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Direct Evidence of Allele-Specific Binding of CTCF and MeCP2 to *Tsix* in a HPRT-Deficient Female F_1 Hybrid Mouse Cell Line

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Key Words
CTCF · MeCP2 · *Tsix* · X chromosome inactivation · *Xist*

Abstract
Mammalian dosage compensation requires silencing of one of the two X chromosomes in females and is controlled by the X inactivation center (Xic). Xic contains many of the regulatory elements for the mutual interplay of X-inactive specific transcript *(Xist)* and *Tsix*, the antisense counterpart of *Xist*. The regulatory elements control X chromosome inactivation (XCI) via the formation of DNA-DNA and DNA-protein complexes with *cis*- and *trans*-acting factors. However, the process-dependent regulation of *Xist/Tsix* by these elements in each XCI process remains largely unknown. In this study, a 6-thioguanine-resistant female F_1 hybrid mouse cell line (designated HOBMSKI2) was constructed from a cross between a female HPRT-deficient transgenic mouse (designated BM3) and a male wild type *Mus spretus* mouse (designated MS), which enabled the direct discrimination of both allele-specific expression of X-linked genes and allele-specific binding of proteins associated with XCI due to DNA polymorphisms between BM3 and MS. Using this cell line, we found that *Tsix* on the active X chromosome (Xa) was not expressed in somatic cells despite the fact that CTCF, which ensures *Tsix* expression in embryonic stem cells, was still bound to the 5′ end of *Tsix* on Xa, implying that CTCF may function differently during each XCI process and its *trans*-activating activity for *Tsix* expression may be lost in the maintenance process. In addition, the monoallelic expression of *Tsix* on Xa was inhibited by epigenetic modification of the chromatin in the maintenance process, which was mediated by protein complexes recruited by MeCP2. The results indicate the value of HOBMSKI2 in directly detecting the allele-specific binding of CTCF and MeCP2 to the 5′ end of *Tsix*. The HOBMSKI2 mouse line is a versatile and useful resource for studying the molecular mechanism of the XCI process.

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In mammals, X chromosome inactivation (XCI) occurs as a means of compensating for a disparity in sex chromosome dosage between XX females and XY males. One of the two X chromosomes in a female cell is transcriptionally silenced during early embryogenesis [Lyon, 1961]. The X chromosome inactivation center (Xic), which is the starting point of XCI, corresponds to a DNA

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sequence of over 1 Mb, located at Xq13, that contains several genomic elements. The gene that plays the main role for XCI in the Xic is the X-inactive specific transcript *(Xist)* [Pontier and Gribnau, 2011]. *Xist* is expressed exclusively from the inactive X chromosome (Xi), producing a 17-kb spliced and polyadenylated transcript, which has no open reading frame and is accumulated *in cis* along the Xi [Carrel and Willard, 1998]. *Xist* functions as a master switch of XCI [Tsai et al., 2008; Tian et al., 2010] and is regulated by its antisense counterpart gene, *Tsix* [Lee et al., 1999]. During differentiation of female embryonic stem (ES) cells, *Tsix* RNA expression is stopped in the future Xi and persists in the future active X chromosome (Xa), implying that *Tsix* is involved in blocking the XCI process by the transcriptional inhibition of *Xist* [Nesterova et al., 2011]. When the 5′ end of *Tsix*, a *cis*-acting center for the choice process, is deleted, *Xist* is always expressed on the deleted X due to the favor of inactivation [Lee, 2002]. Thus, the 5′ end of *Tsix* has been proposed to control *Xist* expression *in cis* at the onset of XCI.

