Dosage Compensation in the Mouse Balances Up-Regulation and Silencing of X-Linked Genes

Hong Lin1, Vibhor Gupta1,2, Matthew D. VerMilyea1, Francesco Falciani2, Jeannie T. Lee3,4, Laura P. O’Neill1, Bryan M. Turner1*

1 Chromatin and Gene Expression Group, Institute of Biomedical Research, University of Birmingham Medical School, Birmingham, United Kingdom, 2 BioInformatics and Systems Biology Group, School of Biosciences, University of Birmingham, Birmingham, United Kingdom, 3 Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts, United States of America 4 Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, Massachusetts, United States of America

Dosage compensation in mammals involves silencing of one X chromosome in XX females and requires expression, in cis, of Xist RNA. The X to be inactivated is randomly chosen in cells of the inner cell mass (ICM) at the blastocyst stage of development. Embryonic stem (ES) cells derived from the ICM of female mice have two active X chromosomes, one of which is inactivated as the cells differentiate in culture, providing a powerful model system to study the dynamics of X inactivation. Using microarrays to assay expression of X-linked genes in undifferentiated female and male mouse ES cells, we detect global up-regulation of expression (1.4- to 1.6-fold) from the active X chromosomes, relative to autosomes. We show a similar up-regulation in ICM from male blastocysts grown in culture. In male ES cells, up-regulation reaches 2-fold after 2–3 weeks of differentiation, thereby balancing expression between the single X and the diploid autosomes. We show that silencing of X-linked genes in female ES cells occurs on a gene-by-gene basis throughout differentiation, with some genes inactivating early, others late, and some escaping altogether. Surprisingly, by allele-specific analysis in hybrid ES cells, we also identified a subgroup of genes that are silenced in undifferentiated cells. We propose that X-linked genes are silenced in female ES cells by spreading of Xist RNA through the X chromosome territory as the cells differentiate, with silencing times for individual genes dependent on their proximity to the Xist locus.

Introduction

In many higher eukaryotes, sex determination mechanisms have evolved in a way that has generated chromosomal differences between the sexes. In eutherian and marsupial mammals and the fruit fly Drosophila, females have two copies of a gene-rich X chromosome, whereas males have one X and one smaller, gene-poor Y. Because monosomy for even the smallest autosome is lethal in mammals, mechanisms have presumably evolved to allow males to tolerate monosomy of the X, as well as to correct a potential imbalance between the sexes in expression levels of several hundred X-linked genes [1,2]. In Drosophila, the situation has been resolved by an overall up-regulation of genes on the single male X, a dosage compensation mechanism that equalises expression both between X and autosomes and between the sexes [3,4]. In mammals, expression in males and females has been balanced by X inactivation, a process by which most genes on one of the two female Xs are silenced early in development [5–7]. However, X inactivation alone exacerbates the Xautosome imbalance, leaving both sexes functionally monosomic for X-linked genes. This problem was highlighted many years ago, and a balancing, 2-fold up-regulation of genes from the single, active X was proposed as a possible solution [2,8]. However, proof of this has been difficult to achieve. Studies of the expression of the Csl4 gene in hybrid mice provided a clue that this might occur [9], but the first indication that genes on the active X are globally up-regulated has come only recently through the analyses of microarray data from a variety of publicly available sources. Comparisons of the mean, overall expression levels of X-linked and autosomal genes in various cell and tissue types, usually from mixtures of male and female, gives an X:autosome expression ratio of approximately 1 [10–12]. Given that both XY male and XX female cells have only a single, transcriptionally active X, and two copies of each autosome, without up-regulation of X-linked genes the mean ratio should be closer to 0.5. The results therefore provide evidence, albeit circumstantial, for a balancing up-regulation of expression from the active X.

We used microarray expression analysis to give a global picture of X-linked gene expression in differentiating mouse embryonic stem (ES) cells, a model system that allows the

Citation: Lin H, Gupta V, VerMilyea MD, Falciani F, Lee JT, et al. (2007) Dosage compensation in the mouse balances up-regulation and silencing of X-linked genes. PLoS Biol 5(12): e326. doi:10.1371/journal.pbio.0050326

* To whom correspondence should be addressed. E-mail: b.m.turner@bham.ac.uk

Academic Editor: Wolf Reik, The Babraham Institute, United Kingdom

Received March 28, 2007; Accepted November 1, 2007; Published December 11, 2007

Copyright: © 2007 Lin et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: ES cells, embryonic stem cells; FDR, false discovery rate; FISH, fluorescence in situ hybridisation; ICM, inner cell mass; RTQ-PCR, real-time quantitative PCR; SNP, single nucleotide polymorphism; Xic, X inactivation centre
In organisms such as fruit flies and humans, major chromosomal differences exist between the sexes: females have two large, gene-rich X chromosomes, and males have one X and one small, gene-poorn Y. Various strategies have evolved to balance X-linked gene expression between the single X and the autosomes, and between the sexes (a phenomenon called dosage compensation). In Drosophila melanogaster, expression from the male X is up-regulated approximately 2-fold, thereby balancing both X-to-autosome and female-to-male expression. In contrast, mammals silence one of the two female Xs in a process requiring the untranslated RNA product of the Xist gene. This balances female-to-male expression but leaves both sexes with only one functional X chromosome. Using mouse embryonic stem cells and microarray expression analysis, we found that dosage compensation in mice is more complex than previously thought, with X-linked genes up-regulated in both male and female cells so as to balance X-to-autosome expression. As differentiation proceeds, female cells show progressive loss of expression from one of the two initially active Xs. Surprisingly, silencing occurs on a gene-by-gene basis over 2–3 week of differentiation; some genes escape altogether, whereas a subgroup of genes, often adjacent to the Xist locus, is silenced even in undifferentiated cells. We propose that female X-linked genes are silenced by progressive spreading of Xist RNA through the X chromosome territory as differentiation proceeds.

