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Hematopoietic Precursor Cells Transiently Reestablish Permissiveness for X Inactivation†

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***Xist* is the trigger for X inactivation in female mammals. The long noncoding *Xist* RNA localizes along one of the two female X chromosomes and initiates chromosome-wide silencing in the early embryo. In differentiated cells, *Xist* becomes dispensable for the maintenance of the inactive X, and its function for initiation of silencing is lost. How *Xist* mediates gene repression remains an open question. Here, we use an inducible *Xist* allele in adult mice to identify cells in which *Xist* can cause chromosome-wide silencing. We show that *Xist* has the ability to initiate silencing in immature hematopoietic precursor cells. In contrast, hematopoietic stem cells and mature blood cells are unable to initiate ectopic X inactivation. This indicates that pathways critical for silencing are transiently activated in hematopoietic differentiation. *Xist*-responsive cell types in normal female mice show a change of chromatin marks on the inactive X. However, dosage compensation is maintained throughout hematopoiesis. Therefore, *Xist* can initiate silencing in precursors with concomitant maintenance of dosage compensation. This suggests that *Xist* function is restricted in development by the limited activity of epigenetic pathways rather than by a change in the responsiveness of chromatin between embryonic and differentiated cell types.**

Mammals achieve dosage compensation by inactivation of one of the two X chromosomes in female cells during early embryogenesis. In the mouse, the paternally inherited X chromosome is inactivated in the cells of preimplantation embryos, giving rise to an imprinted pattern of X inactivation in extraembryonic tissues (41). Concomitant with the establishment of pluripotency in the inner cell mass of the blastocyst, the inactive X chromosome (Xi) is reactivated in cells contributing to the embryo proper (33, 36). Subsequently, the cells of the epiblast possess two active X chromosomes and are not dosage compensated between days 3.5 and 5.5 postcoitum (dpc). During gastrulation, one of the first epigenetic reprogramming events is the establishment of X inactivation to achieve dosage compensation by 6.5 dpc in all cells of the embryo (30, 43). The choice of the X chromosome for inactivation is random, resulting in a mosaic pattern of cells transcribing genes of either X. Female embryonic stem (ES) cells resemble epiblast cells in having two transcriptionally active X chromosomes (37, 42). During the differentiation of ES cells, the process of random X inactivation is recapitulated (21). X inactivation is a multistep process involving an ordered series of chromosomal modifications, which include the specific methylation and ubiquitination of histones, DNA methylation, and the recruitment of Polycomb group proteins (5, 14, 15).

Xist is the trigger for the initiation of chromosome-wide silencing and is required for X inactivation in early embryos (4, 6–8, 34, 37). The mechanism of establishing transcriptional silencing is presently not well understood. The *Xist* gene encodes a long nontranslated RNA that physically associates with the Xi (12). Silencing requires a conserved repeat sequence located at the 5′ end of *Xist*. Deletion of this element results in *Xist* RNA that associates with chromatin and spreads over the chromosome but does not affect transcriptional repression (47). This suggests a function of the 5′ end of *Xist* in binding putative silencing factors. However, it is clear that *Xist* alone is not sufficient for initiation of silencing. Formation of an Xi is dependent on the stage of the cell in development or differentiation. It has been shown that *XIST* cannot establish silent chromatin in differentiated human cells either by employing chromosomal translocations involving the inactive X chromosome (40) or by inducing *XIST* expression from the active X chromosome by DNA demethylation (11, 44). In differentiated cells, the X-inactivation center is not required for maintenance of X chromosome inactivation, and *Xist* RNA, DNA methylation, and histone hypoacetylation cooperate in maintaining the Xi (9, 13). We have previously used an inducible *Xist* expression system in male ES cells to analyze the function of *Xist* in initiating gene silencing during differentiation (46). Induction of *Xist* expression from transgenes integrated on the X chromosome or autosomes led to chromosome-wide silencing and histone H3 lysine 27 trimethylation (H3K27me3) in undifferentiated ES cells (28, 47). *Xist* induction in cells that had differentiated for 2 days or more did not trigger gene repression showing that silencing of the X chromosome depends on a particular cellular context, which subsists in ES cells only for two cell divisions following the onset of differentiation (46). It is not known what restricts *Xist* function in differentiation. One explanation might be differences in chromatin composition and function between embryonic cells and differentiated cells. Chromatin in differentiated cells might have undergone irreversible changes and modifications which impede the establishment of silent chromatin in response to *Xist* expression.

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This view is supported by the observation that the initiation of X inactivation in ES cell differentiation has been linked to a time window when chromosomal silencing is reversible. Alternatively, *Xist* might require factors whose activity is only present in early embryonic cells.

X chromosome inactivation has been assumed to be complete in all cells of female mouse embryos at about 6.5 dpc, and no transcriptional activity from the Xi was found in embryos at 9.5 dpc (30). However, reactivation of the Xi has been observed in primordial germ cells in the female germ line (43). The human Xi displays changes in chromatin marks and function in certain types of cancer. A relationship between *XIST* expression and responses of ovarian cancer to chemotherapy has been reported (24). *XIST* is also expressed in testicular germ cell tumors with multiple inactive X chromosomes, and evidence for initiation of X inactivation in tumor cells has been reported (26, 27, 32). The loss of a normal Xi including the absence of proper *XIST* RNA localization has been observed in breast cancer cells lacking wild-type *BRCA1* (16). Finally, an ectopic human *XIST* transgene has been found to induce chromosome inactivation in human HT-1080 fibrosarcoma cells (19). These observations suggest that cells providing an appropriate context for initiation of silencing by *Xist* could be present in adult mammals. Here, we used an inducible system for ectopic *Xist* expression to identify *Xist*-responsive cells during embryogenesis and in the adult mouse. We observe that silencing of the X chromosome by *Xist* can be initiated in adult mice specifically in immature hematopoietic cells. Deregulated *Xist* expression ultimately leads to lethality due to hematopoietic failure.

MATERIALS AND METHODS

***Xist* induction in mice.** Mice carrying a tetracycline-inducible *Xist* allele (TX) were generated by blastocyst injection of correctly targeted ES cells (47) and were subsequently crossed with $R26^{rtTA/rtTA}$ mice (46). The TX and $R26^{rtTA}$ alleles (46, 47) were genotyped, and the sex of the embryos was determined as previously described (29). The *Xist* gene was induced in TX/Y $R26^{rtTA/rtTA}$ embryos and adult mice by adding doxycycline to the drinking water (1 g of doxycycline [Sigma] and 100 g of sucrose [Fluka] per liter). Water bottles were protected from light and changed every second day. The blood of anesthetized mice was collected by cardiac puncture into heparin-containing vials, and the blood parameters including hematocrit and thrombocytes were determined by a veterinary laboratory (Firma In Vitro, Vienna, Austria).

