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ORIGINAL ARTICLE

X inactivation in a mammal species with three sex chromosomes

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Abstract

X inactivation is a fundamental mechanism in eutherian mammals to restore a balance of X-linked gene products between XY males and XX females. However, it has never been extensively studied in a eutherian species with a sex determination system that deviates from the ubiquitous XX/XY. In this study, we explore the X inactivation process in the African pygmy mouse *Mus minutoides*, that harbours a polygenic sex determination with three sex chromosomes: Y, X, and a feminizing mutant X, named X*; females can thus be XX, XX*, or X*Y, and all males are XY. Using immunofluorescence, we investigated histone modification patterns between the two X chromosome types. We found that the X and X* chromosomes are randomly inactivated in XX* females, while no histone modifications were detected in X*Y females. Furthermore, in *M. minutoides*, X and X* chromosomes are fused to different autosomes, and we were able to show that the X inactivation never spreads into the autosomal segments. Evaluation of X inactivation by immunofluorescence is an excellent quantitative procedure, but it is only applicable when there is a structural difference between the two chromosomes that allows them to be distinguished.

Keywords Histone modification · X inactivation · African pygmy mouse · *Mus minutoides* · Polygenic sex determination · Sex-autosome translocation

Introduction

In eutherian mammals, sex determination involves a genetic system with heterogametic male (XY) and homogametic female (XX), that results in an unequal copy number of X-linked genes between the two sexes. This dosage imbalance of gene expression is resolved by the silencing of one of the two X chromosomes during early embryonic female development through a process called X inactivation (reviews in Heard and Disteche (2006), Veitia et al. (2015), and Marshall Graves (2016)). The silencing of the inactivated X is established through epigenetic factors initiated by the X inactivation centre (Xic) involving in particular the *Xist* gene that codes for a long non-coding RNA which is transcribed exclusively from the future inactivated X. The Xist transcript then spreads in *cis* from the Xic to coat the whole X, recruiting along the way a series of chromatin changes through histone modifications and DNA methylation that trigger the inactivation (review in Heard (2004)). The silenced X is chosen at random, and once established, a specific signature of epigenetic modifications maintains the same inactivated chromosome in all progeny cells. Therefore, females are mosaics for two populations of cells, one with the active maternal X and the other with paternal X (e.g. Heard (2004), Heard and Disteche (2006), Veitia et al. (2015), and Marshall Graves (2016)). In eutherians, there are a few natural exceptions to the standard XX/XY sex determination system (reviews in Fredga (1994) and Parma et al. (2016)), but for now, they have received little attention, in particular regarding X inactivation process. For example, constitutive X0 females known in *Microtus oregoni*, *Tokudaia osimensis*, and *Ellobius lutescens* can be seen as the ultimate evolutionary step of X inactivation with the physically suppression of one X. Nevertheless, the recent analysis of the *E. lutescens* Xic has shown that all genes known to control X inactivation are still present and well conserved (Mulugeta et al. 2016). In *Ellobius tancrei*, *E. talpinus*, and *E. alaicus*, both females and males are XX, and the presence of two X in males raises the question about the need for X inactivation: has the X inactivation been lost in females or been recruited in males as well? Finally, in *Myopus schisticolor*, three sex chromosome complements occur in females, i.e. XX, XX*, and

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X*Y, due to the presence of a third sex chromosome, named X*, that carries a feminizing mutation that prevents the initiation of the male sex-determining program in X*Y individuals. Although limited investigation seemed to suggest random X inactivation of either the X or X* in XX* females (H. Winking, personal communication, in Schempp et al. (1985)), other results showed more striking patterns, with complete non-random X or X* inactivation (100%) in specimens with numerically aberrant X*XY sex chromosome constitution (Schempp et al. 1985). Recently, a novel case of unusual mammalian sex determination system was described in the African pygmy mouse, *Mus minutoides*, a close relative of the house mouse (Veyrunes et al. 2010; Rahmoun et al. 2014; Zhao et al. 2017). In wild populations, up to 75% of females carry a Y chromosome and a peculiar feminizing X* chromosome and are fully fertile. The sex-determining system is in fact very similar to the one of the wood lemming *Myopus schisticolor* and can be characterized as polygenic, with three sex chromosomes, X, Y, and X*, and two sex-determining genes, the regular mammalian male determiner *Sry* on the Y, and a still unknown dominant female determiner on the X*. Like in the wood lemming, females are XX, XX*, or X*Y, while all males are XY (Veyrunes et al. 2010). Some life history and behavioural traits vary along with sex chromosome complement in *M. minutoides*, and interestingly, XX* females are much more similar to XX females than X*Y ones (Saunders et al. 2014, 2016; Ginot et al. 2017). This pattern is at least partly under the direct influence of genes on the sex chromosomes, but considering the paucity of genes on the Y chromosome and that most of them are specialized in male reproduction (Marshall Graves 2006), other mechanisms might be involved, such as a preferential X* inactivation in XX* females (Saunders et al. 2014). The wild-type X and the mutant X* can be cytogenically distinguished owing to important structural rearrangements (Veyrunes et al. 2010). In addition, they are fused to different autosomes, the X to chromosome 1, and the X* to chromosome 16 (while the Y is also fused to chromosome 1; Fig. 1).

