Abstract The molecular mechanism underlying X chromosome inactivation in female mammals involves the non-coding RNAs Xist and its antisense partner Tsix. Prior to X inactivation, these RNAs are transcribed in an unstable form from all X chromosomes, both in the early embryo and in undifferentiated embryonic stem (ES) cells. Upon differentiation, the expression of these unstable transcripts from all alleles is silenced, and Xist RNA becomes stabilised specifically on the inactivating X chromosome. This pattern of expression is then maintained throughout subsequent somatic cell divisions. Once established, the inactive state of the X chromosome is remarkably stable, the only natural case of reactivation occurring in XX primordial germ cells (PGCs) when they enter the genital ridge. To gain insight into the X reactivation process, we have analysed Xist gene expression using RNA FISH in PGCs and also in PGC-derived embryonic germ (EG) cells. XX EG cells were shown to express unstable Xist/Tsix from both X chromosomes. In contrast, no unstable Xist/Tsix transcripts were detected in XX PGCs at any stage. Instead, a proportion of XX PGCs isolated from the genital ridge between 11.5 and 13.5 dpc (the period during which X chromosome reactivation occurs) showed an accumulation of stable Xist RNA on one X. The number of these cells decreased progressively and was nearly extinguished by 13.5 dpc. As a late marker for the inactive state, we analysed localisation of the histone H2A variant macroH2A1.2. Although macroH2A1.2 expression was observed in PGCs, no significant localisation to the inactive X was detected at any stage. We discuss these results in the context of understanding X chromosome reactivation.

Key words X inactivation · Xist · primordial germ cell · EG cells · macroH2A1.2

Introduction

The germ cell lineage is established in the mouse about a week after fertilisation, midway through gastrulation (Lawson and Hage, 1994). Subsequently the primordial germ cells (PGCs) migrate along the hind gut and enter the genital ridges, the site of the future gonads, between 10 and 11 dpc. Germ cell proliferation continues for a few days within the genital ridges, but at about 13.5 dpc the germ cells in female embryos enter meiotic prophase, while those in male embryos cease dividing until after birth (McLaren, 1995). Major epigenetic changes accompany PGC development from pregonadal to advanced gonadal stages, which include global demethylation and genome reprogramming (Tada et al., 1997; 1998). In mature human oocytes, both X chromosomes are active (Epstein, 1969; Gartler et al., 1973), but in XX PGCs during the migratory period one X chromosome is inactivated at random, as occurs in somatic lineages (McMahon et al., 1983). XX and XY germ cells are thus dosage-compensated during migration. Using a sensitive assay for an X-linked enzyme
Regulation of mouse $X\text{ist}$ expression has been studied in early embryos and also in XX embryonic stem (ES) cells which provide a useful in vitro model system. Prior to random X inactivation in undifferentiated cells of the epiblast lineage and in ES cells, there is expression of unstable $X\text{ist}$ transcripts. Expression occurs on both alleles in XX cells and on the single allele in XY cells. As cells begin to differentiate, stable $X\text{ist}$ RNA, transcribed from the major promoters $P_1/P_2$, accumulates on the inactive X chromosome elect. Disappearance of unstable transcripts on the active X chromosome occurs subsequently (Panning et al., 1997; Sheardown et al., 1997). Expression of unstable $X\text{ist}$ RNA is accompanied by transcription of an antisense RNA, $Ts\text{ix}$, that initiates 15 kb downstream of $X\text{ist}$ and extends approximately 5 kb upstream (Lee et al., 1999a; Debrand et al., 1999).

The mechanism for regulation of $X\text{ist}$ RNA stability is unknown although models invoking promoter switching (Johnston et al., 1998), developmental regulation coupled with $Ts\text{ix}$ action (Warshawsky et al., 1999), and RNA concentration thresholds (Wutz and Jaenisch, 2000) have all been proposed.

Previous studies have shown that male germ cells in the postnatal testis have a low level of $X\text{ist}$ expression occurring coincident with programmed demethylation of $X\text{ist}$ promoters at the mitotic proliferation stage (McCarrey and Dilworth, 1992; Salido et al., 1992; Richler et al., 1992; Kay et al., 1993; Norris et al., 1994).