Fluorescence-activated cell sorter (FACS) analysis and cell sorting. The following fluorescein isothiocyanate-, phycoerythrin (PE)-, CyChrome-, or allophycocyanin-coupled antibodies were used for flow cytometry: anti-B220 (RA3-6B2), CD3 (145-2C11), CD4 (L3T4), CD8a (53-6.7), CD11b/Mac1 (M1/70), CD19 (1D3), CD25/interleukin-2Rα (PC61), CD117/c-Kit (2B8), Gr1 (RB6-8C5), immunoglobulin D (IgD; 1.19), IgM (M41.42), Ly5.1 (A20), Ly5.2 (104), macrophage colony-stimulating factor receptor (AFS98), NK1.1 (PK136), Sca1/Ly6A (D7), T-cell receptor β (TCRβ; H57-597), Ter119 (TER119), and Thy1.2/CD90 (53-2.1) antibodies. Nonspecific antibody binding was suppressed by preincubation with CD16/CD32 Fc-block solution (PharMingen). Stained single-cell suspensions were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson) by using a wide forward scatter/side scatter gate, which included all hematopoietic cells except for small erythrocytes. For isolating Lin^- $Sca1^{high}$ $c\text{-}Kit^{high}$ (LSK) cells, the bone marrow was stained with PE-coupled lineage marker antibodies (B220, CD3ε, CD4, CD8, NK1.1, Gr1, Mac1, and Ter119), and Lin^+ cells were eliminated by magnetic cell sorting (MACS) with anti-PE beads (Miltenyi Biotec). Following staining with fluorescein isothiocyanate–anti-Sca1 and allophycocyanin–anti-cKit antibodies, LSK progenitors were sorted with a FACS Aria cell sorter (Becton-Dickinson). Lymphoid progenitors (LPs) were isolated as $C19^-$ $B220^+$ $c\text{-}Kit^+$ cells from the bone marrow.

Competitive bone marrow transplantation. The TX and $R26^{rtTA}$ alleles were crossed for four generations into the C57BL/6 background, before bone marrow transfer experiments were performed as previously described (39). The bone marrow of TX/Y $R26^{rtTA/rtTA}$ $Ly5.2^+$ mice, which were treated with doxycycline for 6 weeks, was mixed at a ratio of 1:10, 1:1, or 10:1 with the bone marrow of wild-type $Ly5.1^+$ C57BL/6 mice prior to injection of 2×10^6 cells into the tail veins of $Ly5.1^+$ C57BL/6 mice 24 h after lethal γ-irradiation (12 Gy). Chimeric mice were analyzed 6 months after transplantation.

Cell culture and RNA analysis. ES cells were cultured as previously described (28). ES cells were established from TX/Y $R26^{rtTA/rtTA}$ blastocysts in Dulbecco's modified Eagle's medium (Biochrome) supplemented with 15% fetal calf serum (Euroclone), 250 U of leukemia inhibitory factor/ml and 50 μM PD98059 (Cell Signaling Technology). $B220^+$ bone marrow cells were isolated by MACS, and pro-B cells were cultured on γ-irradiated ST2 cells in interleukin-7 containing Iscove's modified Dulbecco's medium as previously described (35). $c\text{-}Kit^+$ pro-B cells were isolated by MACS sorting prior to RNA isolation. Allele-specific reverse transcription-PCR (RT-PCR) was performed as previously described (25) using a Superscript One-Step RT-PCR Platinum *Taq* kit (Invitrogen). Northern blot analysis was performed as previously described (46). RNA fluorescence in situ hybridization (FISH) using a directly Cy3-labeled *Xist* cDNA as a probe and immunofluorescence staining was performed as described previously (28). DAPI (4′,6′-diamidino-2-phenylindole) was used to stain the DNA. Magnification of cells varied in order to allow better visibility of *Xist* and H3K27me3 foci.

Methylation analysis. Methylation-specific Southern blotting of the *Xist* promoter was performed by a HpaII/EcoRI digest of genomic DNA and detection of the fragments was performed with an XB1K probe (46). Southern blotting for promoter methylation at the *Mecp2* locus was performed as described previously (50).

RESULTS

Induction of ectopic X inactivation in embryos. We have generated a tetracycline-inducible *Xist* allele, named TX, by inserting a *tet* operator upstream of the *Xist* transcription initiation site in ES cells (Fig. 1A and B) (47). TX/Y mice were obtained and crossed with $R26^{rtTA/rtTA}$ mice expressing the tetracycline-responsive transactivator nls-rtTA (18) from the *ROSA26* locus (46, 49). Hemizygous TX/Y $R26^{rtTA/rtTA}$ males and homozygous TX/TX $R26^{rtTA/rtTA}$ females were born at the expected Mendelian ratio (see Fig. S1A in the supplemental material) and appeared healthy and fertile, demonstrating that the inducible promoter in the absence of doxycycline did not interfere with *Xist* regulation. The inducible TX allele was functional, as a male ES cell line established from TX/Y $R26^{rtTA/rtTA}$ blastocysts showed silencing of X-linked genes and decreased cell viability after 4 days of doxycycline treatment (see Fig. S1B and C in the supplemental material). *Xist* expression was detected by RNA-FISH in TX/Y $R26^{rtTA/rtTA}$ embryos that were exposed, via the drinking water of their mother, to doxycycline for 2 days starting at 9.5 or 13.5 dpc in 90% and 80% of the cells, respectively, and resulted in focal H3K27me3 staining (see Fig. S1 in the supplemental material) (28).

To investigate the ability of *Xist* to initiate gene silencing, we studied the expression of X-linked genes by Northern analysis of TX/Y $R26^{rtTA/rtTA}$ embryos that were treated with doxycycline for 4 days (Fig. 1C). Induction of *Xist* before 9.5 dpc led to the destruction of the embryo within 3 days, precluding a molecular analysis. Induction at 9.5 dpc resulted in clear repression of the X-linked *Hprt* and *Pgk1* genes in 13.5 dpc embryos. This repression became progressively less pronounced in embryos induced at later time points between 9.5 and 12.5 dpc, indicating that the cells of midgestation embryos gradually lose the potential to initiate X inactivation. The conclusion that *Xist*-responsive cells persist in the embryo until 12.5 dpc is furthermore supported by the observation that *Xist* induction at 9.5 dpc resulted in severely malformed embryos at 13.5 dpc (Fig.

