cell death. At the time when the placenta has its greatest growth spurt, proliferation markers are strongly expressed. In a study of 22 placentas, 12 obtained from curettage and 10 at term, it was found that proliferation markers were strongly expressed in cytotrophoblast in early stages of gestation but were decreased in term placentas (Danihel et al. 2002). Apoptosis is an integral part of the developmental program in the placenta. During implantation, apoptosis is important for tissue remodeling of the maternal decidua and invasion by the developing embryo. Yet apoptosis occurs throughout gestation, with frequency highest in third-trimester placentas. There is evidence that an X-linked inhibitor of apoptosis protects first-trimester trophoblast cells from Fas-induced apoptosis (Straszewski-Chavez et al. 2004). Further, pregnancies complicated by fetal abnormalities are associated with increased trophoblast apoptosis. Therefore, it seems that it is not only the stage but also the state of health of the placenta that is relevant. Conceivably, apoptotic changes associated with term placentas and fetal wastage may be involved in the reversal process. In the absence of DNA methylation, either proteases or the loss of other ancillary silencing factors might destabilize X inactivation. One

Table 3

Characteristics of the Inactive X in Human Placental and Control Cells

	Characteristics of the Inactive X Chromosome					
Source of Female Cells	Fetal Age (wk)	AB Dimer	Reversal Competent	*XIST/TSIX* RNA	CpG Methylation[a]	H4 Acetylation[b]
Placenta:						
Newborn	~40	Yes	Yes	Yes/yes	Hypo	Under
Spontaneous abortion	<13	Yes	Yes	ND	Hypo	ND
Surgical abortion	8–14	No	No	ND	Hypo	Under
Fetal tissues	5–6.5	No	ND[c]	Yes/yes	Methylated	ND
Human adult skin	…	No	No	Yes/no	Methylated	Under

Note.—ND = not done.

[a] Hypo = hypomethylated like the inactive X in the other placental specimens; no apparent difference in degree of hypomethylation.

[b] Under = underacetylated like a typical inactive X in adult human cells.

[c] No heterodimers.

would not expect senescent or G0 somatic cells of the embryo proper to undergo reversal of inactivation because the inactive state in these cells is locked in by DNA methylation.

Along with undermethylation, the dual processes of cessation of cell proliferation and onset of apoptosis are also a part of the program of oocyte development. At day 13 of mouse gestation, when male and female germ cells are undergoing mitotic arrest or entry into meiosis, respectively, and when X reactivation has just occurred, apoptotic germ cells are seen for the first time. This programmed cell death continues throughout gestation and is responsible for the loss of a substantial proportion of germ cells before birth. (Coucouvanis et al. 1993).

In summary, we show that not all CV cells are capable of global X reactivation and thereby provide insights into the conditions required for reversal. Our studies preclude obvious differences in levels of DNA methylation, H4 acetylation, and *XIST* expression and suggest that, along with hypomethylation, factors present at the cessation of proliferation and/or at the initiation of apoptosis provide a cell environment permissive for reactivation. The nature of the signals remains to be determined. We suggest that it might be identified by comparing villi in early pregnancy with those at term, perhaps by microarray analysis of proteins involved in cell proliferation and in the apoptotic pathway. In addition, attempts to induce reversal in noncompetent villi cells may shed further light on the process.

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Web Resource

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for G6PD and *HPRT*)

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