No $X\text{ist}$ expression is detectable in mature oocytes, consistent with X chromosome reactivation having occurred (McCarrey and Dilworth, 1992). Here we examine expression of $X\text{ist}$ and also localisation of the variant histone macroH2A1.2 through the course of X reactivation in developing germ cells and in the undifferentiated pluri potent embryonic germ (EG) cells derived from them. Our results demonstrate that $X\text{ist}$ expression is extinguished in XX PGCs during the critical window during which X reactivation occurs and that macroH2A1.2 localisation to the inactive X chromosome does not occur in PGCs during this period.

**Methods**

**EG cells**

XX EG cell line 19G-7 was derived from 11.5 dpc genital ridges from embryos obtained from matings between male 129/Sv-Rosa 26 transgenic mice and female 129/Sv mice (Tada et al., 1998). Hybrid cell line EGFXT7 was produced by fusion between an XX EG cell line from a 12.5 dpc embryo and XX primary thymocytes (Tada et al., 1997). Both cell lines were maintained in culture as previously described.

**Germ cells**

Adult females of the randomly bred MFI strain were mated to males of the same strain. Genital ridges were dissected from the embryos of pregnant females 11.5, 12.5 or 13.5 dpc. For $X\text{ist}$ ex-
pression analysis 12.5 and 13.5 dpc embryo genital ridges were sexed according to morphological features. 11.5 dpc embryos were sexed initially based on Xist expression patterns. Sexing was then confirmed for a subset of embryos by RNA/DNA FISH with DXSmh141 X specific repeat probe (see below). In the case of macroH2A1.2 analysis, sexing was carried out either by amniion analysis (Palmer and Burgoyne, 1991) or by PCR analysis using primers that give distinct bands for the Ube1X and Ube1Y genes (Chuma and Nakatsuji, 2001).

Germ cells were isolated from the ridges by pricking after 15 min incubation in 0.02 % EDTA in phosphate buffered saline (De Felici and McLaren, 1982). Previous analysis has shown that under these conditions PGCs constitute approximately 80 % of isolated cells, the remainder corresponding to contaminating cells from somatic lineages (McLaren and Southee, 1997).

Processing of cells

PGCs released from genital ridge pairs or aliquots of EG cells were placed on glass slides (Superfrost +, BDH) and allowed to adhere by standing at room temperature for approximately 2 hours in a humidified chamber. Subsequent processing of slides, labelling of probes, and RNA FISH have been described in detail elsewhere (Duthie et al., 1999). Xist probes were mx7 and mx8 (Johnston et al., 1998), and DXSmh141 was X chromosome specific probe (Nesterova et al., 2001). TG1 antibody (donated by Peter Beverly) was used to test for the presence of SSEA1 antigen. Double binding sites in 1 % BSA in PBS for 15 min at room temperature, and the antibody was detected with anti-mouse IgG, labelled with FITC (Vector Lab, Inc.).

Indirect immunofluorescence labelling using an affinity purified rabbit polyclonal antiserum against macroH2A1.2 (Costanzi and Pehrson, 1998) was performed on formaldehyde fixed germ cells as rabbit polyclonal antiserum against macroH2A1.2 (Costanzi and IgG, labelled with FITC (Vector Lab, Inc.).

The antibody was detected with anti-mouse

Processing of cells

The cells were incubated with primary TG1 antibody for 5 h at room temperature. The antibody was detected with anti-mouse IgG, labelled with FITC (Vector Lab, Inc.).

Xist expression in primordial germ cells

We went on to analyse Xist expression in freshly isolated PGCs recovered from the genital ridges of embryos 11.5, 12.5 and 13.5 dpc. At all stages a proportion of XX female PGCs exhibited a single stable Xist RNA signal. Examples are shown in Fig. 2a–c. Some cells are likely to be PGCs in which X chromosome reactivation is either underway or recently completed. Unstable Xist/Tsix transcripts were seen in only a very small proportion of XX PGCs. The majority of the remaining cells exhibited no Xist RNA expression at all, indicating that they represent cells in which X reactivation has already occurred. Thus, the Xist expression patterns we observed in EG cell lines are distinct from those occurring in PGCs isolated directly from genital ridges.