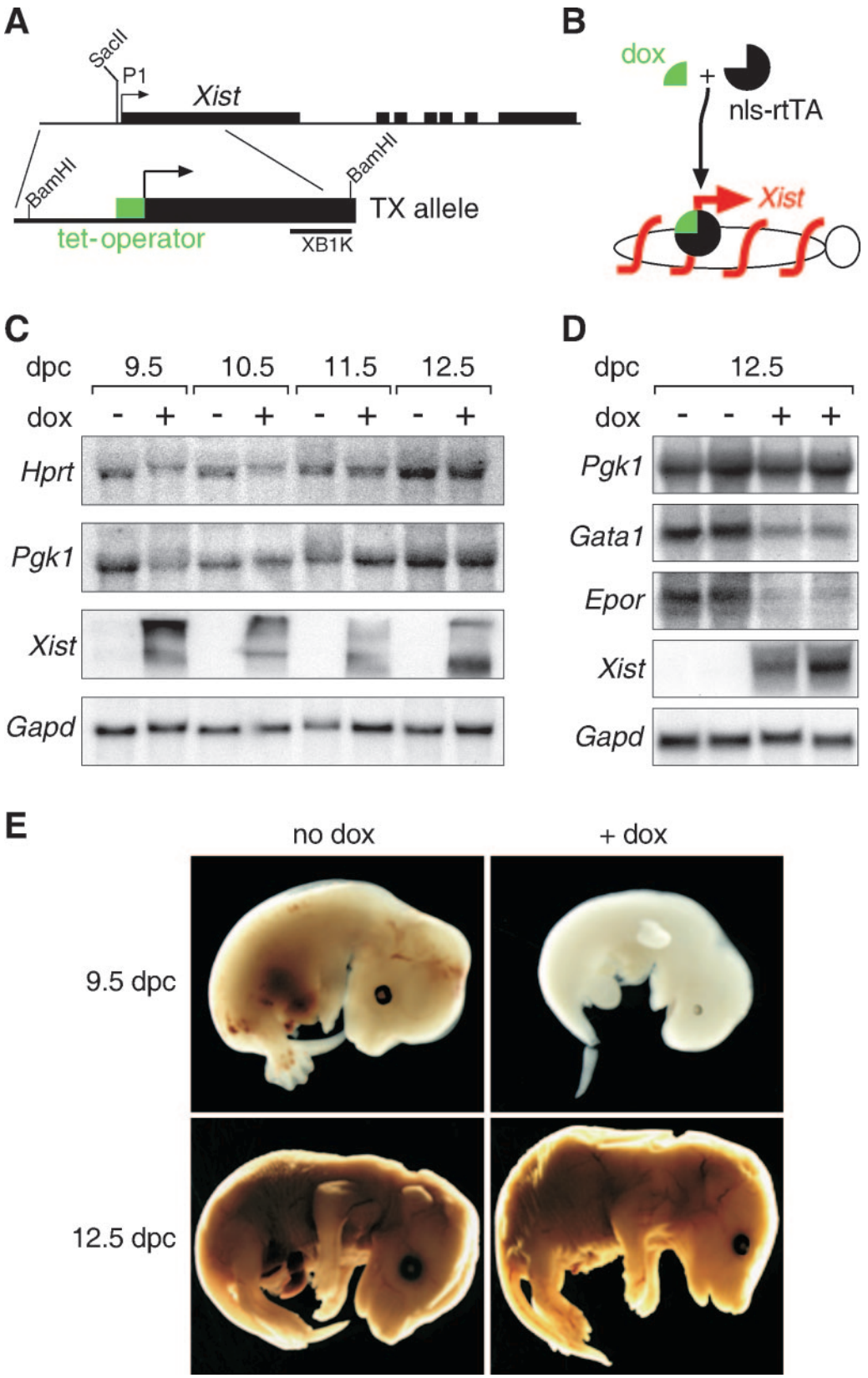

FIG. 1. Ectopic X inactivation upon *Xist* induction in the embryo. (A) Generation of the inducible TX allele. The *tet* operator sequence (green) was introduced into the SacII site upstream of the P1 promoter of *Xist*. The probe XB1K is indicated. (B) Doxycycline (dox)-induced DNA binding of the transactivator protein nls-rtTA results in *Xist* expression. (C and D) Northern blot analysis of X-linked (C) and erythroid cell-specific (D) gene transcripts in male TX/Y $R26^{rtTA/rtTA}$ embryos, in which *Xist* was induced with doxycycline (+) for 4 days starting at the indicated time point. (E) Lateral view of TX/Y $R26^{rtTA/rtTA}$ embryos (+) after *Xist* induction for 4 days after the time point shown. Untreated (−) embryos were used as control.

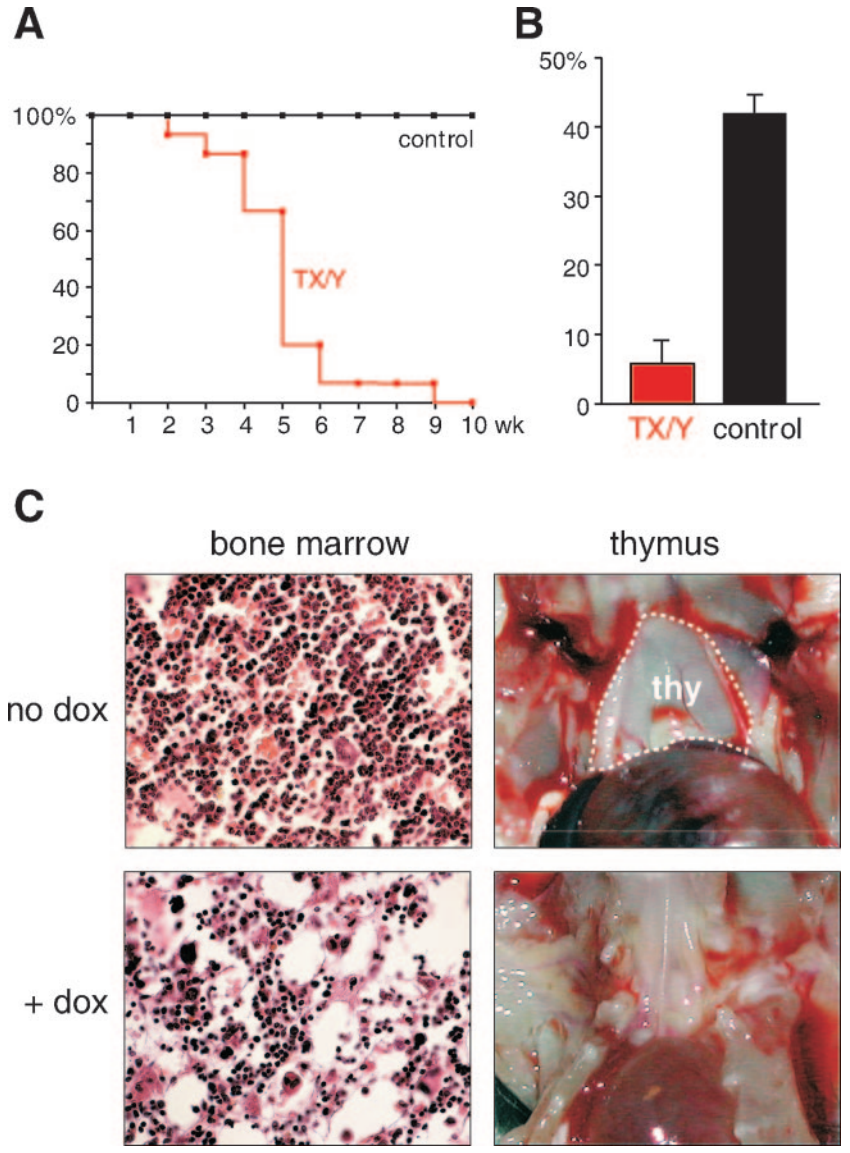

FIG. 2. *Xist* induction leads to anemia in adult mice. (A) Survival of TX/Y $R26^{rtTA/rtTA}$ and control X/Y $R26^{rtTA/rtTA}$ males ($n = 15$ each) after doxycycline treatment for 10 weeks. (B) Hematocrit levels of male TX/Y $R26^{rtTA/rtTA}$ and control X/Y $R26^{rtTA/rtTA}$ mice ($n = 15$ each) after doxycycline treatment for 3 to 6 weeks. (C) Hypocellularity of the bone marrow and absence of the thymus (thy) in a TX/Y $R26^{rtTA/rtTA}$ male after 6 weeks of doxycycline treatment (+). The thymus of an untreated (−) mouse is indicated by a dashed line.