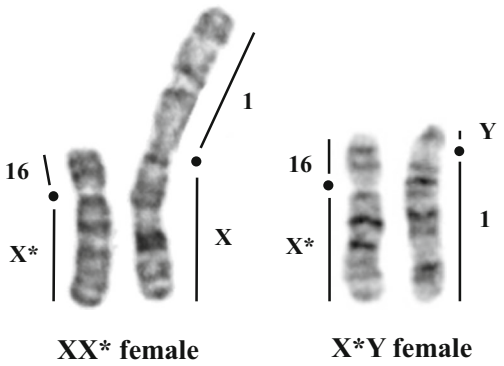

Fig. 1 G-banded sex chromosomes of the XX* and X*Y females. X and Y chromosomes are translocated to autosome pair 1, and X* to autosome 16. Black dots indicate centromere position

The presence of two different X chromosomes in XX* females gives the opportunity to extensively test if both Xs are still randomly inactivated, or if one is preferentially inactivated. We thus investigated the pattern of histone modification characteristic of active and inactive chromatin on metaphase chromosomes from *M. minutoides* XX* female embryo fibroblasts. This work also provided insights into the X histone modifications in rare genomic contexts of importance in human medicine, such as sex-reversed females and X-autosome translocations (e.g. White et al. 1998; Popova et al. 2006).

Materials and methods

Animals and cell culture

The karyotype of *M. minutoides* was published previously (Veyrunes et al. 2004). The karyotype is composed of 18 bi-armed chromosomes, all resulting from Robertsonian fusion events, even the sex chromosomes are fused, but with different autosomes: Rb(Y.1), Rb(X.1), and Rb(X*.16) (Veyrunes et al. 2007). Mice were bred in our own laboratory colony (CECEMA facilities of Montpellier University) established from wild-caught animals (for further details, see Saunders et al. (2014)). Mid-term embryos (between 12 and 16 days post coitum) of pregnant X*Y females (that give birth to XX* and X*Y daughters, and XY sons) were collected. Females were euthanized by CO_2 inhalation. Embryos were first sexed by gonad examination. Female embryos were then genotyped using PCR amplification using the Y-specific *Sry* gene following Veyrunes et al. (2010), to distinguish between XX* and X*Y. Thereby, 11 XX* and two X*Y female embryos were selected for fibroblast cell cultures using half of the body and following standard procedures.

Chromosome preparations

Before harvest, fibroblast cultures (70–90% confluent in a 75-cm^2 flask) were incubated for 2 h with 1% to final volume of KaryoMAX Colcemid (GIBCO). Cells were harvested by trypsinisation and washed with culture medium then with PBS. Cell pellets were resuspended in 1 ml of PBS supernatant before hypotonic treatment by slowly adding of 10 ml of 0.075 M KCl with 0.1% (vol/vol) glycerol and incubated for 20 min in a 37 °C water bath. Swollen cells were kept on ice up to 1 h before spreading. Three hundred to five hundred microlitres of swollen cell suspensions was spun onto slide using Cytospin (300 rpm, 5 min, low acceleration). Metaphase spread quality was highly dependent on the concentration of the cell suspension; slides containing metaphase spreads (area of 6 mm in diameter) were air dried.

