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ORIGINAL INVESTIGATION

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Variability of X chromosome inactivation: effect on levels of *TIMP1* RNA and role of DNA methylation

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Abstract X chromosome inactivation results in dosage equivalency for X-linked gene expression between males and females. However, some X-linked genes show variable X inactivation, being expressed from the inactive X in some females but subject to inactivation in other women. The human tissue inhibitor of metalloproteinases-1 (*TIMP1*) gene falls into this category. As TIMP1 and its target metalloproteinases are involved in many biological processes, women with elevated *TIMP1* expression may exhibit different disease susceptibilities. To address the potential impact of variable X inactivation, we analyzed *TIMP1* expression levels by using an RNase protection assay. The substantial variation of *TIMP1* expression observed in cells with monoallelic *TIMP1* expression precluded analysis of the contribution of the inactive X to total *TIMP1* RNA levels in females, so we examined expression in rodent/human somatic cell hybrids. *TIMP1* expression levels varied more widely in hybrids retaining an inactive X than in those with an active X chromosome, suggesting variable retention of the epigenetic silencing mechanisms associated with X inactivation. Therefore, we investigated the contribution of methylation at the promoter to expression level variation and found that methylation of the *TIMP1* promoter correlated with instability and low level expression, whereas stable *TIMP1* expression from the inactive X equivalent to that seen from the active X chromosome was observed when the promoter was unmethylated. Since all female cell lines examined showed methylation of the *TIMP1* promoter, the contribution of expression from the inactive X appears minimal. However, as women age, they may accumulate cells stably expressing *TIMP1* from the inactive X, with a resulting increase of TIMP1, which may explain some sex differences in various late-onset disorders.

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Introduction

X chromosome inactivation silences most of the genes on one of the two X chromosomes in mammalian females (Lyon 1961), presumably to achieve dosage compensation of X-linked gene products between males and females. This silencing occurs early in development and is maintained in a clonal fashion throughout subsequent cell divisions (reviewed in Avner and Heard 2001). A substantial number of genes on the human X chromosome escape this inactivation and are expressed from both the active (X_a) and the inactive (X_i) X chromosome (Carrel et al. 1999). In addition, some genes have been shown to have variable X inactivation, being expressed from both X chromosomes in some females but subject to X inactivation in others (Anderson and Brown 1999; Carrel and Willard 1999). Of the genes that escape X inactivation, some have homologs on the Y chromosome and thus continue to maintain dosage equivalence between males and females, but for those genes without a Y homolog, the expression from the X_i may generate dosage differences between the sexes (reviewed in Disteche 1999). For a gene that is variable in its inactivation status, expression levels may be different amongst females and between males and females.

Using an allele-specific polymerase chain reaction (PCR) assay to examine expression in heterozygous women with extreme skewing of X inactivation, we have previously demonstrated that the human tissue inhibitor of metalloproteinases-1 gene (*TIMP1*) is one such gene that is variably expressed from the X_i (Anderson and Brown 1999). Human TIMP1 is a multi-functional protein, best known for inhibiting matrix metalloproteinases (MMPs) by binding to their active sites (Denhardt et al. 1993). The MMPs are a family of enzymes involved in the turnover and degradation of the extracellular matrix, which is essential for embryonic development, morphogenesis, reproduction, and tissue remodeling (Nagase and Woessner 1999). Un-

der normal physiological conditions, a balance between matrix-degrading metalloproteinases and the family of TIMPs controls the rate of matrix turnover (Gomez et al. 1997). Disparity between the proteolytic enzymes and their inhibitors has been implicated in a number of pathological conditions. An excess of MMPs, with a relative decrease of TIMP1, is observed in invasive disorders such as rheumatoid arthritis (Yoshihara et al. 2000), Crohn's disease (Sykes et al. 1999), and cancer metastasis (reviewed in Lukashev and Werb 1998). Indeed, malignant progression can be halted by over-expression of *TIMP1* (Sternlicht et al. 1999) and several synthetic inhibitors of MMP activity have been developed for clinical use in these degenerative diseases (Yip et al. 1999). Increased levels of TIMP1 are seen in scarring diseases, such as multiple sclerosis (Lee et al. 1999), liver fibrosis (Arthur et al. 1999), and airway damage in asthma (Vignola et al. 2000). In addition to inhibiting MMPs, TIMP1 has at least three other roles. *TIMP1* was originally identified as EPA (erythroid potentiating activity), and this growth promoting activity is separable from the MMP inhibition domain (Gomez et al. 1997). It has also been shown to inhibit apoptosis, promoting the survival of Burkitt's lymphoma cell lines (Guedez et al. 1998). Finally, TIMP1 also forms a complex with pro-gelatinase-B (MMP-9) to regulate the rate of MMP-9 activation (Itoh and Nagase 1995).