1E). However, ectopic *Xist* expression starting at 12.5 dpc was compatible with the birth of mice that had no major anatomic defects (Fig. 1E) and could feed normally, as indicated by the presence of milk in their stomachs. These mice died within 1 day after birth, possibly due to hematopoietic failure, as the blood hematocrit was severely reduced compared to untreated newborn mice (see Fig. S1D in the supplemental material). Furthermore, the erythroid-specific X-linked *Gata1* and autosomal *Epor* genes were reduced in doxycycline-treated embryos at 16.5 dpc, indicating the loss of red blood cells (Fig. 1D). We therefore conclude that ectopic *Xist* expression during late embryogenesis selectively affects the hematopoietic system.

***Xist* induction causes lethal anemia in adult mice.** We next investigated the function of *Xist* in adult hematopoiesis, as the *ROSA26* locus is expressed in blood cells, allowing for efficient *Xist* activation in all lineages (46, 49). For this, we induced *Xist* expression in 4-week-old male TX/Y $R26^{rtTA/rtTA}$ or female TX/TX $R26^{rtTA/rtTA}$ mice by continuous addition of doxycycline to the drinking water. As early as 2 weeks after *Xist* induction, these mice became weak, and most of them died between 5 and 6 weeks, with no mouse surviving 10 weeks (Fig. 2A). Mice at the first appearance of disease symptoms had a hematocrit of one-tenth of controls (Fig. 2B), suggesting a defect in hematopoiesis. Upon histological examination, the bone marrow was hypocellular, and the thymus was severely reduced or absent at 6 weeks of doxycycline treatment (Fig. 2C), showing that ectopic *Xist* expression affects multiple hematopoietic lineages in adult mice. Importantly, doxycycline had no effect on control X/Y $R26^{rtTA/rtTA}$ males, heterozygous TX/X $R26^{rtTA/rtTA}$ females, or

TX-carrying mice lacking the $R26^{rtTA}$ allele. This shows that deregulated *Xist* expression in adult mice results in lethality due to hematopoietic failure and anemia.

Immature hematopoietic progenitors support *Xist*-mediated silencing. We next performed FACS analysis to study the cellular composition of the bone marrow (20, 38) of TX/Y $R26^{rtTA/rtTA}$ males after 5 and 7 days of *Xist* induction. B lymphocyte development is initiated in pro-B cells and proceeds via pre-B and immature B cells to the mature B-cell stage (10). Pro-B cells ($CD19^+$ c-Kit^+) were reduced twofold by *Xist* induction, consistent with a similar reduction of the bone marrow cellularity (Fig. 3A and G) and with the fact that the proliferation of in vitro cultured TX/Y $R26^{rtTA/rtTA}$ pro-B cells was minimally affected by the presence of doxycycline (see Fig. S2D in the supplemental material). In contrast, the pre-B cells ($CD19^+$ $CD25^+$ IgM^-) were largely eradicated (Fig. 3B and G), and the immature B cells ($B220^+$ IgM^+ IgD^-) were severely reduced after 5 days of *Xist* induction (Fig. 3C and G). The mature B cells ($B220^+$ IgD^+ IgM^+) in the bone marrow (Fig. 3C and G) and spleen (see Fig. S2B and C in the supplemental material) were, however, mainly resistant to *Xist*-mediated killing. Within the erythro-myeloid lineages, the macrophages ($Gr1^{int}$ $Mac1^+$) were most rapidly eliminated, and the cell counts of granulocytes ($Gr1^{hi}$ $Mac1^+$) declined significantly but more moderately (Fig. 3D and G; see also Fig. S2A in the supplemental material), whereas $Ter119^+$ erythroid cells even increased after 7 days of ectopic *Xist* expression (Fig. 3E and G). This expansion of erythroid cells was temporary and specific to more mature cell types as early erythroid progenitors were significantly reduced by 7 days of *Xist* induction (see Fig. S5C and D in the supplemental material). Consistent with this, we found that the erythroid system collapsed after 4 weeks of *Xist* induction, when animals became anemic.

In the thymus, all T-cell subsets rapidly declined upon *Xist* induction (Fig. 3F and H), consistent with a strong reduction of the total cellularity (see Fig. S2C in the supplemental material). The double-positive (DP) pre-T cells ($CD4^+$ $CD8^+$) were the most rapidly eliminated compared to the double-negative (DN) pro-T cells ($Thy1.2^+$ $CD4^-$ $CD8^-$) and the more mature $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes. As an important control, heterozygous TX/X $R26^{rtTA/rtTA}$ females did not reveal any hematopoietic abnormalities after 4 weeks of doxycycline treatment (data not shown). These data therefore indicate that ectopic *Xist* expression severely affects the development of multiple hematopoietic lineages in TX/Y males.

To demonstrate that *Xist*-induced gene silencing and ectopic X inactivation were, indeed, initiated in hematopoietic cells, we analyzed the expression of X-linked genes in cultured TX/Y $R26^{rtTA/rtTA}$ pro-B cells as well as in ex vivo sorted pre-B and mature B cells of TX/Y $R26^{rtTA/rtTA}$ males before and after 4 days of doxycycline treatment. Upon doxycycline treatment, the *Pgk1* and *Hprt* genes were repressed in pre-B cells in contrast to pro-B and mature B cells (Fig. 3I). Moreover, focal H3K27me3 staining was observed in a significant number (>10%) of the doxycycline-treated pre-B cells but was undetectable in pro-B and mature B cells, although *Xist* induction was equally efficient (>80%) in all three cell types (Fig. 3K; also data not shown). Ectopic *Xist* expression also initiated silencing of the *Hprt* and *Pgk1* genes in DP pre-T cells and $CD4^+$ SP thymocytes of TX/Y $R26^{rtTA/rtTA}$ males (see Fig. S2E in the supplemental material). Hence, we conclude that *Xist* induction leads to gene silencing and subsequent cell loss in lineage-committed immature cells of the hematopoietic system.