Xist expression is regulated not only by *Tsix* but also by *RS14*, which is located at the 3′ end of *Xist* and is highly conserved in many species. In contrast, *Tsix* expression is controlled by regulatory elements including X inactivation intergenic transcription element *(Xite)* and the repeat element *DXPas34*, which is located in the vicinity of *Tsix* (online supplementary fig. 1, www.karger.com/doi/10.1159/000341503) [Spencer et al., 2011]. In addition, *Jpx/Enox*, which resides 5′ upstream of *Xist*, activates *Xist* expression in Xi [Johnston et al., 2002; Tian et al., 2010]. Thus, coordination of *Xist/Tsix* expression, which is central to the control of XCI, is regulated by the regulatory elements around *Xist/Tsix*. *Xist/Tsix* expression is also altered by various *cis*- and *trans*-acting factors, which involve insulators and activators that bind to the elements. It is noteworthy that the regulatory elements for systematic control of the mutual interplay of *Xist/Tsix* involve one or more CCCTC-binding factor (CTCF) binding sites, suggesting that CTCF regulates *Xist/Tsix* expression as an insulator that blocks enhancer-promoter interactions or as an activator that induces transcription. However, the multivalent functions of CTCF have obscured the precise role of CTCF in *Tsix* expression. The CTCF-mediated control of *Xist/Tsix* is likely achieved by a coupled interaction of regulatory elements, but their molecular links are little known. Although the molecular aspects of the mutual adjustment between *Tsix* and *Xist* expression have been well-established in counting/choice processes of XCI, differences between counting/choice and maintenance processes concerning the molecular mechanisms associated with the regulation of *Xist/Tsix* expression remain unclear.

One approach to understand these differences is to fully define the mechanism by which the regulatory elements control *Xist/Tsix* expression during each XCI process. In this study, we investigated the binding of CTCF to the 5′ end of *Tsix* on Xa or Xi, which contains several CTCF binding sites, using the HOBMSKI2 female F_1 hybrid mouse cell line. Our results suggest that *Tsix* expression does not affect the maintenance process of XCI and that the expression of *Tsix* on Xa could be regulated by the epigenetic status around *Xist/Tsix* rather than by CTCF binding to the regulatory elements. The results also indicate the value of the HOBMSKI2 cell line in the direct allele-specific identification of X-linked gene expression based on distinct DNA polymorphisms between parental species. The cell line will be applicable for further molecular studies designed to unravel the regulatory aspects of *cis*- and *trans*-acting factors that bind exclusively to Xa or Xi.

Materials and Methods

Cell Culture and Drug Treatment

Immortalized mouse cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Korea) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in 5% CO_2 atmosphere. 5-Azacytidine (5-AzaC; Sigma-Aldrich, Korea) was dissolved in distilled water at a stock concentration of 100 μM and stored at –20°C. Trichostatin A (TSA; Amersham Pharmacia Biotech, USA) was dissolved in absolute ethanol at a stock concentration of 1 mg/ml and stored at –20°C. Cells were seeded in 100-mm culture dishes and cultured for 24 h prior to treatment with drugs. 5-AzaC was added to the medium to a concentration of 1 μM and maintained for 32 h, which was followed by 2 additional treatments with 5-AzaC at the same dose for 16 h each. TSA was added to the medium (50 ng/ml) for 32 h, which was followed by 2 additional treatments with TSA at the same dose for 16 h each.

Construction of Immortalized Mouse Cell Lines

Transgenic mice carrying the $Hprt^{b-m3}$ mutation on both X chromosomes, which were designated BM3, were originally produced by Hooper et al. [1987]. BM3 mice were from E14TG2a ES cells derived from 129/Ola mouse blastocysts and maintained as a homozygous breeding strain in the colony. We obtained F_1 hybrid progenies from a cross between a female $Hprt^{b-m3}/Hprt^{b-m3}$ BM3 mouse and an outbred $Hprt^{a}$/Y male *Mus spretus* mouse (designated MS) and selected a single female mouse from the litter (fig. 1A). The female F_1 hybrid mouse was sacrificed to obtain the kidneys. Kidneys were minced and digested using 0.05% collagenase and then placed onto 100-mm culture dishes to derive primary cultures. Fibroblasts from the tissues grew out and formed colonies and were then trypsinized into single cells after 2–3