Since variations in the MMP-TIMP balance are observed in pathological conditions, it is surprising to see discrepancies among women in their inactivation of *TIMP1*. However, it cannot be assumed that women expressing *TIMP1* from both X chromosomes will have twice as much *TIMP1* RNA, since the expression levels from genes on the X_i are often lower than those on the X_a (e.g., Carrel et al. 1996; Migeon et al. 1982) perhaps because of the surrounding facultative heterochromatin state. The X_i acquires many features of heterochromatin, including hypermethylation, nuclease insensitivity, delayed replication timing, and hypoacetylation of histones (reviewed in Avner and Heard 2001). These features are believed to maintain the stable silencing of the chromosome. Genes escaping X inactivation are often located in clusters along the X chromosome, suggesting the importance of factors that act regionally for the establishment of X inactivation patterns (Disteche 1999; Tsuchiya and Willard 2000). The regions that escape inactivation behave more like the active chromosome, lacking promoter methylation (Goodfellow et al. 1988; Yen et al. 1984), replicating earlier in the cell cycle (Schempp and Meer 1983), and retaining histone acetylation (Jeppesen and Turner 1993) compared with the rest of the X_i chromosome. However, the variable expression from the X_i in the *TIMP1* region is limited to *TIMP1* alone, as two flanking genes, *ARAF1* and *ELK1*, are expressed only from the X_a, even when *TIMP1* expression is observed from the X_i (Anderson and Brown 1999).

To determine whether women expressing two copies of *TIMP1* have increased *TIMP1* mRNA, we analyzed expression levels in male and female cell lines with the RNase protection assay (RPA). Surprisingly, there was considerable variation in *TIMP1* expression from cells with monoallelic expression. The diverse expression levels precluded analyzing the contribution from the X_i to total *TIMP1* RNA in females, so we examined expression in rodent/human somatic cell hybrids. The human X chromosome retains its original X-inactivation status in such hybrids (Migeon 1972), and since the X_a and X_i can be studied independently, hybrids have been a valuable tool for assessing the inactivation status of X-linked genes (e.g., Brown et al. 1997). *TIMP1* expression levels varied more widely in hybrids retaining the X_i than in those with an X_a, perhaps reflecting residual features associated with X inactivation. The gene-specific nature of *TIMP1* expression was suggestive of promoter-related features, so we studied whether methylation, a feature of inactive chromatin correlated with transcriptional repression (Jones and Wolffe 1999), was altered when *TIMP1* was expressed from the X_i. An understanding of what allows some genes to be expressed from an otherwise inactive X chromosome should provide clues about the hierarchy of epigenetic factors that normally result in the stable inactivation of an X chromosome. Furthermore, variable expression of genes from the X_i in some females may alter expression levels sufficiently to result in different susceptibilities to disease.

Materials and methods

Cell culture

Lymphoblast cell lines were acquired from the Coriell Cell Repository and grown in RPMI media 1640 (Stem Cell Technologies) supplemented with 15% fetal calf serum (Cansera), L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen). Cells were harvested by centrifugation 12–26 h after the addition of fresh media. The human/rodent somatic cell hybrids were as previously described (Anderson and Brown 1999) and derived from somatic cell fusion of human cells with *HPRT*-deficient rodent cells. Selection for and against the Xa was originally accomplished by growth in media supplemented with hypoxanthine, aminopterin, and thymidine or 8-azaguanine and 6-thioguanine, respectively. All the hybrids examined in this study were grown in minimal essential media (Invitrogen) supplemented with 7.5% fetal calf serum, L-glutamine, penicillin, streptomycin, and non-essential amino acids (Invitrogen) to sub-confluence before being harvested with trypsin-EDTA (0.25%; Invitrogen). To generate single cell clones, the hybrid cultures were plated to a final concentration of 3–17 cells/60 mm plate. After 5–10 days in culture, well-separated colonies were isolated by trypsinization in cloning cylinders and transferred to new plates.

Expression analysis

RNA was prepared by means of a standard acid-guanidinium extraction protocol (Chomczynski and Sacchi 1987). To remove DNA contamination, the sample was incubated with 1/10 the total volume of RNase-free DNase at 37°C (1 U/μl, Epicentre Technologies) for 1 h and then extracted with phenol followed by ethanol precipitation to remove excess proteins and single nucleotides. RNA concentration was determined spectrophotometrically prior to analysis of the RNA by reverse-transcription-PCR (RT-PCR) or RPA. For RT-PCR, 2–5 μg RNA was reverse-transcribed by random hexamer priming as previously described (Anderson and Brown 1999). The PCR primers used to amplify the cDNA were human-specific, with no amplification of mouse or hamster cDNA being detected. *TIMP1* expression was examined by amplification of the cDNA by *TIMP C1A:C1B*, whereas *XIST C9–4:B2rev* was used to

demonstrate the presence of amplifiable cDNA in the sample (Anderson and Brown 1999). PCR was performed with 1 μM primer, 1.5 mM $MgCl_2$, 20 μM each dNTP, 5 U *Taq* (Invitrogen) for 30 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C.