The context for initiation of *Xist*-mediated silencing is established after stem cell differentiation. To our surprise, the population of LSK (Lin^- $Sca1^{high}$ c-Kit^{high}) progenitors, which includes hematopoietic stem cells (HSCs), was not reduced in the bone marrow of TX/Y $R26^{rtTA/rtTA}$ males but, instead, started to increase in absolute cell numbers after 2 weeks of doxycycline treatment to reach a 20-fold expansion after 4 to 6 weeks in anemic mice (Fig. 4A and C). Similarly, uncommitted LPs ($CD19^-$ $B220^+$ c-Kit^+; pre-pro-B cells) (2, 20, 38) were also 10-fold expanded during the same period, while the cellularity of the bone marrow was moderately reduced in doxycycline-treated versus unstimulated TX/Y $R26^{rtTA/rtTA}$ mice (Fig. 4B and C). Moreover, c-Kit^+ progenitors constituted a majority of the accumulating bone marrow cells after 6 weeks of doxycycline treatment (see Fig. S3A to D in the supplemental material). At this time point, strong *Xist* RNA signals were detected by RNA FISH in 87% of sorted LSK and 74% of LP cells, although no H3K27me3 foci were discernible (Fig. 4D; also data not shown). Finally, the X-linked *Pgk1* and *Hprt* genes were equally expressed in LSK cells of anemic or untreated TX/Y $R26^{rtTA/rtTA}$ males (Fig. 4E). Hence, ectopic *Xist* expression did not initiate X inactivation in uncommitted hematopoietic progenitors, which accumulate possibly by homeostatic expansion due to the loss of the differentiated blood cells. Cells apparently could initiate ectopic X inactivation only once expression of the stem cell marker c-Kit was down-regulated.

The potency of the expanded HSCs was next assessed by competitive bone marrow transfer experiments after crossing the TX and $R26^{rtTA}$ alleles into the C57BL/6 background. Six weeks after doxycycline treatment, the bone marrow of anemic TX/Y $R26^{rtTA/rtTA}$ males expressing the pan-hematopoietic marker Ly5.2 was mixed at a ratio of 1:10, 1:1, or 10:1 with the bone marrow of wild-type $Ly5.1^+$ C57BL/6 mice prior to injection into lethally irradiated $Ly5.1^+$ C57BL/6 recipients. Six months after the transplantation and absence of doxycycline, 76% of the bone marrow cells of a chimeric mouse receiving a 1:1 graft expressed the Ly5.2 marker of the TX/Y $R26^{rtTA/rtTA}$ donor cells (Fig. 4F). Moreover, these $Ly5.2^+$ cells constituted the majority of all hematopoietic cell types in the bone marrow and thymus except for $CD8^+$ SP T cells, which were predominantly derived from the $Ly5.1^+$ HSCs (Fig. 4F; see also Fig. S4 in the supplemental material). The analysis of bone marrow chimeras generated with different graft ratios confirmed that the HSCs were more abundant in the bone marrow of anemic mice compared to wild-type mice (see Fig. S4A in the supplemental material). Importantly, these data indicate that the HSCs of anemic mice are functional, as the *Xist*-induced effects on hematopoiesis were fully reversible. In support of this conclusion, anemic TX/Y $R26^{rtTA/rtTA}$ mice, which were doxycycline-treated for 5 weeks, recovered and showed normalized hematopoiesis 2 weeks after doxycycline withdrawal (data not shown).

Loss of markers of the Xi in immature hematopoietic cells. The observation that *Xist* can initiate gene silencing in immature hematopoietic cells predicts that an epigenetic context enabling initiation of X inactivation is established in these cells. To study if establishment of this context was character-

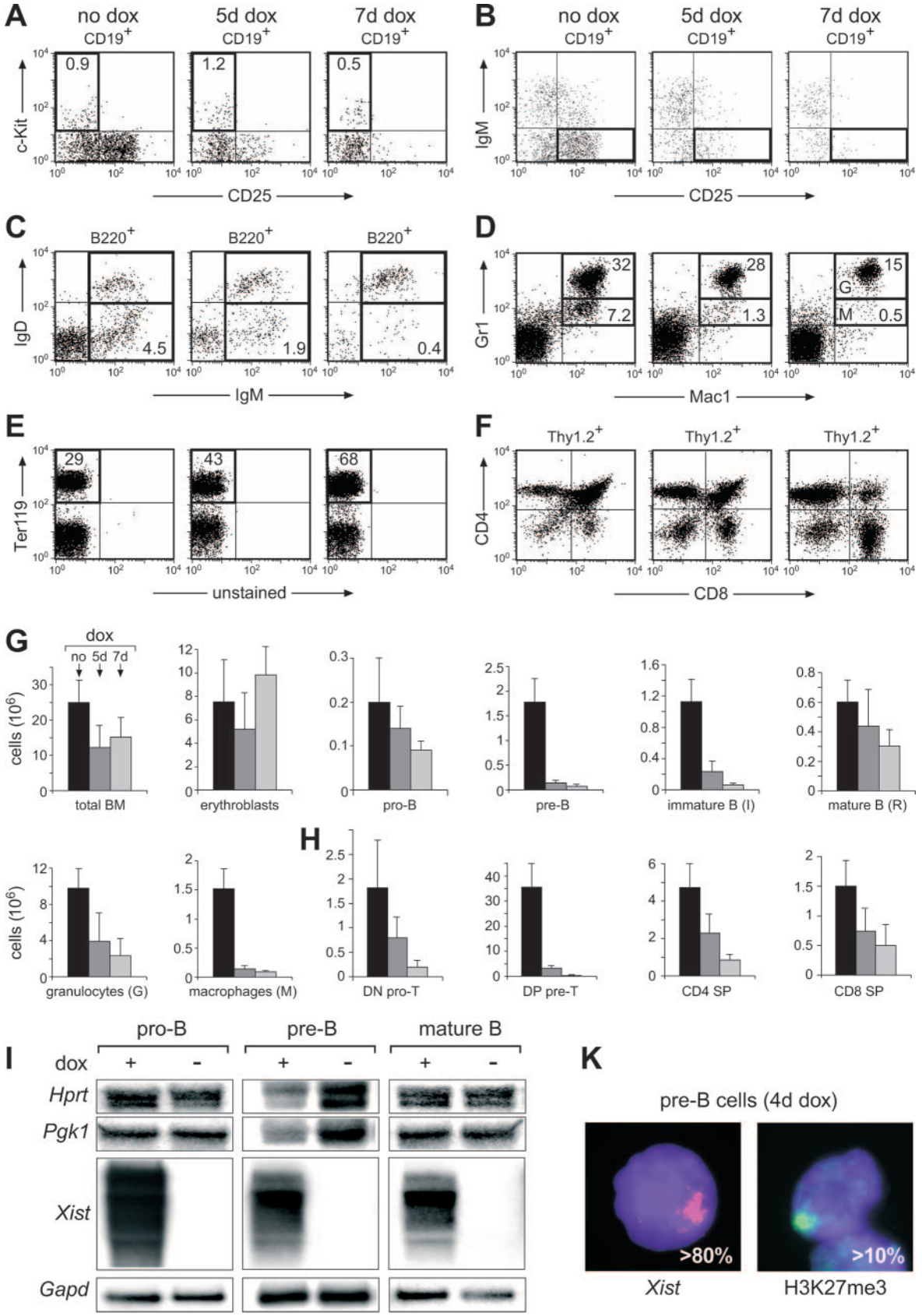

FIG. 3. Depletion of immature hematopoietic cell types by ectopic X inactivation. FACS analysis of the bone marrow (A to E) and thymus (F) of male TX/Y *R26*$^{rtTA/rtTA}$ mice without and with doxycycline treatment for 5 and 7 days (d). Pro-B cells (A), pre-B cells (B), immature and