Antibodies and immunofluorescence

We studied the distribution of two active chromatin markers, namely, histone H4 acetylated at lysine 8 (H4K8ac—Abcam Ab15823) and histone H3 dimethylated at lysine 4 (H3K4me2—Millipore 07-030), and one inactive chromatin marker, histone H3 trimethylated at lysine 27 (H3K27me3—Millipore 07-449), on metaphase spreads (Heard et al. 2001; Plath et al. 2003; Rens et al. 2010). Before use, antibodies specific for modified histones were diluted 1:600 in TKCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris HCl pH 7.5, 0.5 mM EDTA, 0.1% (vol/vol) Triton X-100) with 1.5% BSA (SIGMA). The immunofluorescence protocol was adapted from Rens et al. (2010). Briefly, slides were permeabilised at room temperature with TKCM buffer for 20 min. After removing the TKCM buffer, slides containing metaphases spreads were blocked for 20 min with 5% BSA in TKCM buffer (30 µl onto cell area under plastic film) in humid chamber at room temperature then labelled with 30 µl histone antibodies overnight at 4 °C. After three washes for 4 min each with TKCM buffer, slides were incubated with FITC-conjugated secondary antibody (anti-rabbit IgG from SIGMA) diluted 1:100 in TKCM buffer with 1% BSA for 30 min in humid chamber at room temperature. Slides were washed as previously and fixed during 10 min at room temperature with 5% paraformaldehyde (vol/vol) in TKCM buffer. Slides were washed for 5 min with TKCM buffer and mounted in Vectashield mounting medium containing DAPI (Vector). Slides were examined with a Zeiss Axioplan 2 Imaging epifluorescence microscope equipped with Cytovision capturing software and CCD camera.

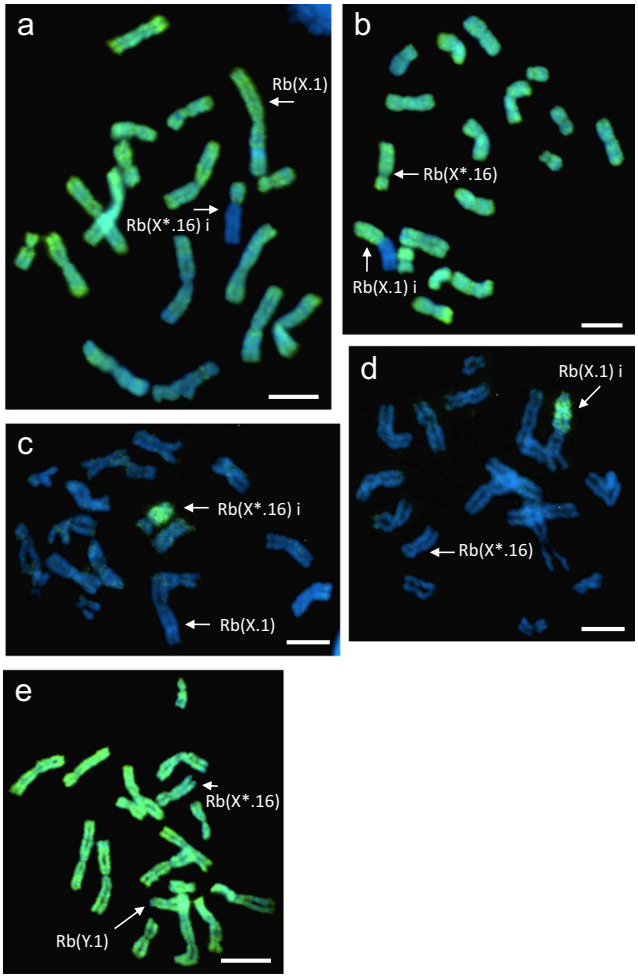

Fig. 2 Immunofluorescence staining for **a** H4K8ac and **b** H3K4me2 histones, both characteristic of the active chromatin, on XX* female metaphase spreads; **c**, **d** H3K27me3 histone, an inactive chromatin marker, on XX* female metaphase spreads; **e** H4K8ac histone on an X*Y female metaphase spread. "i" indicates the inactive X chromosome type. Scale bar = 10 µm

Results

Histone modification characteristic of active chromatin

The distribution of the two active chromatin markers (H4K8ac and H3K4me2) on metaphase spreads of 11 *M. minutoides* XX* was consistent with the one found in the house mouse (Boggs et al. 2002; Chaumeil et al. 2002; Rens et al. 2010), strong signals on the autosomes and one X chromosome, while the other X, supposedly the inactive one, was depleted (Fig. 2a, b). In both sex-autosome translocations, Rb(X.1) and Rb(X*.16), the histone modifications were asymmetrically distributed around the centromere, with a strong signal on the autosomal arm and no staining on the inactive X arm. The X*Y females showed a different hybridization pattern: H4K8ac and H3K4me2 were evenly distributed on all chromosomes including the X* and Y chromosomes (Fig. 2e).

Histone modification characteristic of inactive chromatin

On the same specimens but on different metaphase spreads, we also analysed the repressive modification of the histone H3K27me3 that is a specific mark of the inactivated X in the house mouse (Plath et al. 2003; Rens et al. 2010). On the *M. minutoides* XX* metaphases, there was a strong enrichment on only one single X arm with a clear cut-off limit at the centromere of the sex-autosome translocation (Fig. 2c, d). On the X*Y females, H3K27me3 was not detected (data not shown).