RPA analysis was performed with the RPA II kit (Ambion), following the manufacturer's directions. Probes were generated by cloning PCR products into the pKRX vector, which has T3 and T7 polymerase sites flanking the multiple cloning site. The 182-bp *TIMP1* fragment was generated with *TIMP1 C1A:C1B* primers (Anderson and Brown 1999), whereas the *MIC2* primers (1: cagagccagctgttcagcgt 2: cacagcccatgggaaaccgc) gave a 298-bp fragment. Because of the flanking vector sequences, the unprotected fragments were 243 bp (*TIMP1*) and 367 bp (*MIC2*). Plasmids were purified with the Qiaprep Spin Miniprep kit (Qiagen), and linearized by digestion with *Bam*HI (for *TIMP1*) or *Xho*I (for *MIC2*); 1 μg linear template was used for in vitro transcription with either T7 or T3 polymerase (Invitrogen) with ^{32}P-UTP (3000 Ci/mmol, NEN-US). The labeled probe was denatured (for 5 min at 95°C, for 5 min on ice) and then incubated with 2 U RNase-free DNase (Epicentre Technologies) for 15 min at 37°C followed by 5 min at 95°C to inactivate the enzyme. Aliquots of 35 ng (*TIMP1*) and 60 ng (*MIC2*) of antisense radiolabeled probe were hybridized overnight to 10 μg total RNA, followed by removal of unhybridized probe and sample RNA by RNase digestion. The hybridized product was separated on a native 5% polyacrylamide gel and visualized by autoradiography. To ensure that the probes were in excess, an initial RPA was performed with 10 μg RNA and 8–80 ng (*TIMP1*) or 11–290 ng (*MIC2*) probe. No change in the intensity of the fragments was observed above 10 ng (*TIMP1*) and 25 ng (*MIC2*) probe. The intensity of the protected *TIMP1* fragment was quantitated by phosphoimager (BioRad molecular imager and BioRad FX software) and compared with the intensity of the band detected for *MIC2*. *MIC2* is expressed from both the active and inactive X chromosomes at comparable levels (Goodfellow et al. 1984), controlling for both the amount of input RNA and also for the number of X chromosomes, which can be variable in the hybrids. For hybrids, the *TIMP1* level was determined as: *TIMP1* intensity/*MIC2* intensity; however, since *MIC2* is expressed from the Y chromosome and from the active and inactive X chromosomes, humans should have two expressing copies of *MIC2* for every active X copy of *TIMP1*, so the *TIMP1* level in the human cell lines was calculated as: *TIMP1* intensity/0.5×*MIC2* intensity. All RPA results were normalized to one stock RNA to control for variability between gels.

Methylation analysis

Genomic DNA was isolated from cultured cells with a standard salt extraction protocol. DNA was pre-digested with *EcoRI* at 37°C overnight, followed by incubation with 2 μl RNase at 37°C for 15 min. After phenol extraction and ethanol precipitation, the DNA was quantitated by spectrophotometry. Pre-digested DNA (2 μg) was then incubated overnight at 37°C in a total volume of 20 μl with 20 U of one of the following: mock enzyme (uncut), *Hpa*II, or *Hha*I. An aliquot of 1 μl (100 ng) was then used as a template in the PCRs (as above). All the primers were human-specific and did not flank *Eco*RI sites. The primer sequences are as follows: *TIMP1*: 5'A-cccttgggttctgcactga, 5'B-ccaagctgagtagacaggc (277-bp fragment, spanning 2 *Hpa*II and 2 *Hha*I sites); *ARAF1*: M1-tgccaaagccctaaggtca, M4-cgctgtcgacgatggtct (509 bp; spanning 8 *Hpa*II, 2 *Hha*I sites); *ELK1*: 5'A-gcacagctctgtagggaag, 5'B-agctcacctgtgtgtagcg (293 bp; spanning 1 *Hpa*II, 1 *Hha*I sites); *MIC2*: 5'A-agaggtgcgtccgattctt, 5'B-cgccgcagatggacaattt (375 bp; spanning 3 *Hpa*II, 5 *Hha*I sites); *XIST*: 5'-ttgggtcctctatccatctaggtag, 3'-gaagtctcaaggcttgagttagaag (184 bp, no *HpaII* or *HhaI* sites).