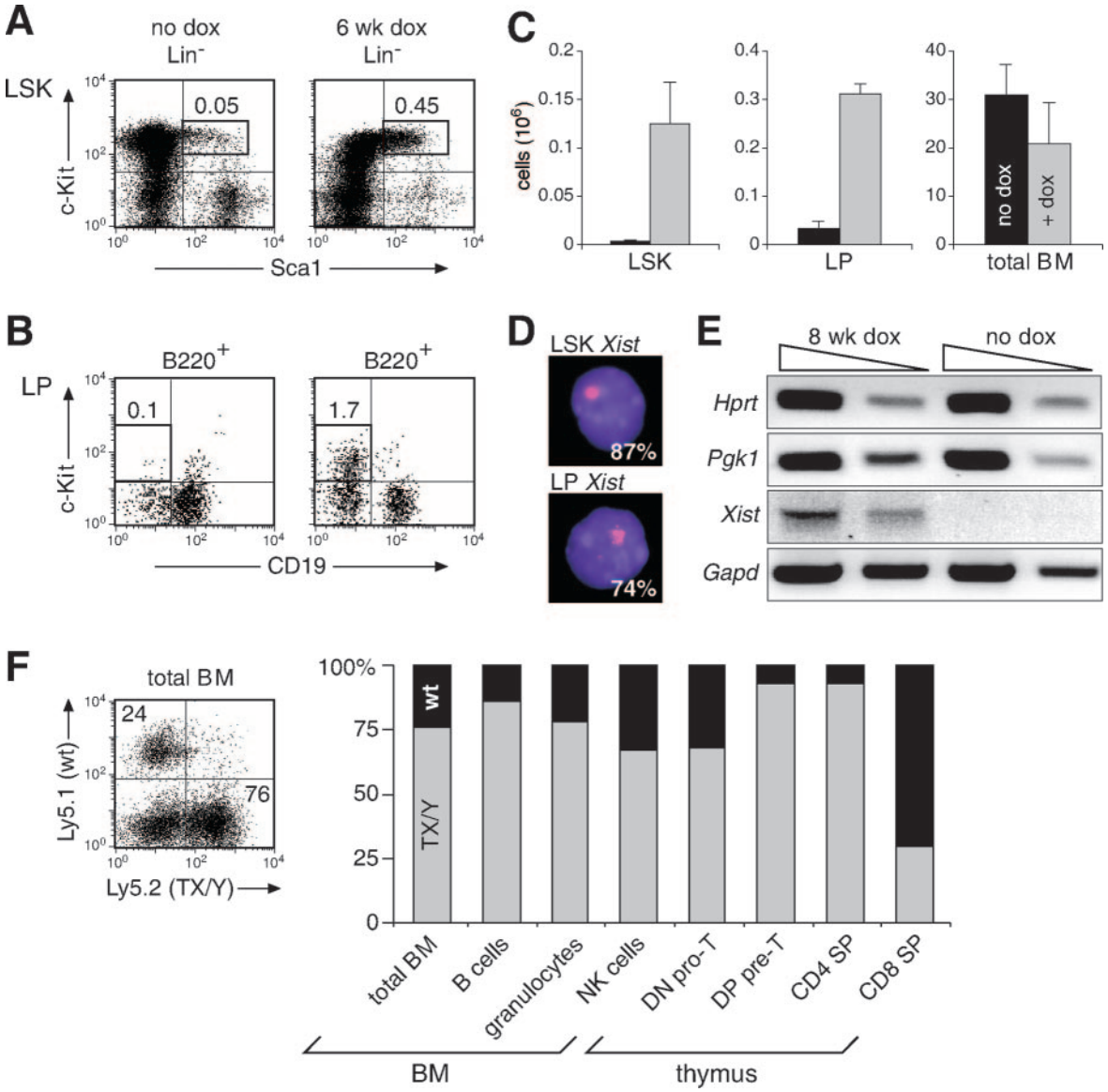

FIG. 4. Absence of ectopic X inactivation in HSCs. (A to E) Male TX/Y $R26^{rtTA/rtTA}$ mice were induced with doxycycline for 6 weeks or left untreated. FACS analysis of multipotent LSK (A) and LPs (B). The percentage of total bone marrow cells in the respective gate is indicated. (C) Absolute cell numbers (10^6) of LSK, LPs, and total bone marrow cells ($n = 5$ each). (D) *Xist* RNA FISH signals with their detection frequency are shown for FACS-sorted LSK and LPs. DAPI staining is shown in blue. (E) RT-PCR analysis of X-linked gene expression in sorted LSK cells. Tenfold cDNA dilutions were analyzed. (F) FACS analysis of Ly5.1$^+$ recipient mice 6 months after transplantation of a 1:1 mixture of bone marrow from a wild-type (wt) Ly5.1$^+$ mouse and a TX/Y $R26^{rtTA/rtTA}$ Ly5.2$^+$ mouse that was treated for 6 weeks with doxycycline. The relative contributions of wild-type (wt) Ly5.1$^+$ HSCs (black bars) and TX/Y Ly5.2$^+$ HSCs (gray bars) to the different lineages are shown. BM, bone marrow; dox, doxycycline.

ized by changes in chromatin composition and function, we characterized the Xi in sorted hematopoietic cells of wild-type female mice by *Xist* RNA FISH and H3K27me3 staining (Fig. 5A and B; see also Fig. S5A and B in the supplemental material). A clear focal *Xist* RNA cluster and one intense H3K27me3 focus were observed in LSKs and LPs. However, the Xi changed in appearance during lymphopoiesis with the loss of focal H3K27me3 staining in pro-B and DN pro-T cells.

mature recirculating B cells (C), granulocytes and macrophages (D), erythroblasts (E), and DN, DP, and SP thymocytes (F) are shown with their percentage in the respective quadrant. Five mice per time point were analyzed to determine the absolute cell number (10^6) of the indicated cell types in the bone marrow (G) and thymus (H). (I) Northern blot analysis of in vitro cultured pro-B cells and ex vivo sorted pre-B cells and splenic IgD$^+$ B cells before (−) or after (+) 4 days of doxycycline treatment. (K) *Xist* RNA FISH and H3K27me3 staining in 4-day-induced pre-B cells of a male TX/Y $R26^{rtTA/rtTA}$ mouse. The percentage of cells ($n > 100$) containing a signal is indicated. DNA is stained by DAPI (blue). BM, bone marrow; I, immature; R, recirculating; G, granulocytes; M, macrophages.