The number of analysed cells and the percentage of inactive X and X* chromosomes for each XX* embryo are summarized in Table 1. The number of metaphases varied between 16 and 126, depending of the cell suspension and the cytospin spreading quality. Arbitrarily, skewed (i.e. non-random) X

Table 1: Number of analysed cells with an inactive X* or X chromosome, and percentage of X* inactivation for each of the 11 XX* embryos

individual	No. metaphases	inactive X*	inactive X	% of X* inactivation
1	**73**	**15**	**58**	**20%**
2	121	40	81	33%
3	111	56	55	50%
4	16	9	7	56%
5	115	65	50	57%
6	126	77	49	61%
7	74	50	24	68%
8	26	19	7	73%
9	**32**	**27**	**5**	**84%**
10	**36**	**32**	**4**	**89%**
11	**53**	**49**	**4**	**92%**
Total	781	439	342	56%

Bold typeface indicates samples that reached skewed X inactivation with a treeshold ratio ≥ 80:20

inactivation is often defined when the ratio is ≥ 80:20% (e.g. Belmont (1996), Plenge et al. (2002), and Talebizadeh et al. (2005)). Interestingly, four of the 11 samples reached the threshold ratio of 80:20, three and one with a highly biased X* and X inactivation, respectively. In total, 781 metaphase spreads have been analysed, 439 (56%) with an inactive X* chromosome and 342 (44%) with an inactive X.

Discussion

X inactivation is a fundamental process in eutherian mammals to restore a balance of X-linked gene products between males and females. However, it has never been extensively studied in a mammal species with an atypical sex determination system which deviates from the ubiquitous XX/XY. In this study, we explore the X inactivation process in the African pygmy mouse, *M. minutoides*, that harbours a polygenic sex determination with three sex chromosomes: Y, X, and X* (Veyrunes et al. 2010). Such a system raises several questions. First, in XX* females, is the X inactivation mechanism still random, or is one type of X preferentially inactivated? Second, in X*Y females, the single copy of X chromosome is not expected to be silenced, could we thus provide direct evidence of the absence of X inactivation? And finally, does the X inactivation spread onto the autosomal arms in the sex-autosome translocations, Rb(X.1) and Rb(X*.16)? Hence, to investigate the X inactivation process in this unusual sex determination system, we conducted immunofluorescence analyses to evaluate histone modification patterns between the two X chromosome types. This appears to be an excellent quantitative procedure to detect any deviation from randomness.

In eutherian mammals, random X inactivation (50:50%) is the norm, but skewed X inactivation patterns have been reported and are most of the time detected in women who manifest severe X-linked genetic diseases (e.g. Van den Veyver (2001), Plenge et al. (2002), and Talebizadeh et al. (2005)). The causes of X inactivation skewing have been extensively discussed (Belmont 1996; Migeon 1998; Brown and Robinson 2000). Particularly, it has been proposed that the main cause for it is somatic selection occurring after random X inactivation. This process may lead to a skewed X inactivation when one of the X chromosomes carries extensive chromosomal rearrangements or a single gene mutation that affects cell survival or growth. Consequently, a majority of cells bearing the inactive mutated X would remain. Other potential causes for X inactivation skewing are mutations on genes that could influence the choice of the inactivated X, such as *Xic*, *Xist*, or *Xce* loci (e.g. Plenge et al. 1997; Migeon 1998). In *M. minutoides*, recent studies comparing the three female genotypes have shown that XX* females are much more similar to XX females than X*Y ones in terms of fertility, behaviour, and morphology (Saunders et al. 2014, 2016; Ginot et al. 2017), and interestingly, it has been hypothesized that these findings could be partly explained by a preferential inactivation of the X* in XX* females (Saunders et al. 2014).

Our results on histone modification patterns support that the *M. minutoides* X* and X chromosomes remain randomly inactivated; they are found to be inactive in 56 and 44% of the 781 analysed metaphase spreads, respectively (Table 1). Hence, there is no evidence of a constitutional X inactivation skewing, as could have been suggested by the observations of behaviour and life history traits. However, the variance among the 11 samples was important. While some showed a perfect random inactivation pattern, e.g. one specimen, with respectively 56 and 55 cells with an inactive X* and X chromosome, four others showed important skews (≥ 80:20%), three in favor of an inactive X* and one for the X (Table 1). Skewed X inactivation can occur by chance as a result of the fact that the X inactivation is a stochastic process and at the time it is established, the progenitor cell population is relatively small. Consequently, it has been estimated that in a control population of healthy women, approximatively 10% present skewed X inactivation (e.g. Plenge et al. (2002) and Talebizadeh et al. 2005). However, in the case of *M. minutoides*, this proportion is much higher (four out of 11 specimens, 36%). One possible explanation is that cell selection took place in the cell cultures. For example, we could have accidentally sampled only one cell population that all derived from the same progenitor cell. To avoid such unbalanced sampling, we began the cell cultures from material representing half of the organism (lower part of the embryo, the other half was used for genotyping).