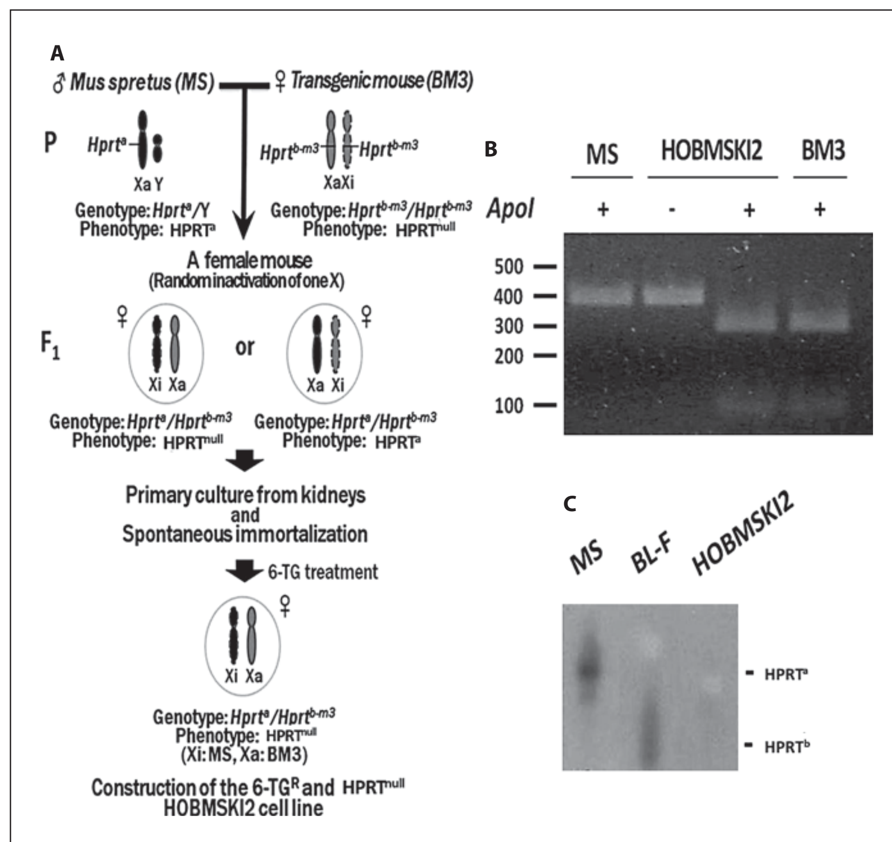

Fig. 1. Construction and characterization of the HOBMSKI2 cell line. **A** Schematic representation of the construction of the HOBMSKI2 cell line. **B** *Apo*I digests of *Hprt* products amplified by RT-PCR. The size of the RT-PCR amplicons for *Hprt* was 397 bp. The PCR products from both BM3 and HOBMSKI2 were digested into 2 fragments by *Apo*I, 301 and 96 bp, which was not observed for MS. The sizes of the fragments in base pairs are indicated to the left. **C** HPRT activity. MS, BL-F and HOBMSKI2 showed $HPRT^a$, $HPRT^b$ and $HPRT^{null}$ isoelectrofocusing patterns, respectively.

weeks. The cells were cultivated at a low density in culture dishes for 2–3 passages and spontaneously immortalized. Homogeneity in the cell population was established by 3–4 more passages after the phenotype change was first evident. The immortalized cells were mosaic for the expression of X-linked genes. In approximately half of the cells the MS X chromosome was active and the BM3 X was inactivated, while in the other half the Xa derived from BM3 and the Xi from MS. These genetically heterogeneous cells were treated with 10 μg/ml of the purine analogue 6-thioguanine (6-TG). After 2–3 weeks, only the HPRT-deficient and 6-TG^R cells (i.e. HOBMSKI2 cells) survived. The same method used for the construction of HOBMSKI2 was applied to obtain MS, BM3, C57BL/6-derived male (designated BL-M) and female (designated BL-F) immortalized cell lines.

Isoelectrofocusing and Isotopic Identification of HPRT Activity

HPRT activity in cells was estimated as previously described [Chapman et al., 1983]. HPRT isozymes were resolved on a horizontal polyacrylamide isoelectric focusing gel over a pH range of 5–8. The gels were stained as described previously using ^{14}C-labeled hypoxanthine (DuPont, USA) [Chasin and Urlaub, 1976].