The proportion of Xist expressing cells decreased progressively between 11.5 and 13.5 dpc. The percentage of Xist expressing cells for individual samples representing PGCs from genital ridge pairs is illustrated on Fig. 2c. Fig. 2f summarises all of the scoring data for XX PGCs. At 11.5 dpc about 40 % of cells from female genital ridges were Xist-negative. This proportion increased pro-
Fig. 1 RNA FISH analysis of Xist/Tsix expression in EG cells and EG × lymphocyte hybrid cells. (a) Schematic representation of the Xist locus indicating the position of promoters P1 and P2 and also the antisense Tsix promoter. The position of probes mx7 and mx8 used for RNA FISH (colours show green or red fluorochrome) is indicated. (b, c) Examples of 19G-7 XX EG cell line. Colocalising pinpoint signals (white/yellow) indicate expression of unstable Xist/Tsix transcripts. Accumulated signal detected with mx7 probe only (red) corresponds to stable Xist RNA. (d) Graph summarises Xist/Tsix expression results, illustrating percentage of cells with different expression patterns. A small circle represents an allele expressing unstable Xist/Tsix RNA, a large circle represents an allele expressing stable Xist RNA, whilst a line indicates an allele with no detectable Xist expression. A total of 215 cells were scored in this experiment. (e) Example of RNA FISH analysis of TMA-58G EG × lymphocyte hybrid cells illustrating a cell expressing unstable Xist/Tsix RNA on three alleles. (f) Example of RNA/DNA FISH used for RNA FISH (colours show green or red fluorochrome) is showing Xist/Tsix RNA detected with mx7 probe (red), and X chromosome DNA detected with the X-specific repeat probe DXSmh141 (green). Original magnification 1000×. (g) Scoring data showing the percentage of cells with different number of alleles expressing Xist/Tsix (n = 120). (h) Percentage of cells with 1, 2, 3 or 4 X chromosomes as judged by DNA FISH with DXSmh141 probe (n = 160). In all examples DNA is counterstained with DAPI (blue).

progressively to a maximum of about 75% at 13.5 dpc. As approximately 20% of cells in PGC preparations are expected to be contaminating somatic cells (McLaren and Southee, 1997) and in an XX embryo these cells will express Xist, our results indicate that Xist expression is extinguished in 95–100% of PGCs by 13.5 dpc. PGCs isolated from XY embryos had no expression of stable Xist RNA (Fig. 2d,g). Unstable Xist/Tsix transcripts were observed in a small proportion of cells. The significance of this small sub-population both in XY and XX cells is unknown.

The progressive extinction of Xist RNA in PGCs presumably reflects germ cell development. To test this further, we determined if there is a correlation between Xist expression and expression of the stage specific embryonic antigen (SSEA1), a marker of early stage developing PGCs (Solter and Knowles, 1978), which is expressed in migratory PGCs, from about 9 dpc, and down-regulated at 11–12 dpc, after entry into the genital ridges. In 11.5 dpc XX PGCs, approximately 50% of cells stained positive for SSEA1 (Fig. 3a). Of these, 80.7% (n = 476) also expressed Xist RNA (Fig. 3b,c). This is significantly higher than the proportion in total 11.5 dpc PGC populations (55.3%, Fig. 2f). This result confirms that Xist RNA extinction correlates with developmental progression in XX PGCs.
Localisation of variant histone macroH2A1.2 in PGCs

We wished to assess a late marker of X inactivation in XX PGCs to compare with the data obtained for Xist RNA localisation. Immunolocalisation studies have demonstrated that the variant histone macroH2A1.2 is concentrated on the inactive X chromosome in differentiated XX somatic cells, appearing in interphase cells as a dense staining region, termed a macrochromatin body (MCB) (Costanzi and Pehrson, 1998). This localisation occurs at a relatively late stage in differentiating XX ES cells, suggesting a role for macroH2A1.2 in maintenance rather than initiation of X inactivation (Mermoud et al., 1999). In addition to inactive X specific localisation, the protein shows a more general diffuse nuclear staining and concentration at the centrosome in a range of cell types, both in XX and XY cells. Multiple macroH2A1.2 foci that correlate with DAPI dense centromeric heterochromatin regions in the interphase nucleus can also be seen in some cells (Mermoud et al., 1999; Costanzi et al., 2000). Initially we used immunolocalisation to analyse
the distribution of macroH2A1.2 in 11.5 and 12.5 dpc XY and XX PGCs. Most cells exhibited either diffuse nuclear staining (Fig. 4a) or multiple macroH2A1.2 foci (Fig. 4b,c). The latter pattern was more prevalent in XX PGCs and increased significantly between 11.5 and 12.5 dpc in both XY and XX cells (Fig. 4e). These observations suggest that appearance of multiple macroH2A1.2 foci may be a marker for germ cell progression towards either meiotic or mitotic arrest. Presence of a single macroH2A1.2 focus, the pattern typically observed in XX somatic cells, was only seen in a very small proportion of XX cells (Fig. 4d). Centrosomal localisation of macroH2A1.2 was observed in PGCs in double labelling experiments using
Fig. 5 MacroH2A1.2 and Xist RNA do not colocalise in the majority of PGCs. (a) Example of an XX cell showing diffuse nuclear staining for macroH2A1.2 (green), and Xist RNA detected with probe mx7 (red). (b, c) Examples of cells with multiple MCBs and non co-localising Xist RNA signal. (d) Example of a cell with diffuse nuclear staining for macroH2A1.2 and a single MCB signal that correlates with the inactive X chromosome as detected by Xist RNA FISH (red + green = yellow). DNA is counterstained with DAPI (blue) in all examples. Original magnification 1000×. (e) Scoring data showing the proportion of cells with none or with one or more MCB at different stages and the fraction of those cells with stable Xist RNA signal. Data for both XX and XY PGCs is shown.