In organisms such as fruit flies and humans, major chromosomal differences exist between the sexes: females have two large, gene-rich X chromosomes, and males have one X and one small, gene-poor Y. Various strategies have evolved to balance X-linked gene expression between the single X and the autosomes, and between the sexes (a phenomenon called dosage compensation). In Drosophila melanogaster, expression from the male X is up-regulated approximately 2-fold, thereby balancing both X-to-autosome and female-to-male expression. In contrast, mammals silence one of the two female Xs in a process requiring the untranslated RNA product of the Xist gene. This balances female-to-male expression but leaves both sexes with only one functional X chromosome. Using mouse embryonic stem cells and microarray expression analysis, we found that dosage compensation in mice is more complex than previously thought, with X-linked genes up-regulated in both male and female cells so as to balance X-to-autosome expression. As differentiation proceeds, female cells show progressive loss of expression from one of the two initially active Xs. Surprisingly, silencing occurs on a gene-by-gene basis over 2–3 week of differentiation; some genes escape altogether, whereas a subgroup of genes, often adjacent to the Xist locus, is silenced even in undifferentiated cells. We propose that female X-linked genes are silenced by progressive spreading of Xist RNA through the X chromosome territory as differentiation proceeds.

The distributions of expression levels of X-linked genes in male and female ES cells are shown as box plots in Figure 1C. In both males and females, expression levels are skewed towards higher expression levels (Figure S2). A similar skewing was seen when the expression of genes on individual autosomes was analysed in the same way (Figure S2). To accommodate this, we have used median rather than mean values for comparison (Figure 1C, horizontal line in each box). The median X:A ratio in undifferentiated (day 0) male ES cells is 0.81 (Figure 1C). This is significantly above the median value of 0.5 that would be expected if X-linked genes were expressed equally to autosomal genes (p = 3.2 × 10−32, n = 249) and demonstrates an overall 1.6-fold up-regulation of expression on the single X. Microarray expression data for three additional male ES cell lines has recently been made available (BL6.9, 129.3, and ES-D3-GL) [15], and we have used this to calculate X:A expression ratios, as above. Values ranged from 0.90 to 1.03, suggesting that an approximately 2-fold up-regulation of X-linked genes is a general property of male ES cell lines.

In XX female cells, equal expression of X-linked and autosomal genes would give an X:A ratio of about 1.0, whereas the measured ratio is 1.39, which is significantly higher (Figure 1C, p = 1.1 × 10−6, n = 249). A very similar X:A expression ratio (1.37) was given by cDNA from the hybrid, female ES cell line 3F1 (see below). Thus, X-linked genes in female ES cells are also up-regulated. After 15 d of differentiation, X:A ratios in both male and female cells were close to 1, which is comparable to the situation in adult cells (Figure 1C).

Expression of X-Linked Genes in the ICM

To test whether the up-regulation of X-linked genes detected in ES cells also occurs in the cells of the blastocyst from which ES cells are derived, RNA was prepared from single ICMs from cultured male and female embryos (distinguished by presence or absence of the Sry gene, Figure 2A), amplified and used to prepare cDNA for labelling of NIA15K arrays. As with expression in ES cells, the expression of X-linked genes in female and male embryos was closely correlated (Figure 2B). The median X:A ratio in male ICMs was 0.86 (Figure 2C), very similar to the value of 0.81 obtained for male ES cells (Figure 1C) and indicative of up-regulation of X-linked gene expression. In female ICMs from the same batch of embryos, the X:A ratio was 0.89 (Figure 2C). This is consistent with up-regulation of genes on Xa in females only if one of the two Xs is inactivated in all or most of the ICM cells used in this experiment. This is certainly possible, because levels of Xist RNA were particularly high in female ICMs (around 8-fold higher than the median expression of autosomal genes) and significantly higher than the Xist RNA levels in male ICM (Figure 2D) and differentiated female ES cells (Figure 1B). Whether this represents persistence of the imprinted paternal X inactivation present from early in development.
development, or random X inactivation, or a combination of the two [17], remains to be determined.

Changes in Expression of X-Linked Genes Proceed throughout ES Cell Differentiation

The dynamics of differentiation-related changes in X-linked gene expression were determined by analysing cDNAs from male and female cells at various times of differentiation between 0 and 21 d. The X:A expression ratio in female cells showed a gradual and progressive decrease until day 15, whereas in male cells, there was little change until day 7, after which there was a progressive increase (Figure 3A). The gradual changes in X-linked gene expression contrast with the relatively early change in distribution of Xist silencing RNA detected by RNA–fluorescence in situ hybridisation (FISH) (Figure 3B) and loss of expression of the pluripotency markers Nanog, Pou5f1/Oct4, and Zfp42/Rex1 (Figure S1B). Xist RNA levels increased through differentiation from day 2 onwards (Figure S1C).