Chromatin Immunoprecipitation (ChIP) Assays for CTCF and MeCP2

For the ChIP assays, 1×10^6 cells were harvested and treated with a protease inhibitor cocktail (Roche, Germany). ChIP was performed using a ChIP assay kit (Millipore, USA) according to the manufacturer's instruction. Antibodies (5 μg) for MeCP2 (Santa Cruz Biotechnology, USA) and CTCF (Bethyl, USA) were used. Experimental controls without antibodies were also performed to demonstrate specific binding to the antibodies. The sites amplified by polymerase chain reaction (PCR) are illustrated in online supplement figure 1. ChIP of site A of the 5′ end of *Tsix* utilized first round primers (forward-1st 5′-TGAGATAGGCTAAGGCACAGAGTA-3′ and reverse-1st 5′-ACAAGCGCAAGAAAGAAACCATT-3′) and nested primers (forward-2nd 5′-GTGTGTCATAGCTAAGAGG-3′ and reverse-2nd 5′-GGAGCCTAAACCTGTGTCTGTC-3′) [Chao et al., 2002]. Forward primer 5′-AGAGCGGAGCGGTGGGTGAG-3′ and reverse primer 5′-TCCCCGGTGGTAGGCATTTTAGTA-3′ were used for PCR-amplification of site C of *Tsix*. PCR-amplifications of *Zrsr1* and PCT12 were performed using previously described primers [Ishihara and Sasaki, 2002].

RNA Purification and Reverse Transcription-PCR (RT-PCR)

Total RNAs were extracted from cultured cells using the High Pure RNA isolation kit (Roche, Germany) according to the manufacturer's protocol. PCR was carried out with an initial denaturation at 94°C for 5 min, followed by 42 cycles at 94°C for 40 s, 55°C for 40 s and 72°C for 40 s. The PCR products were separated by 1% agarose gel electrophoresis and visualized using ethidium bromide staining. The primers used for *Tsix* RT-PCR were forward 5′-TAGGCGTCCCATGAATAATAAAG-3′ and reverse

5′-TCTCTAGCATCCCCACAAAAAT-3′. For RT-PCR of *Gapdh*, forward 5′-TGCCCCCATGTTTGTGATG-3′ and reverse 5′-TGTGGTCATGAGCCCTTCC-3′ primers were used. The primers used in RT-PCR of *Hprt* are forward 5′-TTAAAGCACTGAATAGAAAT-3′and reverse 5′-GCTTTTCCAGTTTCACTAATG-3′.

RFLP Assay of PCR-Amplified Products
PCR products from *Hprt* and site A of *Tsix* were digested with *Apo*I (TAKARA, Japan) and *Mva*I (TAKARA, Japan), respectively. RFLP for *Tsix* site A was performed on the PCR products amplified using the nested primers. Enzyme-digested PCR products were separated by 1% agarose gel electrophoresis and visualized using ethidium bromide staining.

Results

Construction and Characterization of HOBMSKI2
We examined whether HOBMSKI2 possessed Xa from BM3 and Xi from MS by treating the cells with hypoxanthine, aminopterine and thymidine (HAT) medium, which kills HPRT-negative cells. No cells survived after 2–3 weeks of HAT treatment, indicating that HOBMSKI2 had no HPRT activity and consisted of cells that homogeneously harbored BM3 Xa and MS Xi. In addition, the *Apo*I-RFLP assay demonstrated that the *Hprt* allele was exclusively expressed from Xa of BM3 (fig. 1B). The RT-PCR assay for *Hprt* amplification in HOBMSKI2 resulted in an amplicon that was 397 bp in length. The PCR products were digested into 301 and 96 bp by *Apo*I, which was not the case in MS, indicating that the PCR products were from only BM3. Since exons 1 and 2 were deleted from BM3, we used a forward primer targeting base pairs 391 to 410 of exon 3 and a reverse primer targeting base pairs 767 to 787 of exon 9 for *Hprt* amplification. The results of these experiments suggested that BM3 of HOBMSKI2 could transcribe $Hprt^{b\text{-}m3}$ RNA although the translated $HPRT^{b\text{-}m3}$ protein was not structurally normal [Bressler et al., 1993]. It was also confirmed that the HOBMSKI2 cell line showed no HPRT activity in the isoelectrofocusing assay, while MS and BL-F expressed $HPRT^{a}$ and $HPRT^{b}$, respectively (fig. 1C). These results strongly suggested that HOBMSKI2 had an Xa from *Hprt*-deficient BM3 and an Xi from normal $Hprt^{a}$ MS. Karyotype analysis demonstrated that HOBMSKI2 was near-diploid in chromosome number and showed no considerable structural aberrations (data not shown).