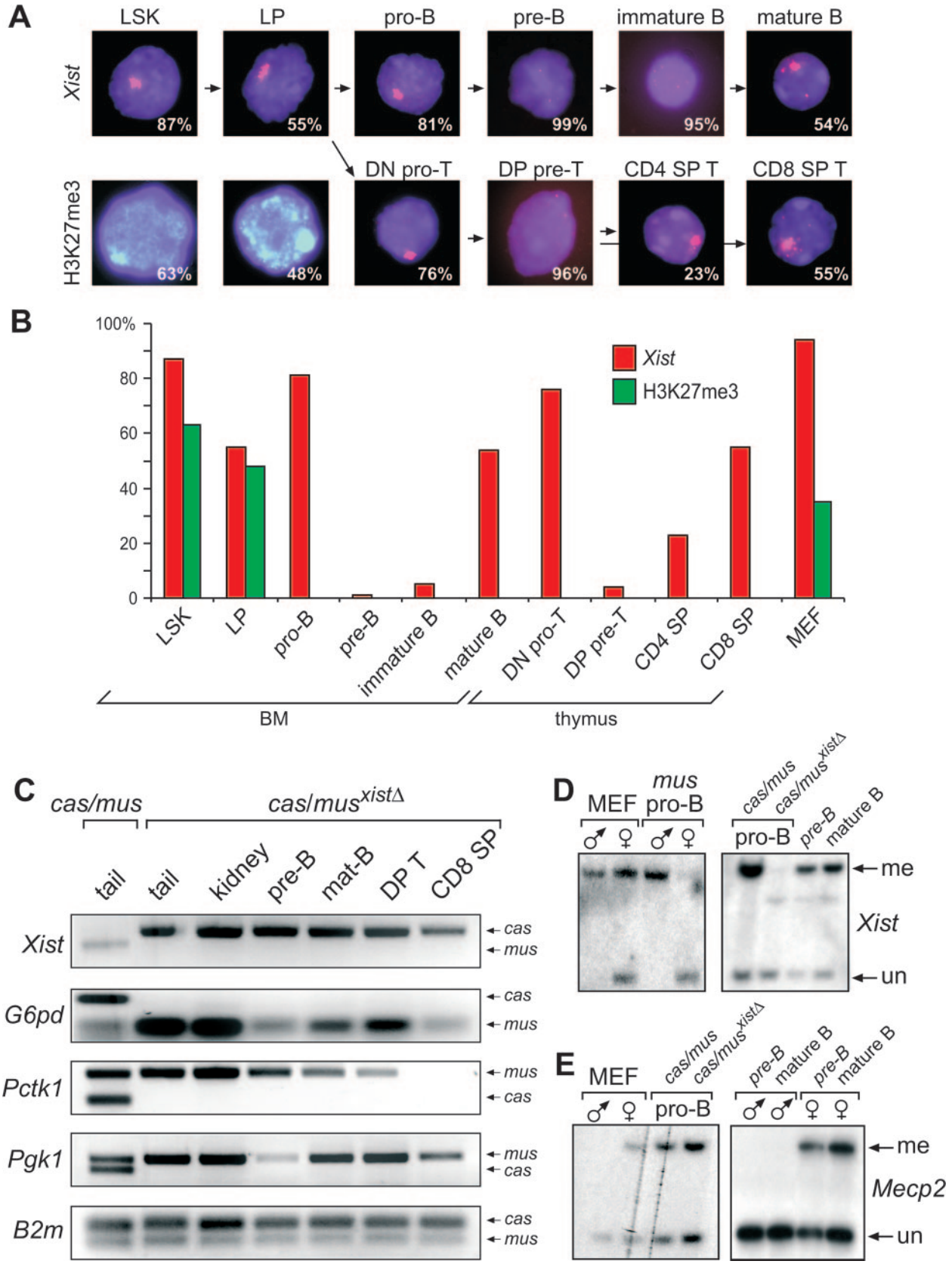

FIG. 5. Absence of markers of the Xi in immature lymphocytes of female mice. (A) *Xist* RNA FISH (red) and H3K27me3 staining (green) in hematopoietic cell types (n = 200 each) of a wild-type female mouse. The percentage of cells is indicated. DNA is stained by DAPI (blue). (B) Statistical evaluation of *Xist* and H3K27me3 signals in hematopoietic cells and mouse embryo fibroblasts. (C) X-linked *G6pd*, *Pgk1*, *Pctk1*, *Xist*,

Moreover, none of the committed cell types downstream of the LPs showed any focal H3K27me3 staining (Fig. 5B; see also Fig. S5B in the supplemental material). Although a focal *Xist* RNA cluster was detected in most pro-B and DN pro-T cells, *Xist* became undetectable in all pre-B cells and was diffusely localized in the majority of DP pre-T cells. Focal *Xist* clusters were again present in a significant fraction of mature B cells as well as $CD4^+$ and $CD8^+$ SP T cells. The absence of *Xist* staining in wild-type female pre-B cells was not attributable to a technical failure, since focal *Xist* staining was detected reproducibly in pre-B cells of TX/Y $R26^{rtTA/rtTA}$ mice after *Xist* induction (Fig. 3K). We conclude that changes in the chromatin of the Xi occur in cell types that become endowed with the potential to initiate X inactivation.

To address the possibility that genes on the Xi might be reactivated in immature lymphocytes, we made use of sequence polymorphisms that distinguish the *Mus castaneus* and *Mus musculus* genes in a genetic setting, where X inactivation is completely nonrandom. We used *M. musculus* × *M. castaneus* (*mus*/*cas*) hybrid mice carrying a deletion in the *Xist* gene on the maternal inherited *M. musculus* X chromosome to force inactivation of the paternally inherited *M. castaneus* X in all cells. Reactivation of the *M. castaneus* Xi could then be detected by RT-PCR. In this genetic setting, *M. castaneus* X-linked genes were not expressed, suggesting that reactivation of genes on the Xi does not normally occur in vivo (Fig. 5C). This finding was corroborated by the observation that DNA methylation was maintained on the promoter of the *Xist* gene on the active X and X-linked genes, such as *Mecp2*, on the inactive X chromosome in cells of the hematopoietic system (Fig. 5D and E; also data not shown). These data are consistent with earlier reports describing stable X inactivation in hematopoietic cells of the mouse and clonal patterns of X inactivation in leukemia (23). We conclude that ectopic X inactivation can be initiated in pre-B cells while dosage compensation is simultaneously maintained.

DISCUSSION

Initiation window for X inactivation in embryogenesis. Using an inducible *Xist* expression system, we have analyzed when silencing of the X chromosome can be initiated during embryogenesis and in the adult mouse. We find that the ability of *Xist* to initiate silencing is progressively lost in cells of the embryo and that *Xist* induction at 12.5 dpc does not interfere with development to term. The initiation phase of X inactivation therefore extends over a considerable time span in embryogenesis from its initiation in gastrula embryos at 6.5 dpc until 12.5 dpc. This finding is compatible with our previous report that *Xist* does not induce silencing in cultured fibroblasts established from embryos at 13.5 dpc (46). *Xist* induction in embryos before 12.5 dpc leads to developmental malformations and cell loss. Histological analysis of the phenotype suggests that differentiated cells are maintained as observed on sections (data not shown). However, the formation of facial structures, limbs, heart, and brain are severely retarded or completely arrested. We believe that this is caused by a loss of multipotent progenitors in the embryo, which results in a depletion of organ-forming cells. Previous studies in ES cell differentiation have indicated that the function of *Xist* for initiation of silencing is lost shortly after its expression is initiated. In this study, we demonstrate that *Xist*-responsive cells are present in the embryo and critically contribute to development between 3.5 dpc and 12.5 dpc.