The observed increase in expression of X-linked genes in differentiating male cells and the decrease in female cells are unique properties of X-linked genes. Expression of genes on each of the 19 mouse autosomes, relative to all genes (designated the n:A ratio) showed no such changes. The n:A ratio varied from one chromosome to another over only a narrow range and did not change with differentiation or differ between females and males to the same extent as did the X:A expression ratio (Chromosome 2 is shown as an example in Figure 3C and all 19 autosomes are shown in Figure S3).

The expression of X-linked genes in differentiating female ES cells can potentially be influenced by the following three parallel processes: (i) silencing through X inactivation, (ii) up-regulation through dosage compensation on the active X, and (iii) differentiation-related expression changes that are unconnected to the dosage compensation process. Given the close correlation between expression levels of X-linked genes in male and female cells throughout differentiation (Figure 1A and 1B), the latter two processes are likely to occur to a similar extent in both male and female cells. This being the case, changes in the female:male expression ratio of X-linked genes should reflect progression of the X inactiva-
tion process alone. With this in mind, we co-hybridised red/green labelled cDNAs from female and male cells, at the same stage of differentiation, to the same slide, and we calculated red:green or green:red ratios as a log2 M value, as described [18,19]. In undifferentiated, cells the M value is around 0.65 (Figure 4A), corresponding to a linear female:male expression ratio of about 1.6. M values derived from undifferentiated cells and cells at later stages of differentiation were all normally distributed (Figure 4B). There was no detectable fall in M value for the first 7 d of differentiation, with a small increase at days 2–4. Thereafter, there was a progressive decrease, culminating in an M value close to 0, which reflects equal expression of X-linked genes in female and male cells by day 21 (Figure 4A and 4B). It seems that a net loss of expression of female X-linked genes occurs later than previously concluded on the basis of single-gene analyses [13,20].

To determine the consistency of these findings between ES cell lines, we assayed X-linked gene expression in the hybrid (m. mus domesticus X m. mus castaneous) ES cell line 3F1. There is a close correlation in expression of X-linked genes between these two very different lines (Figure 4C). Further, co-hybridisation of 3F1 and male (CCE/R) cDNAs from the same stages of differentiation to the same slides showed a relatively late decrease in the expression of female X-linked genes relative to male, with no detectable decrease in female:male expression ratio (M value) after 2 d of differentiation, and complete equalisation only after 15 d (Figure 4D).

By studying the change in M value with differentiation time for individual genes, it became clear that some genes consistently showed a relatively early loss of activity in female cells, while others inactivated later, or not at all. Differences in silencing times were confirmed by real-time quantitative (RTQ)-PCR assays, in which expression levels in differentiating embryoid bodies were expressed relative to levels at day 0 (examples are presented in Figure S4). As a first test of whether genes that inactivated relatively early in one ES cell line also inactivated early in others, we co-hybridised cDNA from undifferentiated cells and cells differentiated for 7 d to the same slide and calculated the day 7:day 0 expression ratio as an M value. Genes showing reduced expression in PGK12.1 cells after 7 d of differentiation...
tended also to show reduced expression in 3F1 cells, with a good overall correlation between the two cell lines in the change in expression of X-linked genes after 7 d of differentiation (p, 0.001, Figure S5).

We subjected the PGK12.1 M value dataset (Figure 4A) to cluster analysis using the TIGR programme [21]. The programme grouped expression data from 252 X-linked clones into selected numbers of clusters, based on the manner in which expression (M value) changed during differentiation. Figure 5A shows the results of an analysis in which the data were resolved into four clusters, each shown as a graph plotting the median value at each stage of differentiation tested. Figure 5B shows the corresponding heat maps. Clusters 1, 2, and 3 showed similar patterns of change, starting at an M value of 0.6–0.8 at day 0 (corresponding to a linear F:M ratio of 1.54–1.75) falling to around 0 by day 21, but differing in the stage at which M values first fell significantly, i.e., day 4–7 for cluster 1 (46 genes), day 7–12 for cluster 2 (74 genes), and day 12–21 for cluster 3 (64 genes). In contrast, the 21 genes in the fourth cluster behaved differently, with M values close to 0 in undifferentiated cells (median = −0.14) and generally increasing on differentiation (Figure 5A). We note that irrespective of how many clusters the programme was asked to resolve, there was always one that showed essentially the same pattern as that of cluster 4 and that stood out from the rest. The genes in cluster 4 are listed in Table S1. They include Xist, a gene known to show increased expression in female cells as they differentiate [22] (Figure S1C). Ontology analysis using Fatigo+ [23] showed that none of these four gene clusters was significantly enriched in genes associated with specific functional categories or cell lineages (unpublished results).