Absence of Expression of Tsix *on Xa in Somatic Cells*
We examined *Tsix* expression in the BL-M and BL-F mouse cell lines. RT-PCR assays for BL-M and BL-F produced no positive bands at the expected size (data not shown), consistent with the previous results [Stavropoulos et al., 2005]. The results indicated that the somatic cell lines did not express *Tsix*. In undifferentiated ES cells, *Tsix* expression ensures the active status of the future Xa by blocking *Xist* expression [Tian et al., 2010]. However, presently *Tsix* expression was completely blocked by an unknown mechanism in somatic cells, suggesting that *Tsix* might function differently concerning the regulation of *Xist* on Xa between somatic and undifferentiated ES cells (i.e. between the counting/choice and maintenance processes of XCI).

Monoallelic Binding of CTCF to the 5′ End of Tsix *on Xa in Somatic Cells*
Since CTCF was shown to function as an insulator of *Xist* in the activation of *Tsix* expression, we next assayed whether CTCF still bound to the 5′ end of *Tsix* in vivo, using BL-M and BL-F somatic cell lines which do not express *Tsix*. In the 5′ end of *Tsix*, there are 4 regions for CTCF binding (see online suppl. fig. 1). Chao et al. [2002] showed that CTCF bound to the A and C sites using ChIP assay in female mouse fibroblasts. They, however, did not examine the binding of CTCF to B and D sites, because those sites are tandemly repetitive. We tried to examine the binding of CTCF to the B and D sites but could not obtain any specific PCR amplicons using primers within B and D sites, because those sites consist of several tandemly repeated CCCTC regions which correspond to the CTCF binding site. That is, such tandemly repetitive CTCF binding sites within the B and D sites, which span over 2 kb, prohibited us from obtaining the specific PCR products, resulting in the varied-sized PCR products due to sequence homology among several template regions hybridized with primers. In addition, it was also found that primers, which were hybridized with the outside sequence from 5′ and 3′ of the B and D sites and span over 2 kb in the expected size of amplicon, could not produce the specific PCR products because the templates for PCR ranged from 300 bp to 1 kb in size after being sheared by sonication. For those reasons, we selected only the A and C sites of the 4 CTCF binding sites and examined the binding of CTCF to the sites using ChIP assay.

ChIP assay was performed with goat polyclonal anti-CTCF IgG (fig. 2A). A 140-bp fragment from site A and a 593-bp fragment from site C of *Tsix* were amplified from immune-precipitated DNA in both BL-M and BL-F, indicating that CTCF was recruited to those regions. The obtained PCR products from sites A and C suggested that CTCF still bound to *Tsix* in BL-M and BL-F somatic cells,