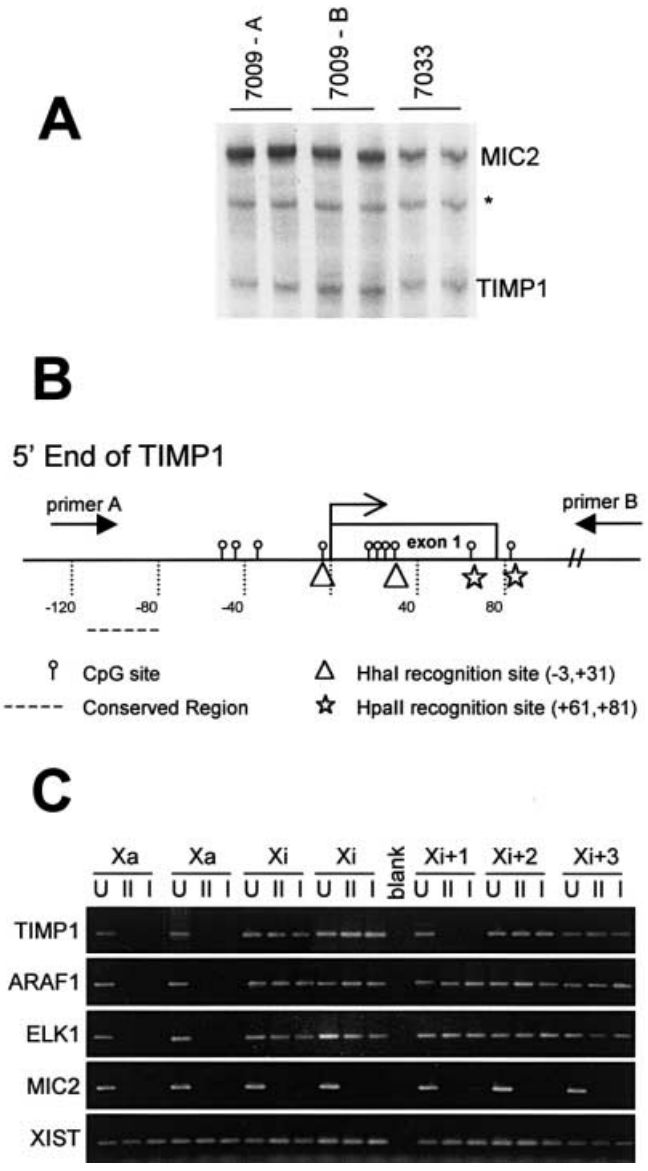

Fig. 1 **A** Representative RNase protection assay results in two male cell lines. *Bottom band* Protected *TIMP1* fragment, *top band MIC2* control for equal amounts of input RNA, *middle band* (*asterisk*) artefact band consistently appearing with the *TIMP1* probe. There were a minimum of two analyses per RNA (two lanes per RNA sample); *7009-A*, *7009-B* different RNA samples from the same cell line. **B** Schematic map of the 5' end of *TIMP1*. The primers flank the minimal promoter from –122 to +155 (Clark et al. 1997). The conserved region is a 38-bp region with 37 bp 100% conserved between human, mouse, and rat, and includes AP-1 and Ets sites required for basal transcription. The methylation of CpG sites (*lollipops*) within *Hpa*II (*star*) and *Hha*I (*triangle*) recognition sites were examined. **C** Products from amplification of DNA without digestion (*U*), after *Hpa*II digestion (*II*), or after *Hha*I digestion (*I*). The presence of a band after digestion (*II* or *I lanes*) indicates that those enzyme sites were methylated and resistant to digestion. *ARAF1* and *ELK1* flank *TIMP1* in Xp11.23. *MIC2* primers were used to control for complete digestion, whereas *XIST* was used to confirm the presence of DNA. The cell lines are (listed *left to right*): active X hybrids (X_a), AHA-11aB1 and t60–12; inactive X hybrids (X_i), t48–1a-1Daz4a and t11–4Aaz5; inactive X hybrids that express *TIMP1* (X_i^+), t75–2maz34–1a, t86-B1maz1b-3a, and t81-az1D

Results

Cell lines show a range of *TIMP1* expression levels

To quantitate the amount of *TIMP1* RNA, we performed RPA with probes for the *TIMP1* and *MIC2* genes. *MIC2* is an X-linked gene known to escape X inactivation and was used as an internal control for RNA levels from the X chromosome. The intensity of the protected *TIMP1* fragment was compared with the intensity of the *MIC2* band to determine the relative levels of *TIMP1* RNA in each sample (Fig. 1A, Table 1). Whereas the variability between replicates of the same RNA preparation was 7.4%, different RNA preparations from the same cell line showed a larger variability (average difference of 13%), which we attributed to cell-cycle differences because the cells were not synchronized.

We established the levels of *TIMP1* RNA from the active X chromosome by examining expression in either male cells or female cells previously demonstrated to express *TIMP1* from only the active X by using an allele-specific assay (Anderson and Brown 1999). *TIMP1* RNA levels in the blood of six healthy unrelated males showed a 4-fold range of expression (Table 1). The level of *TIMP1* did not correlate with the expressed allele or the age of the donor. As the *TIMP1* gene responds to numerous cellular signals, this variability may reflect differences in cellular environments, so subsequent studies were limited to cultured cell lines in which the environmental influences could be minimized. In these cell lines, there was a 1.7-fold range of expression (Table 1). This variability was appreciably greater than the differences between different RNA preparations from the same cell line and made it difficult to determine whether *TIMP1* RNA levels were elevated in females expressing two copies of *TIMP1*. Whereas the RNA level was increased in HSC593, a cell line previously demonstrated to express *TIMP1* from the X_i, this difference was not highly significant (P=0.2). Because the allele-specific PCR assay used to identify cells expressing *TIMP1* from the X_i requires a heterozygous female with non-random X inactivation, it is difficult to obtain informative female samples. Therefore, we also examined RNA levels in female cell lines whose X inactivation status could not be established with our PCR assay because they were homozygous for the *TIMP1* polymorphism (Anderson and Brown 1999). One cell line (GM7005) showed significantly higher expression (P=0.004) and it is tempting to suggest that this reflects biallelic expression of *TIMP1*. However, the other three cell lines of unknown X inactivation status had expression levels within the upper range of X_a levels and may or may not express *TIMP1* from the X_i. Therefore, the variability possible in RNA levels within a cell line and between cell lines monoallelically expressing *TIMP1* precludes the identification of cell lines with biallelic *TIMP1* expression based on RNA levels.