Transient potential for initiation of X inactivation in adult hematopoiesis. We observe that the hematopoietic system contains cells that are capable of initiating silencing of the X chromosome in the adult mouse. *Xist* induction in mice led to a loss of blood cells, causing lethality due to anemia after 4 to 6 weeks. We show that cells, which can support *Xist*-mediated gene silencing, are present and critical for survival in adult mice. This demonstrates that deregulated *Xist* expression can be pathological leading to a fatal condition. All hematopoietic lineages were severely affected by *Xist* induction, with the pre-B cells, the DP pre-T cells, and the bone marrow macrophages being lost most rapidly. Notably, we find that lymphoid lineage cells before and after the pre-B- and pre-T-cell stages were either less or not at all responsive to *Xist* induction. Hence, immature precursor cells of the B- and T-cell lineages temporarily reestablish epigenetic pathways, as seen in ES cells, and thus become *Xist* responsive. The epigenetic context for initiation of X inactivation is therefore established throughout the lifetime of the mouse during normal hematopoiesis after commitment of the HSCs to differentiation and lineage choice.

In ES cells, initiation of *Xist*-mediated silencing is restricted to an early time point in differentiation, when silencing is reversible. In contrast to embryonic cells, pre-B and pre-T cells can initiate X inactivation although these cells maintain proper dosage compensation. In support of this notion, no cells with two active X chromosomes were detected in female mice beyond 9.5 dpc (30, 43). Reactivation of the X-linked *Pgk1* gene was also not observed in the blood, bone marrow, or thymus of female mice with a translocation-induced nonrandom pattern of X inactivation (42). Our results, using mice with a nonrandom pattern of X inactivation due to a deletion in the *Xist* gene, support the view that non-dosage-compensated cells are extremely rare or absent in the adult mouse. Yet we demonstrate the loss of markers of the Xi in pre-B and pre-T cells, which both have the potential to initiate X inactivation. In somatic cells, the Xi is maintained by redundant mechanisms including DNA methylation (13). Maintenance of the Xi together with the ability to initiate silencing might parallel the situation in ES cells, when X inactivation is initiated and imprinting marks are maintained. It has been shown that main-

and autosomal control *B2m* expression was analyzed by allele-specific RT-PCR in the tissues indicated from an F_1 *cas*/*mus*(*xist*Δ) female mouse, containing a maternal *M. musculus* X bearing a deletion of the *Xist* gene and a paternal *M. castaneus* X, and from a control wild-type *cas*/*mus* female. (D and E) Methylation analysis of the *Xist* (D) and *Mecp2* promoter (E) in the indicated cell types. Mouse embryo fibroblasts and pro-B cells of all indicated sexes and genotypes were cultured, while pre-B and mature B cells were sorted ex vivo. MEF, mouse embryo fibroblast.

tenance of genomic imprinting is dependent on DNA methylation (31, 45). We observe maintenance of DNA methylation on the promoters on the Xi, when other marks of the Xi are lost at the pre-B- and pre-T-cell stages.

Xist becomes competent to induce gene silencing at a stage in lymphoid development, which is characterized by the establishment of allelic exclusion at the immunoglobulin heavy chain (*Igh*) or *TCR*β loci, respectively. Functional V(D)J (indicating variable, diversity, and joining regions, respectively) rearrangement of one of the two *Igh* alleles during pro-B development leads to the expression of the Ig(μ) protein as part of the pre-B-cell receptor (3). Likewise, the generation of a functional *TCR*β gene in DN pro-T cells leads to cell surface expression of the pre-TCR. Transient signaling of the pre-B-cell receptor or pre-TCR promotes differentiation to pre-B or DP pre-T cells and establishes allelic exclusion, which prevents further V-to-DJ rearrangement at the second incompletely rearranged *Igh* or *TCR*β locus, respectively (3, 17). The responsiveness of pre-B and pre-T cells to *Xist* could thus result from a change in cellular signaling, which likely activates pathways controlling long-range gene activity and *Xist* function. V(D)J recombination of the antigen receptor loci is under epigenetic control and regulated by the accessibility of the DNA segments subject to allelic exclusion (3, 17). It is tempting to speculate about a mechanistic link between allelic exclusion of the antigen receptor genes in lymphocytes and X inactivation in the embryo. Counting and choice in X inactivation could be regarded as an allelic exclusion mechanism before *Xist* activation and initiation of chromosomal silencing in early embryonic development. Recently, understanding of counting and choice in random X inactivation has been advanced by the observation of X inactivation center pairing in ES cells at the onset of X inactivation (1, 48). It will be interesting to see if similarities in the mechanisms between the choice in X inactivation and allelic exclusion at the antigen receptor loci will be uncovered in the future. In conclusion, *Xist* can initiate chromosome wide-silencing in hematopoietic cells that are still able to maintain the Xi.

Difference in responsiveness to *Xist* between embryonic and HSCs. Blood cell production is maintained throughout the lifetime of the mouse by HSCs, which differentiate into all blood cell types. Stem cell maintenance is of critical importance, and the cellular identity of the HSC must be stably maintained. At the same time, the cell identity has to be changed once the stem cell has been activated and cellular differentiation progresses to produce mature blood cells. Our data show that HSCs and uncommitted progenitors, like most somatic cells, do not have the ability to initiate X-linked gene silencing upon ectopic *Xist* expression and are thus resistant to *Xist*-mediated killing. The Xi in female HSCs and multipotent progenitors appears to be stable, as revealed by the presence of an *Xist* RNA cluster and a corresponding H3K27me3 staining similar to other somatic cells. This highlights a difference between adult and embryonic stem cells. Only following differentiation of HSCs do the lineage-restricted hematopoietic precursors establish an epigenetic environment resembling that of embryonic stem cells. It needs to be shown if the establishment of a context for initiation of X inactivation is paralleled by a general ability for epigenetic reprogramming of these hematopoietic precursors. Our work identifies a change in epigenetic activity at a time in hematopoietic development when epigenetic patterns may be fixed after reprogramming of gene expression at lineage commitment. Previously, reprogramming of the X chromosome after gastrulation has been observed in cells that enter the germ line. The absence of markers of the Xi was also observed in certain human tumors (16). A question that needs to be addressed in the future is whether an epigenetic context for reprogramming the X chromosome is also established during tumorigenesis. Recently, it has been shown that ectopic expression of the transcription factor *Oct4*, which is associated with pluripotency of ES cells and the early embryo, induces reversible hyperproliferation of progenitor cells of the intestinal epithelium (22). Finally, the identification of developmental transitions during which cells gain and lose their ability to initiate *Xist*-mediated gene silencing will facilitate efforts to find yet elusive factors involved in the earliest steps of X inactivation and gene silencing.

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