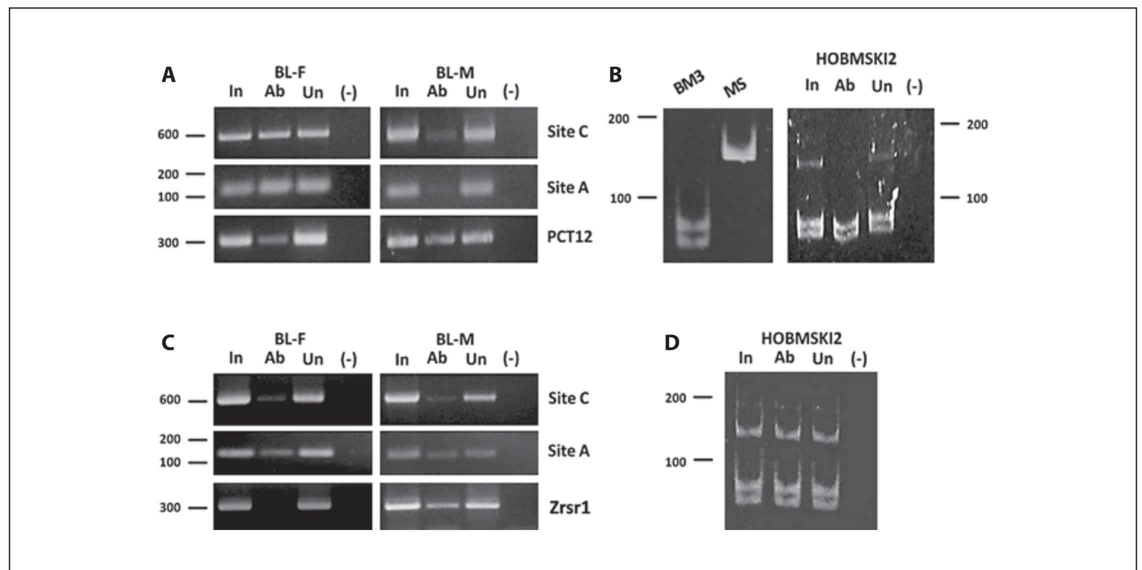

Fig. 2. ChIP for binding of CTCF and MeCP2 to sites A and C of *Tsix* on Xa in somatic cell lines. **A** ChIP assays with primers for sites A and C of *Tsix* and with antibodies against CTCF in BL-F and BL-M cell lines. PCR products for sites A and C were 362 and 593 bp in size, respectively. For amplification of site A, nested PCR was performed using the 2nd-forward and 2nd-reverse primers after the first round of PCR. The amplified products were 140 bp in size. PCT12, the potential CTCF-binding site between H19 and L23 mitochondrial-related protein (L23mrp), was used as a positive control to confirm the reliability of the experiments. **B** Nested PCR-RFLP for site A was carried out after the ChIP assay and the products were digested using the restriction enzyme *Mva*I. Products obtained from the nested PCR of HOBMSKI2 were digested using *Mva*I, which produced 2 fragments of 74 and 66 bp in size. **C** ChIP assays with primers for sites A and C of *Tsix* using antibody against MeCP2 in the BL-F and BL-M cell lines. ChIP assay for zinc finger, RNA-binding motif and serine/arginine rich 1 *(Zrsr1)*, which has been reported to be associated with MeCP2, was used as a positive control to confirm the reliability of experiments. **D** Nested PCR-RFLP for site A was carried out after the ChIP assay using the 2nd-forward and 2nd-reverse primers and the restriction enzyme *Mva*I. Products obtained from the nested PCR were digested by *Mva*I, which produced 2 fragments of 74 and 66 bp in size. *Mva*I-RFLP of Ab-ChIP clearly showed both BM3 and MS patterns in HOBMSKI2. In = input control presenting amplification in sheared chromatin; Ab = PCR products from amplification of chromatin bound with the antibody against CTCF; Un = unbound control presenting amplification from chromatin remained in supernatant after precipitation with salmon sperm DNA-protein A agarose slurry with CTCF antibody; (-) = antibody-free control was amplified from chromatin that had been precipitated by a salmon sperm DNA-protein A agarose slurry devoid of antibody against CTCF.

corresponding to the maintenance process of XCI, even though *Tsix* is not expressed in these cells. Unbound control was amplified from chromatin that was not precipitated with antibody against CTCF. The antibody-free control was amplified from chromatin that had been precipitated by a salmon sperm DNA-protein A agarose slurry devoid of antibody against CTCF, which resulted in an absence of non-specific precipitated chromatin.

Considering that CTCF bound to *Tsix* in both male and female somatic cells, it was thought that CTCF could bind to *Tsix* on Xa alone or on both Xa and Xi. Thus, we used the HOBMSKI2 cell line to determine if CTCF could bind to *Tsix* on Xa or Xi. ChIP assay for site A of *Tsix* was followed by the *Mva*I-RFLP assay (fig. 2B). The HOBMSKI2 DNA to which CTCF antibody bound showed only a BM3 *Mva*I-digested pattern. This result demonstrated that CTCF exclusively bound to only the A site of BM3 X in HOBMSKI2, implying that CTCF bound to the 5′ end of *Tsix* on only Xa in somatic cells, corresponding to the maintenance process of XCI. The results were consistent with the idea that CTCF might exert different functions in regard to *Tsix* expression between the counting/choice and maintenance processes.