Table 1 Normalized *TIMP1* expression levels in human cells. BL1–BL6 are blood samples from unrelated males; the remainder are cultured lymphoblast cells. The ages of the men BL1–BL6 are 52, 57, 54, 42, 24, and 35 years, respectively

Individual/ cell line	*TIMP1* expression[a]	Expressed allele	Level of *TIMP1*
BL1	X_aY (1)	C	0.71±0.003
BL2	X_aY (1)	T	0.62±0.14
BL3	X_aY (1)	T	0.52±0.19
BL4	X_aY (1)	C	2.07±0.32
BL5	X_aY (1)	C	0.55±0.26
BL6	X_aY (1)	T	1.01±0.38
GM7033	X_aY (1)	C	1.01±0.27
GM7057	X_aY (1)	T	0.92±0.13
GM11200	X_aY (1)	T	1.13±0.10
GM7059	$X_aX_i^-$ (1)	C	0.66±0.09
GM7348	$X_aX_i^-$ (1)	T	1.04±0.07
3149	$X_aX_i^-$ (1)	C	0.87±0.13
2659	$X_aX_i^-$ (1)	T	0.92±0.002
3251	$X_aX_i^-$ (1)	T	0.79±0.002
HSC593	$X_aX_i^+$(2)	C, T	1.43±0.22
GM11198	$X_aX_i^?$ (?)	T[b]	0.97±0.01
GM7005	$X_aX_i^?$ (?)	T[b]	2.27±0.00
GM7350	$X_aX_i^?$ (?)	C[b]	1.25±0.27
GM01416D	$X_aX_i^?$ (?)	T[b]	0.96±0.06

[a]The number of alleles expressing *TIMP1* is shown in *brackets*; some of the females have previously been demonstrated to have mono-allelic (X_i^-) or biallelic (X_i^+) expression, whereas for females who are homozygous (see [b]), it is unknown whether there is expression from the X_i ($X_i^?$)
[b]Homozygous females

In order to assess the potential contribution of the X_i to total *TIMP1* levels, we analyzed expression in rodent/human somatic cell hybrids retaining either the human X_a or X_i. The expression levels of *TIMP1* in X_a-containing hybrids were similar to the cells expressing one copy of *TIMP1* with a comparable range of expression (Table 2). The RPA was repeated by using mouse *Actin* as a control for levels of RNA, and comparable results were observed (data not shown), confirming that the variability observed was attributable to different amounts of *TIMP1* RNA, not *MIC2*. Combining the data from the X_a-containing hybrid cell lines and lymphoblast cell lines expressing *TIMP1* only from the X_a, we found a range of normalized *TIMP1* expression from 0.58 to 1.13, with an average expression of 0.88 and a standard deviation of 0.17.

Levels of *TIMP1* RNA were determined by RPA for X_i-containing hybrids previously shown to express *TIMP1* by RT-PCR (Table 2). The span of expression levels from the X_i (at least 5-fold) was greater than that observed for the X_a cell lines, primarily because of the very low expression in X_i^+3. In this line, no expression was detected above the resolution limit for the RPA (0.25). RT-PCR can detect as little as 1/100 of the *TIMP1* expression from the X_a (data not shown); thus, the RNA level for t81-az1D can be estimated to be between 0.01 and 0.25. We hypothesized that low expression from an X_i might reflect a residual epigenetic feature of X inactivation and thus examined methylation of the promoter region.

Table 2 Normalized *TIMP1* expression levels in the human/rodent somatic cell hybrid cell lines containing an active (X_a) or inactive (X_i) chromosome. The X_i^+ cell lines have been shown to express *TIMP1* from the X_i by RT-PCR (*NS* not significant above background)

Cell line	X content and *TIMP1* expression	Expressed allele	Level of *TIMP1*
AHA-11aB1	X_a	T	1.00±0.20
t60–12	X_a	T	0.67±0.24
A23–1aC15	X_a	C	0.58±0.04
tHM-34–2A41B	X_a	T	0.96±0.06
t75–2maz34–1a	X_i^+	C	1.40±0.10
t86-B1maz1b-3a	X_i^+	C	0.59±0.10
t81-az1D	X_i^+	T	NS
t11–4Aaz5	X_i^-	–	NS

Variable loss of methylation in X_i-containing hybrids

The methylation status of the 5' end of *TIMP1* was determined by PCR after methylation-sensitive digestion with *Hpa*II or *Hha*I, which examines the methylation status of four of 11 CpG sites in the *TIMP1* minimal promoter (Fig. 1B). If the DNA was methylated, the enzymes would not be able to cut, and a fragment would be amplified by PCR. No band after *Hpa*II or *Hha*I digestion indicated that at least one of the enzyme recognition sites was unmethylated. The *XIST* 3':5' primers had no enzyme recognition sites and consequently controlled for the presence of DNA after digestion, whereas amplification of the *MIC2* promoter region was used to demonstrate complete digestion.