Some X-Linked Genes Show Mono-Allelic Expression in Undifferentiated Female ES Cells

One explanation (among several) for the finding that some genes are equally expressed in XX female and XY male ES cells is that they are expressed from only one allele in female cells. This could result from the failure to reactivate the paternal allele which, for most X-linked genes, is selectively silenced in the preblastocyst embryo but reactivated in the inner cell mass during blastocyst maturation [17,24]. Alter-
natively, it could reflect initiation of (random) X inactivation before the onset of ES cell differentiation. To explore these possibilities, we assayed the expression of paternal and maternal alleles using the hybrid ES cell line 3F1, derived from a m.mus domesticus (129/sv) × m.mus castaneous hybrid backcrossed to 129 [25]. The 129 X chromosome is maternally derived, whereas the castaneous X chromosome is paternal [26]. In 3F1 cells, the 129 X chromosome carries a loss-of-function Tsx mutation, such that when 3F1 cells differentiate, Xist is always up-regulated on the 129 X chromosome, which is therefore always inactivated [25].

We identified single nucleotide polymorphisms (SNPs) in three cluster 4 genes (Jarid1c, Gm784, and Acsl4) that distinguished the 129 and castaneous alleles and that could be selectively restriction digested so as to generate cDNAs that are distinguishable electrophoretically. Remarkably, for all three genes, expression in undifferentiated 3F1 cells was exclusively from the castaneous allele in undifferentiated 3F1 cells; a second, Ogt, showed expression that was strongly skewed towards the castaneous allele; whereas three cluster 4 genes (Jarid1c, Gm784, and Acsl4) that distinguished the 129 and castaneous alleles and that could be selectively restriction digested so as to generate cDNAs that are distinguishable electrophoretically. Remarkably, for all three genes, expression in undifferentiated 3F1 cells was exclusively from the castaneous allele in undifferentiated 3F1 cells; a second, Ogt, showed expression that was strongly skewed towards the castaneous allele; whereas three cluster 4 genes (Jarid1c, Gm784, and Acsl4) that distinguished the 129 and castaneous alleles and that could be selectively restriction digested so as to generate cDNAs that are distinguishable electrophoretically. Remarkably, for all three genes, expression in undifferentiated 3F1 cells was exclusively from the castaneous allele in undifferentiated 3F1 cells; a second, Ogt, showed expression that was strongly skewed towards the castaneous allele; whereas
the third, Brodl, showed bi-allelic expression (Figure 6B). Two genes whose female:male expression ratios in undifferentiated cells showed the expected female bias by microarray analysis (Ptk1 and Zfp185) showed clear biallelic expression, as did a third gene (Pgr15l), for which a suitable SNP was available but which was not present on the NIA15K array (Figure 6B, supplementary Figure S6). The conclusion from these results is that a subpopulation of X-linked genes in female ES cells is mono-allelically expressed before differentiation and that in 3F1 cells, where inactivation is 100%
skewed towards the maternal X, it is always the paternal (castaneous) X that is expressed. Thus, mono-allelic expression is not due to failure to reactivate the paternal allele from its preblastocyst silent state, but instead is due to the onset of “random” X inactivation before differentiation.

For all genes tested that showed biallelic expression in undifferentiated cells (Brdrl, Zfp185, Ogt, Pck1, and Pgr151), allele-specific analysis confirmed the microarray data, showing that inactivation of X-linked genes occurs over a wide range of differentiation times and that individual genes have characteristic times of inactivation (Figure 6B and Figure S6).

Proximity to the X inactivation centre (Xic) Correlates with Mono-Allelic Expression in Undifferentiated Female ES Cells

In searching for possible reasons for the gene-to-gene differences in inactivation rate, we asked whether position on the X chromosome, and specifically proximity to Xist and the Xic, was of any relevance. To do this, we tabulated the distribution of genes in each cluster across seven X chromosome regions of similar gene content (Table S2). Genes in clusters 1–3 are distributed across the X chromosome with no clear enrichment or depletion in any single region, nor any clear differences between clusters. In contrast, cluster 4 showed a significant enrichment in the region (85–108 Mb) that contains the Xic (8 of 21 genes, p = 0.038, Fisher’s exact test). Six of these eight genes are within 6 Mb (94.8–100.5 Mb) of the Xic (Tables S1 and S2).

The tendency of genes silenced before ES cell differentiation to be located adjacent to the Xic suggests that Xist RNA plays a role in their silencing, even before its increased expression early in differentiation. To test this, we took advantage of the finding that Xist transcript levels in undifferentiated 3F1 cells (carrying a mutation of the Tsix gene) are about 3-fold higher than in the 16.6 hybrid line, from which 3F1 was derived [25] and which has a functional Tsix gene [22]. In 16.6 cells, inactivation is 80% skewed towards the 129 X chromosome as a result of differences in strength of the Xce alleles in the parental strains [27,28]. In undifferentiated 16.6 cells, two of the four genes that are mono-allelically expressed in 3F1 cells (Gm784, Acs4) were also expressed exclusively from the castaneous allele. However, the two that were most distant from the Xic (Phka2, Jarid1c) were bi-allelically expressed (examples shown in Figure 6C), consistent with the possibility that silencing reflects local spreading of Xist RNA.