Biallelic Binding of MeCP2 to the 5′ End of Tsix *in the Maintenance Process of XCI*

Since a mutation in methyl CpG binding protein 2 gene *(Mecp2)* leads to nonrandom XCI, MeCP2 is thought to be an epigenetic factor controlling XCI [Takagi, 2001]. Appropriately, we assessed whether MeCP2 bound to the

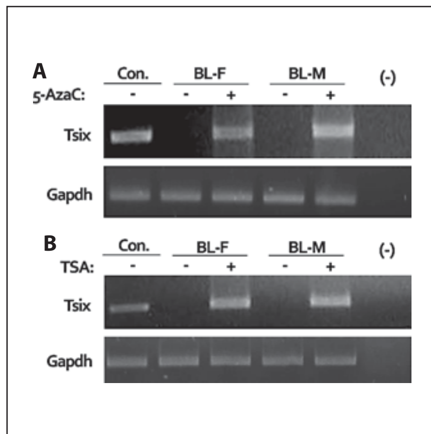

Fig. 3. RT-PCR for *Tsix* amplification after treatment with 5-AzaC (**A**) and TSA (**B**) in somatic cell lines. *Tsix* expression was restored in both cell lines treated with either 5-AzaC or TSA, indicating epigenetic control of *Tsix* expression. + and - indicate the RT-PCR results from cells treated with and without chemicals, respectively. PCR products were 550 bp in size. P19, a male mouse embryonal carcinoma cell line, was used as a positive control (Con) for *Tsix* expression. *Gapdh* was used as positive control for RT-PCR. (-) indicates the PCR negative control, which was performed without templates.

5′ end of *Tsix* and if this binding inhibited *Tsix* expression during the maintenance process of XCI in somatic cells. In these experiments, MeCP2 bound to the 5′ end of *Tsix* in both BL-M and BL-F somatic cells (fig. 2C). This result suggested that MeCP2 might directly or indirectly block *Tsix* expression by the formation of a protein-protein complex with epigenetic factors. However, whether the binding was restricted to only Xa or whether it occurred on both Xa and Xi was not resolved.

To determine if MeCP2 bound to the 5′ end of *Tsix* on Xa or Xi, the ChIP assay was performed using goat polyclonal IgG against MeCP2 in HOBMSKI2. *Mva*I-RFLP showed that both MS and BM3 alleles were produced from input control representing amplification from input chromatins, unbound control and MeCP2-antibody (fig. 2D). This result indicated that the amplified products originated from both BM3 and MS alleles, implying that MeCP2 bound to the 5′ end of *Tsix* on both Xa and Xi.

Re-Expression of Tsix *by Change of the Epigenetic Status*

MeCP2 bound to the 5′ end of *Tsix* of both Xa and Xi, implying that the role of CTCF for upregulating *Tsix* expression on Xa might be blocked by binding of MeCP2 to the 5′ end of *Tsix* and subsequent recruiting of epigenetic factors to the region. To determine whether MeCP2 inhibited *Tsix* expression in somatic cells, RT-PCR was carried out after the cells were treated with the demethylating agent 5-AzaC, which was expected to induce the release of epigenetic factors, including MeCP2, from the 5′ end of *Tsix*. Indeed, *Tsix* was re-expressed in both BL-M and BL-F cell lines when the cells were exposed to 5-AzaC (fig. 3A). Thus, it was likely that *Tsix* on Xa lost its transcriptional activity due to epigenetic changes, including methylation at the 5′ end of *Tsix*, which occurred during the maintenance process of XCI.

MeCP2 interacts with co-repressor molecules such as SIN3A and histone deacetylase (HDAC) to inhibit gene expression. To test the functional relevance of HDAC in the context of MeCP2-mediated *Tsix* repression, we examined *Tsix* expression in the presence of TSA, a known inhibitor of HDAC activity. *Tsix* was expressed in both BL-M and BL-F cells treated with TSA (fig. 3B). These results indicated that MeCP2 could epigenetically block *Tsix* expression by forming a complex with co-repressors, including HDAC, in the maintenance process of XCI.