No PCR amplification was observed after either *Hpa*II or *Hha*I digestion of the X_a-containing hybrids, demonstrating a lack of methylation. PCR amplification was observed for the X_i-containing hybrids that failed to express *TIMP1*, indicating that the enzyme sites were methylated (Fig. 1C). However, when *TIMP1* was expressed from the inactive chromosome, various methylation states were observed. The t75–2maz34–1a hybrid ($X_i^+{}_1$) was unmethylated at the *TIMP1* promoter, whereas methylation was present in the t86-B1maz1b-3a ($X_i^+{}_2$) and t81-az1D ($X_i^+{}_3$) hybrids. *ARAF1* and *ELK1* flank *TIMP1* and are silent in all X_i hybrids studied (Anderson and Brown 1999). *ARAF1* is 5' to *TIMP1* with ~11 kb of intervening DNA, whereas *ELK1* is ~35 kb downstream. After methylation-sensitive digestion, PCR products were observed for both genes in all X_i-containing hybrid cell lines, but for none of the X_a-containing hybrids (Fig. 1C), indicating that both *ARAF1* and *ELK1* promoters are methylated when silent and unmethylated when expressed. The *TIMP1* gene is found within intron 5 of the *SYN1* gene, and although the *SYN1* gene is primarily expressed in neuronal tissues, we detected variable low level expression in human lymphoblasts, fibroblasts, and both X_a and X_i hybrids. This expression did not correlate with the expression of *TIMP1*. The *SYN1* promoter was unmethylated in male cells indicating hypomethylation of the Xa, but it was methylated in both Xa and Xi hybrids showing no correlation with *TIMP1* or low level *SYN1* expression (data not shown).

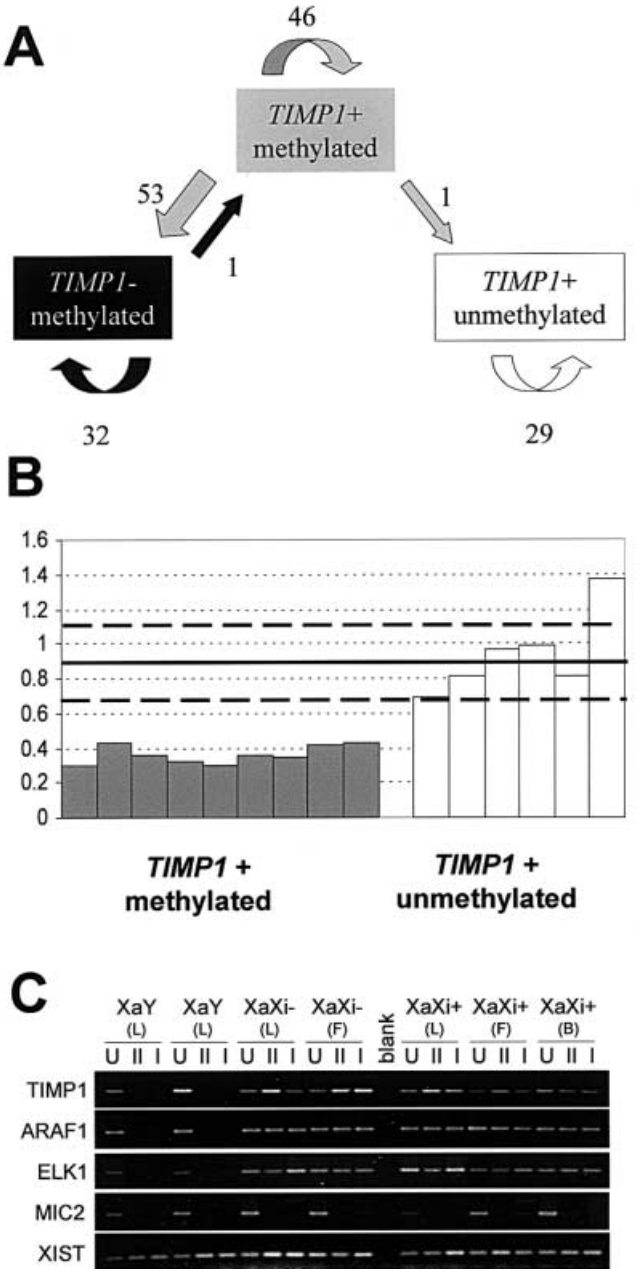

Fig. 2 **A** The expression and methylation status for 162 subclones. Three categories of subclones were obtained: *TIMP1-* and methylated (*black box*), *TIMP1+* and methylated (*gray box*), or unmethylated (*white box*). *Arrows* start at the category of culture from which the subclones were derived and end at the category to which the derived subclones belong. *Numbers near arrows* Number of subclones observed for each type. **B** Expression levels in single cell clones positive for *TIMP1* expression by RT-PCR from two sibling cultures, differing only in their methylation status. There is a consistent increase in RNA level when methylation is absent. *Solid line* Average X_a expression level (0.88), *dotted lines* standard deviation (±0.17). **C** Methylation analysis of the human cell lines. PCR products from uncut DNA (*U*), after *Hpa*II digestion (*II*), or after *Hha*I digestion (*I*). The presence of a band after digestion (*II* or *I lanes*) indicates the presence of methylation. The DNA was isolated from (*left to right*): X_aY male GM07009 and GM07033; $X_aX_i^-$ GM07059, $X_aX_i^-$ GM02859 fibroblast, $X_aX_i^+$ HSC593, $X_aX_i^+$ 1813, $X_aX_i^+$ 07 (*L* lymphoblast cell line, *F* fibroblast cell line, *B* blood)