If silencing of X-linked genes reflects the progressive spreading of Xist RNA through the X chromosome territory, then one would predict that genes that are close together on the chromosome should be silenced at similar times. To test this, we prepared a list of X-linked gene pairs separated by progressively increasing distances, and we asked whether members of each pair were found in the same cluster (i.e., any one of clusters 1–4, Figure 4) more often than expected by chance. For this analysis, the four clusters are taken as broad indicators of inactivation timing. We find that gene pairs separated by up to 40 kb (Table S3) are in the same cluster (p < 0.05, statistical procedures used are outlined in Text S1 and Figure S7). The five closest gene pairs, from 0 to 2.8 kb apart, were always present in the same cluster (p < 0.05, Table S3).

Discussion

The results presented here use microarray expression analysis to show that dosage compensation in mouse ES cells involves up-regulation of X-linked genes in both males and females, together with the progressive, differentiation-dependent silencing of genes on one of the two female X chromosomes. We find that up-regulation is present prior to
ES cell differentiation and is present also in male (and most likely female) ICMs. Up-regulation of X-linked gene expression in male and female cells is consistent with the overall ~1:1 expression ratio of X-linked and autosomal genes calculated from analysis of publicly available microarray expression data for a wide range of mouse and human cell types [11].

Progressive Silencing of X-Linked Genes in Differentiating Female ES Cells

Increasing the level of Xist RNA transcripts early in differentiation of female ES cells, is a key event in silencing, in cis, of X-linked genes [6,22]. The more extensive Xist signal detected by RNA-FISH, following up-regulation, has been thought to represent “coating” of the X chromosome, an event that triggers gene silencing. However, the data presented show that global silencing of X-linked genes in differentiating ES cells is not contiguous with the onset of Xist up-regulation, but is put in place progressively over several weeks of differentiation. Indeed, we find no general relationship between transcriptional silencing and any one of the chromosome-wide changes that first become apparent on Xi at specific stages of ES cell differentiation, including Xist coating and H3 lysine 9 methylation (days 1–2), histone deacetylation (days 5–7), and incorporation of the histone variant macroH2A (days 8–12) [20,29–31].

The slow, progressive fall in X-linked transcripts is not easily attributable to experimental or technical factors. Falls in transcript levels inevitably lag behind transcriptional silencing and will show a spread of values that reflects how promptly individual cells begin to differentiate, but such effects cannot account for the consistent variation from one gene to another in the stage at which transcript levels fall. Nor can differences in RNA turnover or stability account for the gene-to-gene variation in silencing time. An unstable transcript will vanish as soon as transcription stops, while a transcript will vanish as soon as transcription stops, while a

Differential reconfiguration of X chromosome territories in differentiating ES cells is not contiguous with the onset of Xist RNA early in ES cell differentiation leads to the formation of a distinct Xist domain within the X chromosome territory, into which genes are placed as they are silenced [20,35]. The results presented here are consistent with an Xist domain, but we suggest that the domain expands through the X chromosome territory as differentiation proceeds, and that genes are silenced as they come into contact with the spreading Xist RNA. The progressive increase in Xist transcript levels during differentiation of female, but not male, ES cells (Figure S1C) is consistent with an expanding Xist domain. The stage at which any gene is silenced will therefore depend on its position within the X chromosome territory relative to the Xist locus. These positions will depend on how the X chromosome is folded within its territory, and for all genes except those most proximal to Xist, the folding need bear little relationship to the gene’s (linear) position on the chromosome, which we find to be the case (Table S2). We do find, as predicted by the spreading model, that genes that lie close together on the chromosome (within about 40 kb) tend to be silenced at about the same time, as measured by cluster analysis (Table S3). None of this evidence is inconsistent with the possibility that some genes at least may be actively drawn into the Xist domain [20]; it is possible that Xist spreading and gene repositioning occur in parallel.

In the model we propose, the pattern of gene silencing through differentiation is critically dependent on the configuration of the X chromosome territory, specifically the positioning of the Xist locus and of other loci relative to it. Differential reconfiguration of X chromosome territories in female cells prior to the onset of X inactivation, or even changes in their intranuclear location, may be a crucial initial step in the X inactivation process [22,36]. The fact that XY male ES cells express low levels of Xist RNA prior to differentiation, but do not inactivate genes proximal to Xist (e.g., Gm784, Acs14, Figure 6), indicates that Xist RNA is not the sole determinant of inactivation. Perhaps configuration of the X chromosome territory or chromatin conformation in undifferentiated male ES cells is such as to preclude contact between Xist RNA and critical X-linked loci. Our previous observation that X-linked genes in female ES cells carry levels of histone modifications associated with transcriptional activity that are higher than those in males [37] raises the intriguing possibility that chromatin modifications might help determine susceptibility to Xist silencing. In this respect, it is interesting that the dosage compensation complex in D. melanogaster, which includes roX RNAs, preferentially targets transcriptionally active genes, possibly through their distinctive histone modifications [3,4,38].