Discussion

Xite, Jpx, DXPas34 and *RS14*, which are regulatory elements for *Xist/Tsix* expression, lie in the genomic regions around *Xist/Tsix* and form the CTCF-mediated chromatin loops with *Xist/Tsix* [Stavropoulos et al., 2005; Spencer et al., 2011]. Since there are so many CTCF binding sites at the regulatory elements around *Xist/Tsix* regions, it was assumed that CTCF plays an important role in the regulation of *Xist/Tsix* (fig. 4). Indeed, CTCF is a *trans*-acting factor that plays an important role in the XCI process [Chao et al., 2002]. However, presently, the function of CTCF on Xa was blocked by epigenetic factors which formed a protein complex with MeCP2 in somatic cells during the maintenance process of XCI. Put another way, CTCF lost the capability of up-regulating *Tsix* in somatic cells, suggesting that blocked *Xist* expression in Xa could persist without CTCF-mediated *Tsix* up-regulation after XCI had been initiated. Since CTCF is multifunctional in various genomic regions, the function of CTCF at specific target sites has proved difficult to define [Lutz et al., 2000]. Presently, CTCF showed a process-limited function in *Tsix* during XCI. CTCF induced *Tsix* up-regulation during counting/choice processes, but its action decreased after the initiation process of XCI had begun and was terminated in the maintenance process.

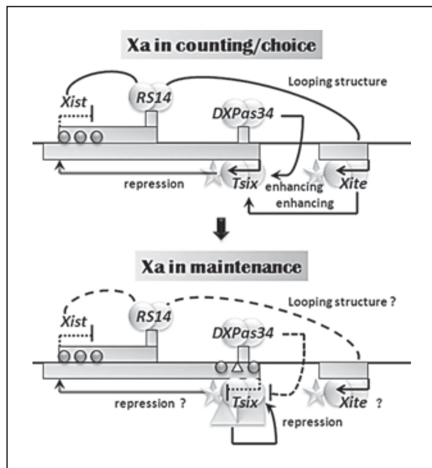

Fig. 4. Model for process-dependent control of XCI by cooperative interaction of regulatory elements in Xic. Mutual regulation of *Xist/Tsix* requires many *cis-* and *trans*-acting regulatory elements which lie in Xic. Regulatory elements including *Xist/Tsix/Xite/RS14/DXPas34* were regulated not only by DNA-binding proteins such as CTCF and OCT4 but also by epigenetic modifications. However, the comprehensive interplay between regulatory elements with *cis-* and *trans*-acting factors that modulate the epigenetic environment was obviously different between the counting/choice and maintenance processes of XCI. Small circles = methylation; large circle = CTCF; large triangle = MeCP2; small triangle = acetylation; square = HDAC; star = OCT4.

It is still not clear why *Tsix* was not expressed on Xa of differentiated ES and somatic cells, since CTCF still bound to *Tsix* on Xa. The difference in the function of *Tsix* on Xa concerning *Xist* down-regulation before and after differentiation implies that blocking of *Xist* expression might not be achieved by persistence of *Tsix* transcription during the maintenance process, but rather by a change in the epigenetic environment due to the binding of epigenetic factors including MeCP2 and HDAC to the 5′ end of *Tsix*. To ascertain whether MeCP2 could bind *Tsix*, a ChIP assay was performed using HOBMSKI2. This analysis showed that MeCP2 bound to the 5′ end of *Tsix* in both Xa and Xi (fig. 2D). In addition, MeCP2-mediated blocking of *Tsix* expression was eliminated by 5-AzaC and TSA, suggesting that MeCP2 might play a role as a transcriptional repressor against *Tsix* on Xa in a methylation-dependent manner and by interaction with HDAC (fig. 3).

Together, our data implies that CTCF cannot induce *Tsix* expression in the maintenance process of XCI and that blocking of *Xist* expression on Xa might be achieved not by *Tsix* expression, but rather by a change in the epigenetic status caused by DNA methylation and histone methylation/acetylation and subsequent binding of epigenetic factors (fig. 4). However, it is still not known how the regulatory elements control the mutual interplay of *Xist/Tsix* in a process-dependent manner and how the epigenomic environment around *Xist/Tsix* is modulated by *cis-* and *trans*-acting factors.

We previously reported, using an interspecies F_1 hybrid mouse cell line, that *Zfx*, *Rps4* and *Ube1* are all subject to XCI, but that these 3 genes escape from XCI in humans [Bressler et al., 1993]. Thus, it is obvious that these interspecies mouse cell lines are useful resources for defining the allele-specific expression of X-linked genes. Indeed, HOBMSKI2 will provide the opportunity to identify the novel regulatory factors for XCI and to unravel the molecular mechanism of the XCI process.

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