antibody to gamma tubulin (not shown) as reported previously for a variety of cell types (Rasmussen et al., 2000; Mermoud et al., 2001).

To correlate different macroH2A1.2 staining patterns with Xist RNA expression we carried out double staining for Xist RNA and macroH2A1.2 (Fig. 5). The proportion of XX germ cells expressing Xist RNA was seen to decline between 11.5 and 12.5 dpc, consistent with earlier results. The majority of Xist expressing cells, however, showed diffuse localisation of macroH2A1.2 throughout the nucleoplasm (Fig. 5a). Also, in cells with multiple macroH2A1.2 foci the Xist RNA signal generally did not colocalise with any of the foci (Fig. 5b,c). Cells that did show colocalisation were usually in the small subpopulation with a single non-centrosomal macroH2A1.2 focus (Fig. 5d). These cells may be representative of the low level somatic cell contamination that occurs in PGC preparations. Quantitation of the data is shown in Fig. 5e. In summary, our results indicate either that macroH2A1.2 does not associate with the inactive X chromosome in XX PGCs, or that it is removed at a very early stage, prior to or immediately after entry of PGCs to the genital ridge.

Discussion

Whilst the fact of X chromosome reactivation in XX germ cells on entering the genital ridge was firmly established by studies that used first, an X-linked enzyme assay (Monk and McLaren, 1981) and subsequently, a LacZ transgene carried on the X chromosome (Tam et al., 1994), both of these approaches rely on protein products of X-chromosome genes. We set out to examine expression of Xist, the primary initiator of X inactivation during the critical period when reactivation occurs. Our data indicate that at 11.5 dpc the XX germ cells that are more immature (as judged by SSEA1 expression) and have entered the genital ridge more recently still express stable Xist transcripts. Other germ cells have already started to extinguish Xist expression, a process which appears to be virtually complete by 13.5 dpc. The dispersal of RNA signal that we observed in a proportion of cells may occur either because the Xist RNA dissociates from the X chromosome or because the reactivated chromatin decondenses. Tam et al. (1994) reported that reactivation of an X-linked lacZ transgene...
was detectable in a proportion of XX PGCs at 11.5 dpc and was essentially complete by 13.5 dpc. The extinction of Xist expression therefore correlates well with these findings.

Coating of the inactive X chromosome with Xist RNA is the earliest marker for establishment of the inactive state, but Xist RNA is not required for the maintenance of X inactivation (Brown and Willard, 1994; Csankovski et al., 1999). Thus, loss of Xist RNA in XX PGCs may not play a causative role in the reactivation process. It is plausible, in fact, that X reactivation occurs prior to Xist RNA extinction and that the dispersed Xist signal we observed in a large proportion of XX PGCs occurs as a result of decondensation of the heterochromatic inactive X structure. In support of this, we found that nascent transcripts for both alleles of two different X linked genes, Pkg-1 and Brx, can be seen in some XX PGCs with Xist RNA signal (our unpublished observation).