Evolutionary Considerations

Dosage compensation is a rapidly evolving process, and the mechanisms by which it is accomplished vary from one
Dosage Compensation in Embryonic Stem Cells

organism to another [1,10,38]. It is interesting to ask whether evolution is driven predominantly by a need to equalise overall X-linked and autosomal expression levels, or whether transcript levels of key individual genes exert the major selection pressure. Recent studies on expression of Z-linked genes in birds, in which females are heterogametic (ZW) and males homogametic (ZZ), throw some interesting light on this [39]. For a representative group of genes in various tissues in two species (zebra finch and chicken), the expression of Z-linked genes was consistently and significantly higher in ZZ males—where the Z:autosome expression ratio was around 1—than in ZW females—where the Z:autosome ratio ranged from 0.7 to 0.9 depending on the tissue. These findings indicate that dosage compensation is incomplete in birds, and that higher eukaryotes can tolerate significant overall differences in gene expression between the sexes and between X-linked and autosomal genes. It now seems that the three model organisms commonly used to study dosage compensation: fruit fly (D. melanogaster), mouse (Mus musculus), and the nematode worm Caenorhabditis elegans have all adopted up-regulation of X-linked gene expression in XY (or XO) males as a means of balancing X:autosome expression levels [1,38]. In mouse and C. elegans [40,41] there is also an overall suppression of X-linked transcription in XX females/hemaphrodites. The extra complexity of the mammalian, and worm, mechanisms is likely to reflect their evolutionary histories. It is generally accepted that the gene-poor Y chromosome is the evolutionary result of progressive degeneration of one of two originally homologous chromosomes, one of which (the proto-Y) carried a sex-determining allele [42,43]. Restricted crossing-over at and around the sex-determining locus, which is necessary to prevent the formation of intersex states, allows the progressive spread of mutations and the loss of functional genes along the proto-Y by reducing the selection pressure to which they are subjected [8]. For many mutated genes, selection pressure will favour up-regulation of the remaining functional allele to restore the original transcript levels. The magnitude of this selection pressure will depend on the sensitivity of the gene product’s function to transcript level. If in mammals (and C. elegans), unlike Drosophila, the newly evolving up-regulation mechanism were expressed from the beginning in both males and females, then a female-specific silencing mechanism would need to evolve in parallel to suppress damaging overexpression [38,42]. The fact that the up-regulation of X-linked genes in Drosophila is male-specific, whereas in the mouse is not, suggests that the mechanisms by which up-regulation is achieved may be fundamentally different in the two organisms, despite the presence in mammals of homologues of several of the Drosophila dosage-compensation complex components [44,45]. Unravelling the up-regulation mechanism in mammals and defining how it interacts, if at all, with Xist-mediated silencing to optimise expression of X-linked genes are now questions of particular interest.

Materials and Methods

Cells and cDNA preparation. The mouse ES cell lines PGK12.1 (129 × PKG hybrid) [46], CCER (129svs) [47], and 3F1 (129sv × castaneus hybrid) [25] were cultured as previously described [37]. Differen-
tiation was induced by replating on nonadherent plastic dishes in the absence of leukaemia inhibitory factor (LIF). Adult control cells were thymic lymphocytes from 4-wk-old Balb/c mice. ICM cells were prepared from cultured Balb/c mouse embryos at the early blastocyst stage by the immunosurgery procedure of Solter and Knowles [48], as previously described [49]. Embryos were sexed by testing (by PCR) the trophectodermal material remaining after immunosurgery for presence of DNA encoding the male-specific, Y-linked antigen Sry.

Total RNA was extracted from ES cells using the RNeasy mini kit (Qiagen). For ICM, RNA was extracted with the RNAqueous-Micro kit and amplified with the MessageAmp II aRNA amplification kit (both from Ambion). cDNA was prepared with RT-Superscript-III (Invitrogen), purified with the Qiagen PCR purification kit, and labelled with Cy3 or Cy5 (Amersham) using Invitrogen Bioprime labelling kits (see Text S1 for details).

cDNA microarrays. The NIA 15K mouse cDNA library [14,50] was purchased through the UK Medical Research Council and printed in duplicate onto glass slides by the Genomics and Proteomics Laboratory, University of Birmingham (http://www.genomics.bham.ac.uk) using an Advalytix Automated Hybridization Station. The library contains 15,247 cDNA clones with an average insert size of 1.5 kb. The ES cell data presented here are derived from 252 X-linked clones (corresponding to 180 named genes) and 6,945 autosomal clones (corresponding to 5,085 named genes) that consistently gave above-background signals with ES cell cDNAs. cDNAs from female and male ES cells at the same stage of differentiation were labelled with Cy3 and Cy5, and equal amounts (80–120 pmol) were co-

Figure S1. Expression of Xist/5 and Xist/3b during ES Cell Differentiation

(A) Microarray-derived expression of the Xist/5 and Xist/3b genes in male (CCER) and female (PGK12.1) ES cells, as indicated, at different days of differentiation.

(B) Microarray-derived expression of the Nanog, Pou5f1, and Zfp42 genes in female (PGK12.1) ES cells at different days of differentiation. (C) Microarray-derived expression of the Xist gene in female (PGK12.1) ES cells at different days of differentiation. Expression is relative to autosomal genes (log2 X:A ratio).

Found at doi:10.1371/journal.pbio.0050326.sg001 (38 KB PPT).

