Identification of regulatory elements flanking human XIST reveals species differences

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Abstract

Background: The transcriptional silencing of one X chromosome in eutherians requires transcription of the long non-coding RNA gene, XIST. Many regulatory elements have been identified downstream of the mouse Xist gene, including the antisense Tsix gene. However, these elements do not show sequence conservation with humans, and the human Tsix gene shows critical differences from the mouse. Thus we have undertaken an unbiased identification of regulatory elements both downstream and upstream of the human XIST gene using DNase I hypersensitivity mapping.

Results: Downstream of XIST a single DNase I hypersensitive site was identified in a mouse undifferentiated ES cell line containing an integration of the human XIC region. This site was not observed in somatic cells. Upstream of XIST, the distance to the flanking JPX gene is expanded in humans relative to mice, and we observe a hypersensitive site 65 kb upstream of XIST, in addition to hypersensitive sites near the XIST promoter. This -65 region has bi-directional promoter activity and shows sequence conservation in non-rodent eutheria.

Conclusions: The lack of regulatory elements corresponding to human TSIX lends further support to the argument that Tsix is not a regulator of XIST in humans. The upstream hypersensitive sites we identify show sequence conservation with other eutheria, but not with mice. Therefore the regulation of XIST seems to be different between mice and man, and regulatory sequences upstream of XIST may be important regulators of XIST in non-rodent eutheria instead of Tsix which is critical for Xist regulation in rodents.

Background

X-chromosome inactivation results in transcriptional silencing of one of the two X chromosomes in female mammals, thereby ensuring dosage equivalence of most X-linked genes between males and females. The X inactivation centre (XIC) is a single locus on the X chromosome that is required in cis for X-chromosome inactivation [1]. The XIC contains the X Inactive Specific Transcript (XIST/Xist) gene that produces a large (approximately 17 kb) non-coding RNA [2-4] that is necessary and sufficient to induce X inactivation [5-7]. X inactivation occurs very early in mammalian development, making analysis of the initial events challenging, particularly in humans. Most analyses have therefore been done in mouse, where ES cells not only provide an in vitro system modelling X inactivation, as female ES cells undergo random X inactivation upon differentiation [8]. Detailed analyses of the genomic region downstream of the mouse Xist locus have revealed several cis-acting regulatory elements for Xist (see Figure 1A), including Tsix [9], DXPas34 and Xite [10].

The Tsix gene encodes an untranslated RNA antisense to Xist that is transcribed across the Xist locus, extending beyond the promoter of the sense strand [9,11]. A critical role for Tsix in regulating Xist expression was demonstrated by augmented Tsix expression resulting in inhibition of Xist accumulation [12] while deletion of the Tsix promoter resulted in primary non-random inactivation of the mutant X in females [13]. DXPas34, a 1.2 kb CG-rich region located 750 bp downstream of the Tsix major promoter, is critical for Xist and Tsix regulation, and shows bi-directional promoter activity [14,15]. Xite, named as the X-inactivation intergenic transcription element, was identified by DNase I
Figure 1  Mapping DNase I hypersensitive sites downstream of XIST. A schematic of the mouse (A) and human (B) Xist/XIST genes and surrounding regulatory elements shows exons (black boxes) for Xist/XIST and other genes in the region (arrows indicate the direction of transcription). Grey dots show the location of conserved blocks between mouse and human [15], and triangles mark DNase I hypersensitive (DHS) sites (filled triangles - sites in undifferentiated cells; empty triangles - sites in differentiated cells; HS1, HS5 - HS7 [40], HS2 - HS4 [46,47], HS8 - HS15 [55]). DNA from nuclei of ES-10, L1.10.1 and Xa and Xi-containing hybrids exposed to DNaseI was digested with restriction enzymes (ScaI (10 kb); SapI/BpiI (10.2 kb); NstI (11.1 kb); XmnI (9.3 kb); NcoI (11.1 kb) and BamHI (6.4 kb)) for Southern analysis. All experiments were carried out at least twice and a representative Southern blot is shown for each fragment including a lane with no DNase I (parental fragment shown as black arrow) followed by six lanes of increasing amount of DNase I treatment. One HS site (grey arrow), was detected in the SapI/BpiI fragment of ES-10 cells, with a size of ~7 kb. No HS site was detected in the XmnI fragment which encompasses the four previously reported transcription start sites of TSIX [32]. No HS site was detected in either the Xa or Xi hybrid downstream of XIST.
hypersensitive transcription, and is also a site of bi-directional transcription, although the transcripts themselves are not necessary for Xite to promote Tsix persistence on the active X [10]. Homologous pairing of a region downstream of Xist, encompassing Tsix, DXPas34 and Xite, is necessary for the initiation of X inactivation [16,17]. This pairing can be recapitulated by subfragments which contain a high density of CTCF sites, and CTCF as well as transcription is essential for the establishment of pairing [16].