The two methylated *TIMP1*-expressing cell lines ($X_i^+{}_2$ and $X_i^+{}_3$) showed low levels of *TIMP1* expression. Thus, we hypothesized that the methylated and expressing cell populations might reflect a mixed cell population, with some unmethylated expressing cells contributing to the low level of expression and some methylated silent cells being amplified with the PCR-based methylation assay, which can readily detect 10% of the total DNA as uncut/methylated (data not shown). To address this possibility, single cell clones of the three X_i^+ hybrids were examined for methylation and expression. The $X_i^+{}_1$ hybrid was originally unmethylated with high level *TIMP1* expression, and all 13 subclones were *TIMP1+* and unmethylated. Several subclones from the methylated *TIMP1+* hybrids (5/16 $X_i^+{}_2$ and 3/9 $X_i^+{}_3$) failed to express *TIMP1*, supporting the idea that low level expression was attributable to the presence of a mixed population of silent and expressing cells. These *TIMP1–* clones were all methylated; indeed no clone has ever been observed that is *TIMP1–* but unmethylated. Surprisingly, the *TIMP1+* subclones also remained methylated. To address whether there was ongoing instability of expression or inactivation, we generated further single cell clones from individual *TIMP1+* and *TIMP1–* clones. All subcloning results are summarised in Fig. 2A. Methylated clones never arose from an unmethylated (TIMP+) clone, and methylated silent clones rarely gave rise to expressing clones (1/33). Subclones from the methylated expressing cells were much more diverse, including a high frequency of methylated silent clones and rare unmethylated expressing clones.

Methylation correlates with expression levels

To determine whether the low level expression was a characteristic of the cell lines or attributable to the presence of methylation at the promoter, we examined the expression levels of subclones of two sister cultures that expressed *TIMP1* but differed in their methylation states. These cultures arose during the second subcloning of $X_i^+{}_2$ from a methylated *TIMP1+* clone. The only difference between these sibling subclones should be epigenetic characteristics, because they arose from the same culture. When the clone was unmethylated at the promoter, 16/16 subclones expressed *TIMP1*, and expression was in the range seen for an X_a (Fig. 2B). The methylated *TIMP1+* culture continued to show instability, with only 13/31 (42%) of its clones remaining *TIMP1+*, and those that were positive showed low expression levels (Fig. 2B). Therefore, we examined methylation of the *TIMP1* promoter in females to determine whether expression from the X_i is likely to alter significantly the total expression of *TIMP1*.

The male lymphoblast cell lines examined had only the one active X chromosome and were unmethylated at the *TIMP1* promoter (Fig. 2C), and female cell lines that inactivated *TIMP1* (X_i^-) showed methylation. Females are generally a mosaic of cells with different X chromosomes active, and therefore methylation would be expected if either X chromosome were subject to inactivation. However, the three females previously shown to have expression from the X_i had extremely skewed X inactivation (>90% inactivation of one X) and thus could be considered a monoclonal population with expression from both X chromosomes. DNA from these females (derived from blood, a fibroblast culture, and a lymphoblast culture) showed amplification after *Hpa*II and *Hha*I digestion, indicating that the *TIMP1* promoter on the *TIMP1*-expressing X_i remained methylated.

Discussion

X inactivation is an extraordinary example of co-ordinate gene control, silencing most of the ~2000 genes on one of the two X chromosomes of females to achieve dosage compensation with hemizygous males. However, more than 10% of X-linked genes escape this inactivation and are expressed from both X chromosomes (Carrel et al. 1999). Dosage compensation is maintained when these genes have functional Y homologs (Disteche 1995), but when there is no functional Y homolog, females may have higher amounts of that gene product. For some genes, overexpression in females may be necessary and may play a role in normal female development; for other genes, it may have no impact, but dosage is clearly important for a subset of X-linked genes. For example, abnormal duplication of *DAX-1* in males results in sex reversal, whereas deletions lead to X-linked congenital adrenal hypoplasia (Goodfellow and Camerino 2001). The dosage of the *PLP* gene is also strictly regulated, since both duplication and deletion of the gene cause Pelizaeus-Merzbacher disease, an X-linked demyelination disorder (Sistermans et al. 1998). However, expression from the X_i does not necessarily cause a significant increase in gene product, because gene expression from the X_i is often lower than that from the active chromosome, as observed for the *STS* gene (Migeon et al. 1982). Since TIMP1 is involved in a delicate biological balance with MMPs, any alterations may disrupt normal physiology, so we have examined *TIMP1* RNA levels to determine the contribution of expression from the X_i chromosome.