Figure S2. Distribution of Expression Levels of X-Linked and Autosomal Genes in Male and Female ES Cells

X-linked gene expression in undifferentiated and differentiated ES cells shows a distribution skewed towards higher expression levels. In undifferentiated cells only, the median expression is higher in females than in males. Autosomal genes show a similar distribution
and skewing, but with no distinction between males and females, or change with differentiation; the distributions for genes on Chromosomes 2 and 10 are shown as examples.

Found at doi:10.1371/journal.pbio.0050326.sg002 (45 KB PPT).

**Figure S3.** Expression of Genes on Individual Autosomes by Microarray Analysis

Expression levels of genes on individual Chromosomes 1–10, relative to expression from all autosomes (nA ratio), in female (red) and male (blue) ES cells through differentiation are shown. None of the autosomes show any consistent difference between males and females or any change with differentiation, with the exception of Chromosome 11, whose genes are consistently more strongly expressed in males, and Chromosome 12, whose genes are consistently more strongly expressed in females. However, these expression differences are small compared to those shown by X-linked genes.

Found at doi:10.1371/journal.pbio.0050326.sg003 (60 KB PPT).

**Figure S4.** Fold Change in Expression of Some X-Linked Genes During Female ES Cell Differentiation

(A) Array-derived expression ratios of *Muaal*, *Pepsi1*, *Svr4*, and *Snc11* at different days of differentiation in ES cell. Data for each gene was normalised to day 0.

(B) Real-time PCR validation of array-derived expression ratio. *ActB* was used as an endogenous, internal control and the data was normalised to day 0 values thereafter, as in (A).

Found at doi:10.1371/journal.pbio.0050326.sg004 (34 KB PPT).

**Figure S5.** Correlation between Genes Inactivated in PGK12.1 and 3F1 ES Cells after 7 d of Differentiation

The graph shows how levels of expression of individual X-linked genes change after 7 d of differentiation in two female ES cell lines: PGK12.1 (x-axis) and 3F1 (y-axis). For each cell line, cDNAs from undifferentiated and differentiated cells were co-hybridised to the same slide, and the difference in expression was calculated as an M value (day 7/day 0, ratio, log2 scale). Pearson product moment correlation coefficients (r value) and probabilities of chance correlation (p) are shown. These experiments were carried out with an NIA15K “half library”, and the number of data points is less than shown in other figures.

Found at doi:10.1371/journal.pbio.0050326.sg005 (34 KB PPT).

**Figure S6.** Allelic Expression of Zfp185 during Female ES Cell Differentiation

(A) Two forward primers (Table S4) hybridize selectively to the SNP allele by day 7 of 3F1 differentiation.

(B) Allele-specific analysis shows reduced expression of *Zfp185* from the 129 allele by day 7 of 3F1 differentiation.

Found at doi:10.1371/journal.pbio.0050326.sg006 (63 KB PPT).

**Table S1.** Clones and Corresponding Genes in Cluster 4

| Chromosome location from the online data provided for the NIA15K array (http://www.nia.nih.gov) |
|-----------------------------------------------|
| Found at doi:10.1371/journal.pbio.0050326.st001 (37 KB DOC). |

**Table S2.** Gene Distribution along the X Chromosome

(A) Distribution along the X chromosome of clones in clusters 1–4.

(B) Distribution along the X chromosome of genes in clusters 1–4.

(C) Distribution along the X chromosome of clusters 1–4 as a proportion of genes in each cluster.

Found at doi:10.1371/journal.pbio.0050326.sg002 (52 KB DOC).

**Table S3.** Relationship between the Proximity of Genes within a Gene Pair and Their Presence within the Same or Different Clusters

For the purposes of statistical analysis, pairs were counted as one if they were within the same cluster and zero if they were in different clusters; see also Figure S7.

Found at doi:10.1371/journal.pbio.0050326.sg003 (46 KB DOC).

**Table S4.** Primer Pairs Used for Real Time PCR Validation of Microarray Data

Found at doi:10.1371/journal.pbio.0050326.sg004 (35 KB DOC).

**Table S5.** Primers and Restriction Enzymes Used for Allele-Specific Analysis of Selected X-Linked Genes

Found at doi:10.1371/journal.pbio.0050326.sg005 (42 KB DOC).

**Text S1.** Details of Statistical Procedures Used for Analysis of Microarray Data and Technical Procedures Used for cDNA Preparation, PCR, and SNP analysis

Found at doi:10.1371/journal.pbio.0050326.sg006 (46 KB DOC).

**Accession Numbers**

The National Center for Biotechnology Information (NCBI) unigene cluster IDs (http://www.ncbi.nlm.nih.gov) for the genes mentioned in the text are as follows: Acsd4 (Mm.391357), Brodl (Mm.100112), Gm784 (Mm.298000), Jarid1e (Mm.142655), Nanog (Mm.440503), Ogt (Mm.259191), Petk1 (Mm.102574), Pgr151 (Mm.356164), Phks2 (Mm.350712), Pou5f1(osk+4) (Mm.17051), Sry (Mm.377114), Tisx (Mm.435573), Xist (Mm.435573), Xbro (Mm.336117), Xir5 (Mm.435653), Xbr5c (Mm.255790), Xbr5d (Mm.435653), Zfp421(Ex1) (Mm.285848), and Zfp185 (Mm.1161).