While the mouse system provides an excellent framework from which we can understand the process of X inactivation, how human fits into this framework is not clear. XIST and Xist share sequence homology [3,4] and are each sufficient to initiate silencing [18,19]; however, there are substantial differences between the two species in other aspects of X-chromosome inactivation. Significantly, X inactivation is imprinted in mouse extraembryonic tissues [20] but not in humans (reviewed in [21]) and Tsix is very different from TSIX in patterns of expression and extent of transcription across the sense strand [22-24]. Human TSIX has been observed in embryoid bodies and a human embryonal carcinoma cell line, but also in chorionic villus cells [22,24]. TSIX is also expressed in mouse ES-10 cells carrying a multi-copy integration of a 480 kb XIST-containing human YAC transgene [25] and in L1.10.1, a clone of a somatic male cell line transfected with a human XIST-containing PAC, which contains at least 50 kb of flanking genomic DNA [26]. While these cells did not show identical initiation sites for the TSIX transcript, in all cases the TSIX transcript truncated well before the 5′ end of XIST. As human ES cells have been variable in their X inactivation status (e.g. [27]), mouse ES cells with a human transgene remain one of the best models for human X inactivation. Multi-copy integrations of the 480-kb transgene containing human XIST display partial X inactivation center function upon in vitro differentiation of male mouse embryonic stem cells, including activation in some cells of the endogenous mouse Xist locus [28,29].

Given the importance of XIST for X inactivation, it is perhaps surprising that the X inactivation center region shows little sequence conservation surrounding XIST between mouse and human [30,31]. In addition to XIST, the region contains several conserved genes and a number of non-coding RNAs and pseudogenes. Downstream of Xist the closest gene is the testes-specific Tsx gene in mouse, however TSX is a pseudogene in human [32]. The closest gene upstream of XIST is the Jpx (also known as ENOX) non-coding RNA gene. Interestingly, the region between Jpx and XIST is ~90 kb in human, which is approximately 9 times larger than that of mouse. Thus rearrangements downstream of XIST where the mouse regulatory elements are found may have been compensated for by regulatory regions upstream of the gene. To identify such regulatory elements in the absence of substantial sequence conservation we have used DNase I hypersensitive site mapping as an unbiased approach to identify cis-acting regulatory elements in humans. Genomic regions hypersensitive to DNase I digestion (DHS sites) have been shown to harbour cis-regulatory elements critical for gene regulation [33], and both of the mouse Xist regulatory regions DXPas34 and Xite show DNase I hypersensitivity [12]. Here, we report the identification of three previously unknown hypersensitive sites surrounding the human XIST locus.