Undifferentiated XX or XY ES cells resemble pregastrulation epiblasts in producing unstable Xist/Tsix RNA transcripts, visualised as punctate signals (Panning et al., 1997; Sheardown et al., 1997). EG cells resemble ES cells in being pluripotent and capable of indefinite proliferation in vitro if maintained in appropriate culture conditions that discourage differentiation. The 11.5 dpc XX EG cells that we examined gave punctate signals indicative of unstable Xist/Tsix transcripts. The same was true for Xist alleles in hybrid cells formed by fusion of XX lymphocytes and a 12.5 dpc XX EG cell line. The presence of some presumptive differentiated cells expressing stable Xist RNA further indicates that EG cell lines are more similar to ES cells than to the 11.5–12.5 dpc PGCs from which they were derived. However, it is not known whether the XX EG lines we analysed were derived from a PGC that had already undergone X reactivation and/or Xist extinction, and this may be important in terms of the EG cell Xist expression status. A previous RT-PCR-based study demonstrated absence of Xist expression in XX EG cells, derived from 8.5 dpc pre-migratory PGCs (Stewart et al., 1994), a stage when most of the XX PGCs would be expected to have undergone X-inactivation. Xist expression was detected following differentiation into embryoid bodies. It is plausible that unstable Xist/Tsix transcripts were also expressed in these EG lines in the undifferentiated state but that PCR conditions were not sufficiently sensitive to detect them.

Expression and accumulation of stable Xist transcripts is not sufficient for X inactivation, since in early preimplantation embryo transcripts accumulate on the paternal X chromosome before differentiation and X inactivation take place (Sheardown et al., 1997; Johnston et al., 1998). Similarly, expression of stable human Xist RNA in undifferentiated mouse ES cells or fusion hybrids does not cause inactivation (Heard et al., 1999; Yoshida et al., 1997). Presumably developmentally regulated silencing factors downstream of Xist that are required for the maintenance of the inactive state are absent in these situations. One candidate for this function is the variant histone macroH2A1.2. Our analysis indicates either that XX PGCs fail to incorporate macroH2A1.2 at the onset of X inactivation or, alternatively, that the protein is initially incorporated and then dissociates prior to the 11.5 dpc stage of development. If the former is true then this could reflect a less stringent foundation for X inactivation in XX germ cells that in turn could be important for facilitating X reactivation. In support of this view, CpG islands on the inactive X chromosome are not methylated in PGCs, contrasting with XX somatic lineages (Grant et al., 1992). The alternative possibility, that macroH2A1.2 initially incorporates on the inactive X chromosome in XX PGCs and is then subsequently displaced, may suggest a role in the reactivation process. However, in XX somatic cells conditional deletion of Xist results in delocalisation of macroH2A1.2 from Xi, and neither event leads to X chromosome reactivation (Csankovski et al., 1999). Thus reactivation probably requires reversal of alternative/additional epigenetic silencing mechanism(s).

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References


Clemson, C.M., McNeil, J.A., Willard, H.F. and Lawrence, J.B. (1996) XIST RNA paints the inactive X chromosome at inter-
phase: Evidence for a novel RNA involved in nuclear chromo-
Costanzi, C. and Pehrson, J.R. (1998) Histone macroH2A1 is con-
potential stem cells derived from migrating primordial germ cells. Differentiation 68:220–226.
Durcova-Hills, G. and McLaren, A. (2001) Germ cells and pluri-
potential stem cells in the mouse. Repro Fert Dev (in press).
Heard, E., Clerc, P. and Avner, P. (1997) X-chromosome inacti-
Lee, J.T., Lu, N.F. and Han, Y. (1999b) Genetic analysis of the mouse X inactivation center defines an 80-kb multifunction do-
Matsui, Y., Zsebo, K. and Hogan, B.L. (1992) Derivation of pluri-
Nesterova, T.B., Barton, S.C., Surani, M.A. and Brockdorff, N. (2001) Loss of Xist imprinting in diploid parthenogenetic preim-
Palmer, S.J. and Burgoyne, P.S. (1991) The Mus musculus dom-
esticus Tdy allele acts later than the Mus musculus Tdy allele: a basis for XY sex-reversal in C57BL/6-YPOS mice. Development 113:709–714.
Resnick, J.L., Bixler, I.S., Chang, L. and Donovan, P. (1992) Long-
term proliferation of mouse primordial germ cells in culture. Na-
Stewart, C.L., Gadi, I. and Bhatt, H. (1994) Stem Cells from pri-
mordial germ cells can reenter the germ line. Dev Biol 161:626–628.
Warshawsky, D., Stavropoulos, N. and Lee, J.T. (1999) Further ex-
amination of the *Xist* promoter-switch hypothesis in X inactivation: Evidence against the existence and function of a P0 promoter. Proc Natl Acad Sci USA 96:14424–14429.