**Acknowledgments**

We thank Neil Brockdorff for generously providing ES cell lines and FISH probes, Graham Anderson for the CCE/R cell line, and Brian Charlesworth for comments on the manuscript.

**Author contributions.** BMT, HL, LPO conceived and designed the experiments. HL, VG, and MDVM performed the experiments. HL, VG, and FF analyzed the data. JTL contributed reagents/materials/analysis tools. BMT, HL, LPO wrote the paper.

**Funding.** This work was supported by the Medical Research Council (MRC), Biotechnology and Biological Sciences Research Council (BBSRC), Cancer Research UK, and the National Institutes of Health (ROI-GM58839, JTL). LPO is a Royal Society Research Fellow.

**Competing interests.** The authors have declared that no competing interests exist.

6. Heard E, Clerc P, Avner P (1997) X-chromosome inactivation in mammals. Annu Rev Genet 31: 571–610.

7. Lyon MF (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature 190: 372–373.

8. Charlesworth B (1978) Model for evolution of Y chromosomes and dosage compensation. Proc Natl Acad Sci U S A 75: 5618–5622.

9. Adler DA, Rugarli EI, Lingenfelter PA, Tsuchiya K, Poslinski D, et al. (1997) Evidence of evolutionary up-regulation of the single active X chromosome in mammals based on CFe expression levels in *Mus spretus* and *Mus musculus*. Proc Natl Acad Sci U S A 94: 9244–9248.

10. Gupta V, Parisi M, Sturgill D, Nottall R, Doctolero M, et al. (2006) Global analysis of X-chromosome dosage compensation. J Biol 5: 3.

11. Nguyen DK, Distech CM (2006) Dosage compensation of the active X chromosome in mammals. Nat Genet 38: 47–53.

12. Talebizadeh Z, Simon SD, Butler MG (2006) X chromosome gene...
expression in human tissues: male and female comparisons. Genomics 88: 673–681.
14. Kargul GJ, Dudekula DB, Qian Y, Lim MK, Jaradat SA, et al. (2004) Verification and initial annotation of the NIA mouse 15K cDNA clone set. Nat Genet 28: 17–18.
15. Sharova LV, Sharov AA, Piao Y, Shaik N, Sullivan T, et al. (2007) Global gene expression profiling reveals similarities and differences among mouse pluripotent stem cells of different origins and strains. Dev Biol 307: 446–459.
16. Raefski AS, O’Neill MJ (2005) Identification of a cluster of X-linked imprinted genes in mice. Nat Genet 37: 620–624.
17. Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 303: 644–649.
18. Smyth GK, Speed T (2003) Normalization of cDNA microarray data. Methods 31: 263–273.
19. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30: e15.
20. Chaumeil J, Le Baccon P, Wutz A, Heard E (2006) A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced. Genes Dev 20: 2223–2237.
21. Lee Y, Tsai J, Sunkara S, Karamycheva S, Pertea G, et al. (2005) The TIGR Gene Indices: clustering and assembling EST and known genes and integration with eukaryotic genomes. Nucleic Acids Res 33: D71–74.
22. Sun BK, Deaton AM, Lee JT (2006) A transient heterochromatic state in Xpreempts X inactivation choice without RNA stabilization. Mol Cell 21: 617–628.
23. Al-Shahrour F, Minguez P, Tarraga J, Medina I, Alloza E, et al. (2007) Fatigo ++: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. Nucleic Acids Res. 35 (Web Server issue): W91-W96.
24. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, et al. (2004) Integration with eukaryotic genomes. Nucleic Acids Res 33: D71–74.
25. Lee JT, Lu N (1999) Targeted mutagenesis of Tsix leads to nonrandom X inactivation. Cell 99: 47–57.
26. Mlynarczyk-Evans S, Royce-Tolland M, Alexander AA, Kalantry S, et al. (2006) X chromosomes alternate between two states prior to random X-inactivation. PLoS Biol 4: e159. doi:10.1371/journal.pbio.0040159.
27. O’Neill LP, Randall TE, Lavender J, Spotswood HT, Lee JT, et al. (2003) X-linked genes in female embryonic stem cells carry an epigenetic mark prior to the onset of X inactivation. Hum Mol Genet 12: 1783–1790.
28. Straub T, Becker PB (2007) Dosage compensation: the beginning and end of generalization. Nat Rev Genet 8: 47–57.
29. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, et al. (2004) Integration with eukaryotic genomes. Nucleic Acids Res 33: D71–74.
30. Marini I, Siegal ML, Baker BS (2000) The evolution of dosage-compensation mechanisms. Bioessays 22: 1106–1114.
31. Potocnik AJ, Nielsen PJ, Eichmann K (1994) In vitro generation of Xist RNA exhibits a banded localization on the inactive X chromosome. J Cell Biol 127: 217–227.
32. Omura S, Osawa T, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
33. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
34. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
35. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
36. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
37. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
38. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
39. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
40. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
41. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
42. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
43. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
44. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
45. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
46. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
47. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
48. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
49. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.
50. Osawa T, Omura S, Takahashi A, et al. (2007) Dynamic networks of X-inactivation associated with development of mouse embryos. Genes Dev 21: 2922–2933.