**Results**

**Identification of DHS sites 3′ to human XIST**

The critical timing for XIST regulation is early in development and thus for mapping of DHS sites 3′ to human XIST we used the mouse undifferentiated embryonic stem cell line ES-10 which contains a human XIST transgene. We also examined the L1.10.1 cell line which is a somatic male HT1080 cell line transfected with a PAC containing the XIST region, and which expresses XIST as well as TSIX. Additionally, we examined mouse/human somatic cell hybrids containing either the human active (Xa) or inactive (Xi) chromosome, which provide a unique opportunity to compare the chromatin structure between the Xi and Xa independent of each other without requiring allele-specific detection. We generated three probes downstream of XIST that were able to detect six overlapping restriction fragments allowing the analysis of a 43 kb region (see Figure 1B). Only one DHS site, located approximately 12 - 13 kb downstream of XIST, was identified in the ES-10 cells. This DHS site does not correspond to the cluster of four TSIX transcription start sites described in these cells, which are located approximately 14 kb further from the 3′ end of XIST [25]. The DHS site is close to the antisense transcription start in L1.10.1 cells. Interestingly, however, the DHS site was not found in L1.10.1 (Figure 1B), nor were any others, despite previous detection of antisense transcript both with RT-PCR and FISH in this cell line [24]. We confirmed the ongoing presence of antisense transcript in the L1.10.1 cells used for DHS mapping by RT-PCR (data not shown). DHS mapping in both Xi and Xa hybrids did not show the presence of any DHS site downstream of XIST, including at the region identified to contain a DHS in ES-10 cells (Figure 1B). It thus appears that, unlike the situation in mouse where there are both developmental-specific and constitutive DHS sites downstream of Xist, in humans there is only a single developmental-specific DHS site downstream of XIST.
Identification of DHS sites 5' to human XIST

The lack of putative regulatory elements 3' to XIST, where many of the mouse transcriptional regulatory sites are located, led us to examine the region 5' to XIST which is larger in humans than it is in mouse [30]. We generated three probes to examine the region upstream of human XIST for DHS sites. Almost 80% of the just over 90 kb region upstream of XIST is comprised of repetitive elements, predominantly LINE1 (39.5%) and ALU (27.3%) as identified by repeatmasker (http://www.repeatmasker.org/[34]). This high repeat content precluded examination of the entire region and the restriction fragments assessed by the three probes interrogate a total of 39 kb in the 90 kb region.

We again performed Southern analysis with DNA isolated after increasing DNase I treatment from cells of ES-10, an Xa-containing, and an Xi-containing somatic cell hybrid (Figure 2). The presence of multiple bands for proximal but not distal probes in ES-10 can be explained by the individual copies of the XIST transgene present in the multi-copy integration transgenes not containing the same amount of DNA sequence flanking the XIST locus. We observed one or more DHS site(s) immediately upstream of XIST in ES-10, as well as in the Xa-containing hybrid where XIST is silenced. These sites were observed variably in Xi-containing hybrids where XIST continues to be expressed (Figure 2). While no other hypersensitive sites were detected upstream of XIST in ES-10, one DHS site was found approximately 65 kb upstream of the XIST transcription start on both the active and inactive X chromosomes in somatic hybrids (Figure 2).

The location of the -65 DHS site was refined by repeating the DHS mapping with two other restriction enzyme digests for the Xa-containing hybrid (Figure 3A), thereby refining the location of the site to between 73,053,323 and 73,053,866 (hg18). A dot-plot sequence comparison of human to cow, and human to mouse sequences showed that within the region approximately 60 kb upstream of XIST there is a region of

![Figure 2 DNase I hypersensitivity mapping upstream of XIST. Below the schematic of the XIST region (see legend in Figure 1) are Southern blots of DNA from ES-10, an Xa hybrid, and an Xi hybrid. Small rectangular boxes are probes for Southern blotting of overlapping restriction fragments (from left to right: PstI (13.1 kb); BsmI (10.3 kb); NdeI (7.2 kb); SapI (6.7 kb); Spel (6.3 kb); Scal (3.8 kb)). All experiments were carried out at least twice. One HS site, highlighted by the open arrow, was detected just upstream of the transcriptional start site, within the Spel fragment, at somewhat variable positions in the three cell lines. Another HS site was found within the BsmI fragment approximately 65 kb upstream of the XIST transcription start in both Xa and Xi hybrids (grey arrow). No other DNase I hypersensitive sites were observed.](image)
approximately 10 kb that is relatively conserved between human and cow, but not mouse (Figure 3B and 3C). This region is also conserved in dog (data not shown). The novel -65 DHS site identified with both the Xa and Xi in somatic hybrids is located within this conserved region.