When determining the baseline level of *TIMP1* expression, we discovered considerable variation in *TIMP1* RNA levels. Cell-cycle variability and/or culture conditions probably account for many of the differences between RNA preparations of the same cell line, because the *TIMP1* gene responds to a variety of growth factors (Denhardt et al. 1993). Surprisingly, however, beyond the variation within a cell line, there was an almost 2-fold range of *TIMP1* RNA between cell lines expressing only one copy of the gene. Diverse expression levels from the active X chromosomes could reflect sequence differences that lie at the *TIMP1* promoter and that alter transcription factor binding sites. For instance, a single nucleotide polymorphism in the MMP-1 promoter creates a binding site for the Ets gene family of transcription factors and enhances expression (Rutter et al. 1998). The *TIMP1* minimal promoter contains both AP-1 and Ets sites and changes in these

binding sites could modify transcription, but the promoter sequence (–122 to +600 in Dean et al. 2000) is the same between the active hybrids with the highest and lowest expression levels (data not shown). The intrachromosomal variability does not correlate with the expressed polymorphism (Tables 1, 2; Anderson and Brown 1999). However, other sequence differences beyond the minimal promoter and exon 5 polymorphism may influence expression levels. *TIMP1* is regulated at the RNA level, suggesting that RNA levels are a major determinant of protein levels (Doyle et al. 1997), but it is possible that there are translational mechanisms to provide a consistent amount of protein across cells. Since the TIMP1 protein is an inhibitor of MMPs, it is the balance of TIMPs to its target proteins (not absolute amounts) that is important in determining proteinase activity. Therefore, variable *TIMP1* RNA levels between cell lines may reflect the amount of MMPs present within cell lines.

Expression levels from the X_i chromosome showed greater variability than levels from the X_a, because of very low level expression in some X_i^+ hybrids. The low level expression observed in X_i^+ hybrids could be characteristic of these cell lines or attributable to residual silencing by epigenetic features associated with X inactivation such as methylation. By analyzing sibling cultures differing in methylation, we determined that low level expression was not inherent to the cell line but associated with the presence of methylation at the *TIMP1* promoter. The observation of methylation and expression in single cell clones was unexpected since methylation generally correlates with silencing of X-linked genes. It is possible that there are critical sites required to silence *TIMP1* that we have not examined. For example, it has recently been demonstrated that there are three crucial sites determining expression status for the X-linked *HPRT* gene (Chen et al. 2001). However, methylation of the four sites analyzed in the *TIMP1* minimal promoter region does show strict correspondence with inactivation status in cells that have never expressed *TIMP1* from the X_i.

We have hypothesized that *TIMP1+* methylated cultures are composed of a mixture of silent methylated cells and expressing unmethylated cells. This could also explain the lower level expression in the presence of methylation, presumably because of the lack of contribution from the methylated cells. However, the outcome of our subcloning experiments suggests a more complex composition (Fig. 2A). Both the unmethylated *TIMP1+* state and the methylated silent clones are quite stable, indicating that the premise of a simple mixture of unstable cells is unlikely as methylated *TIMP1+* clones persist. This suggests that *TIMP1* promoter methylation and gene expression may not be mutually exclusive. When the *TIMP1* promoter is methylated, expression from the X_i is significantly lower, close to or below the RPA assay background level of 0.25. These results are consistent with the findings for the *HGNC* (choroideremia) gene where single cell analysis has shown low-level expression from most cells, although methylation has not been analyzed (Carrel and Willard 1999).

TIMP1 is not always expressed from the X_i, and comparing cell lines with and without this X_i expression may reveal which epigenetic factors are required to maintain the inactive state. The X_i is associated with the hypermethylation of promoters, nuclease insensitivity, delayed replication timing, and the hypoacetylation of histones. These epigenetic features are most likely redundant, and we propose that one of these features can be lost without disrupting dosage compensation. For example, cell lines from patients with ICF immunodeficiency syndrome have a hypomethylated X_i chromosome, but a gene is expressed from the X_i only when replication timing is advanced similar to the X_a homolog (Hansen et al. 2000). In the X_i^+ cells, one of the epigenetic controls may be absent at *TIMP1*, allowing low level expression from the X_i before methylation is disrupted. However, once methylation is lost, *TIMP1* stably expresses at a high level, which could result in the loss of dosage equivalence. It will be interesting to study the other features normally associated with X inactivation to determine whether they differ when *TIMP1* is expressed from the inactive chromosome.

Cells from the three females with expression from the X_i showed methylation at the *TIMP1* promoter. The small amount of *TIMP1* RNA expressed from the X_i in the presence of ongoing methylation is unlikely to be biologically significant given the variability of *TIMP1* expression from the active chromosome. In the hybrid cell lines, there was a slight trend toward loss of methylation amongst TIMP1+ cells (see Fig. 2A). If this trend also occurs in females, then as women age, they may accumulate cells that express higher levels of *TIMP1* from the X_i. If TIMP1 rises above an accepted range of expression, it may lead to aberrant regulation of metalloproteinase activity. A controlled balance of the MMPs and the TIMPs are required for normal tissue remodeling processes such as pregnancy, development, and wound healing. Increased amounts of TIMP1 may reduce overall MMP activity, predisposing women to fibrotic disorders but potentially protecting them from some forms of metastasis. Expression from the X_i chromosome with the eventual rise of a gene product above a normal range may help explain observed sex differences in the susceptibility to some late-onset disorders, such as arthritis and multiple sclerosis.

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