However, we cannot rule out the possibility that the cells grew out from only one or a few small tissue pieces before the first culture passage and harvest. Overall, our results suggest that the X* chromosome, although highly rearranged (Veyrunes et al. 2010), does not affect X inactivation, and thus that the genes involved in X inactivation have not been affected by the rearrangements.

As expected, no histone modifications were detected on metaphase spreads of X*Y females (Fig. 2), since in eutherian mammals, a counting mechanism exists to maintain only one active chromosome (Monkhorst et al. 2008; Augui et al. 2011). This was first revealed in humans with abnormal numbers of X chromosomes: females with Turner syndrome (XO) show no inactivation of their unique X, while XXX females and even Klinefelter males (XXY) display respectively two and one inactive X chromosomes. Another remarkable feature of the *M. minutoides* karyotype is the presence of two X-autosome translocations, Rb(X.1) and Rb(X*.16) (Veyrunes et al. 2004, 2007). X-autosome translocation is considered as one of most deleterious rearrangements in mammals, since it can generate important perturbations of gametogenesis and gene expression, notably due to the spreading of X inactivation into the adjacent autosome, that causes the silencing of autosomal genes (review in Dobigny et al. (2004)). This phenomenon has been demonstrated in humans and mice (e.g. Rastan (1983), Schanz and Steinbach (1989), White et al. (1998), Hall et al. (2002), and Popova et al. 2006) and has been shown to generally lead to severe phenotypic effects (Waters et al. 2001). Fixation of X-autosome translocations is nevertheless not that uncommon in mammalian karyotypes, and it has been suggested that the cost of such rearrangement can be overcome by the addition of a large block of heterochromatin between the X and the autosomal segments that would serve as a boundary to functionally isolate the two components. Support for this hypothesis is found in almost all mammal species known to possess X-autosome translocations, in which a large block of heterochromatin is systematically identified between the two chromosomal segments (review in Dobigny et al. (2004)). However, very few studies have actually demonstrated its role as insulation buffer. In the common shrew *Sorex araneus* and in rodents of the genus *Taterillus*, replication banding pattern after BrdU treatment showed that a late-replicating segment, supposedly inactive, was limited to the ancestral X compartment (Pack et al. 1993; Dobigny et al. 2004), and in the marsupial potoroo *Potorous tridactylus*, hypomethylation was restricted to one of the X chromosome arms of the X-autosome translocations (Rens et al. 2010). Here, with modern technique of immunofluorescence to detect histone modifications, we confirmed that the X inactivation never extents to the translocated autosome in *M. minutoides* (Fig. 2). These results are even more interesting in *M. minutoides* since no blocks of C-positive heterochromatin were found near the centromeres of Rb(X.1) and Rb(X*.16) (Veyrunes et al. 2004), and there is no pseudoautosomal region that could create a natural barrier preventing the spreading (the sex chromosomes being asynaptic; Britton-Davidian et al. 2012). These observations support our assumption that in this species, the isolation of the X and autosomal compartments may involve other types of repetitive sequences to act as a buffer (Veyrunes et al. 2004; Colomina et al. 2017), or some genomic characteristics, such as a low density of LINE-1 elements, as they are suggested to act as booster elements of the X inactivation propagation (Lyon 1998; Bala Tannan et al. 2014; Cotton et al. 2014).

In summary, we explored the X inactivation process in a mammalian model that presents a unique sex determination system with two types of X chromosomes. We provided direct evidence that in the XX* females, the mutant feminizing X* and the ancestral X are randomly inactivated, and that in X*Y females, inactivation does not occur. Evaluation of X inactivation by immunofluorescence is an excellent quantitative procedure, but it is only applicable when there is a structural difference between the two chromosomes that allows them to be distinguished. However, we warned about cell selection that could occur in cultures and bias the results. We also concluded that in the sex-autosome translocations found in this species, the X inactivation does not spread into the autosomal arm. More extensive molecular studies (e.g. RT-qPCR, methylation-specific PCR) on autosomal genes at the junction with the X arm would be required to confirm the chromosome-wide histone modification pattern and the absence of X inactivation spreading.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experimental protocol was performed in accordance with European guidelines and with the approval of the Ethical Committee on Animal Care and Use of France (no. CEEA-LR-12170).

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