To determine the biological relevance of this conserved sequence that results in a DHS in somatic cells we cloned several regions of XIST into the pGL4 series of plasmids to assay promoter and enhancer activity by monitoring luciferase reporter activity after transient transfection into HT1080 somatic cells (Figure 4). We cloned three ~700 bp regions of the XIST promoter region (named -3, -2, -1) as well as a 1,087 bp region at the -65 DHS site in both orientations. There was a notable orientation bias for regions around the XIST promoter, and in fact -3 could not be cloned in one orientation. As pGL4.10 lacks a minimal promoter, assaying luciferase activity monitors promoter activity; while pGL4.23 contains a minimal promoter and therefore monitors enhancer activity. In both assays the -65 region showed significant activity, when cloned in both orientations. Thus, like many of the mouse regulatory regions, the -65 DHS seems to have bi-directional promoter and enhancer activity.

Discussion

X-chromosome inactivation in both humans and mice requires the presence in cis of the X-inactivation center, and the XIST/Xist gene contained therein. Interestingly, the XIST gene appears to have evolved in eutherians from a protein-coding gene, Lnx3 [31], and while critical regions such as the 5’ A repeats required for silencing are conserved [19,35], other regions of XIST are more variable amongst the eutheria [36]. Extensive studies in mouse models have defined a wide variety of regulatory elements for Xist including Tsix, DXPas34 and Xite. These elements are all located 3’ to Xist, between Xist and the adjacent testes-specific Tsx gene. In humans TSX is a non-expressed pseudogene, and the blocks of sequence conservation previously reported between humans and mice 3’ to XIST are ancestral TSX exons [31,32]. The human TSX region lacks an equivalent to the mouse CpG island that was shown to be essential for function of Tsix [13,32,37]. Other significant differences between mouse and human Tsix/TSIX include a lower level of human TSIX transcription and termination of human TSIX prior to the XIST promoter [24,32], while antisense transcription across the mouse Xist promoter region is necessary for antisense function [38]. In addition, Migeon et al. (2002), using RNA FISH for cellular localization of transcripts, showed that human TSIX transcripts are co-expressed with XIST from the inactive X throughout human embryonic development,
suggesting that this antisense is unable to repress XIST [22], leading to the argument that Tsix regulation of Xist may be specific to mouse [39].

Upstream of XIST/Xist the adjacent gene is JPX/ENOX, which is conserved in humans and mice [30], although the distance to the JPX CpG island and first exon is larger in humans (~90 kb) than in mice (less than 10 kb) [30]. JPX is a non-coding RNA gene including different repetitive elements in different species, and the promoter of the mouse Jpx gene has been shown to interact with the Xist promoter in undifferentiated ES cells [40]. No sequence conservation between humans and mice is found in the region between Jpx and Xist; however, there is conservation between humans and cows (and also dogs, data not shown), including an exon of the Ras11c pseudogene [31].

The lack of sequence conservation between human and mouse in the region flanking XIST/Xist, as well as the differences in the TSIX/Tsix transcript, led us to undertake this study to identify potential regulatory elements for XIST by using DHS mapping both downstream and upstream of XIST. Downstream of XIST in the region where most regulatory elements are observed for mouse Xist we find only a single DHS, and this is only observed in undifferentiated mouse ES cells containing a human XIC transgene. While this site mapped close to one of the previously described human TSIX transcription start sites, the presence of the DHS did not correlate with TSIX transcription, and thus we consider it more likely that this DHS is reflective of a developmental event not regulating antisense transcription. As previously reported there is no substantial sequence conservation between cow, human and mouse downstream of XIST [24,30], and dog also fails to show homologous regions (data not shown). In this region downstream of XIST the human sequence is enriched for LTR class repetitive elements, while mouse and dog are enriched in LINE elements. It has been demonstrated that in addition to a cis-regulatory role in XIST regulation, the mouse Tsix and Xite regulatory elements are also involved in a trans-regulation involving a transitory pairing of homologous X chromosomes proposed to establish the mutually exclusive choice of the future active and inactive X chromosomes in females [41]. As
the human XIC transgene in the ES-10 cells studied is capable of inducing expression of the single Xist gene in these male ES cells [29], it is plausible that the DHS identified in the transgene 3’ to XIST is involved in such trans-interactions. There is now genome-wide mapping of DNase I hypersensitive sites [42] as well as histone modifications and CTCF binding sites, which often mark promoters and enhancers [43,44] in a number of cultured cell lines or human CD4+ cells. The genomewide mapping of CTCF sites in somatic cells did not identify any enrichment in the region of this 3’ DHS. However, we also did not observe this DHS in somatic cells. Enhancer sequences have been reported to show cell-type specific patterns [45], so this region may contain an enhancer specific to undifferentiated cells.

We found evidence for DHS sites near the transcriptional start site of XIST in both somatic and ES-10 cells. XIST is expressed in the ES-10 cells prior to differentiation, however it is not expressed in the Xa-containing hybrid cells, thus these sites are observed independent of XIST expression. We did not refine the localization of these sites, as they likely correspond to the minimal XIST promoter and regulatory elements. The minimal mouse promoter has been shown to have a cluster of DHS sites [46,47]. In agreement with the previously defined human minimal promoter [48] we detected strong promoter activity in our transient luciferase assay using 1 kb of DNA surrounding the XIST transcription start site (data not shown). Although a CTCF binding site has previously been defined at -43 bp of the XIST promoter [49], genome-wide mapping in CD4+ T cells only identified CTCF binding further upstream [43]. This would correspond to our fragment 3 which showed limited promoter or enhancer activity in the ‘reverse’ orientation, but was unable to be cloned in the ‘forward’ orientation where it would be aligned with the test promoter in the same orientation it is aligned to XIST in the human genome. Genome-wide mapping of DHS sites identified a DHS site approximately three kb upstream of the promoter, in the vicinity of the CTCF site, as well as sites further within XIST that our analysis would not have detected [42]. Thus it appears that there is a regulatory element for human XIST ~three kb upstream of the XIST promoter which includes CTCF binding sequences.

Further 5’ to XIST we find a DHS site in a region sharing sequence conservation with cow and dog (data not shown). A DHS site in this region can also be observed in the genome-wide mapping in CD4+ T cells, and furthermore genome-wide H3K4me1 enrichment, which is characteristic of enhancers, flanks the site [45]. We refined the location of the DHS to between 73,053,323 and 73,053,866, while the DHS site identified by the global mapping is slightly proximal at 73,053,237- 73,053,138. This might reflect subtle discrepancies in mapping, or between cell types. The region cloned for subsequent enhancer and promoter activity analysis was 73,054,787 -73,053,643 and would contain the H3K4me1 marked regions [45] and the majority of the region to which our DHS site was mapped, but be just upstream of the DHS site mapped in CD4+ cells [42].

Overall we find fewer regulatory elements in human than have been identified in mice. This could be due to higher repetitive element content in human which made analyzing the whole region challenging. Furthermore, the repetitive elements themselves might harbour regulatory elements. Indeed, the TSIX transcription starts mapped by Migeon et al. were to MERS5B, AluY and L2 class repetitive elements [32], and conservation of repetitive elements between the mouse Tsix transcription start and humans was noted by Cohen et al. [15]. While acquisition of repetitive elements may have led to an expansion of the XIC region in humans compared to mice, the conservation upstream of XIST between humans and other eutheria, including homology to an exon of the Rasl gene of chicken [31] suggests that this region was likely lost in rodents.

While many XIST regulatory elements do not appear to be conserved between humans and mice, many of the basic events required for X-chromosome inactivation must be conserved (reviewed in [21]). The 3’ DHS site might demarcate a developmental-specific regulatory region that participates in the trans pairing interactions involved in initiation [17,41]. It has been proposed that a critical function of TSIX is to partition chromatin domains in the XIC [50]. Perhaps the 5’ regulatory regions we have identified are capable of recapitulating such a function in humans. However we did not observe a consistent difference between the active and inactive X chromosomes for these DHS sites, and genome-wide chromatin mapping in somatic cells does not show evidence for a chromatin domain ending at the -65 DHS. Ultimately testing whether human X inactivation involves regulatory processes related to those detailed in mouse will require the challenging investigation of human XIST expression during early development. It has been shown that X chromosome inactivation is initiated in human preimplantation embryos [51]; however, human ES cells have shown considerable variability (e.g. [27]), making mouse ES cells with human XIST transgenes one of the best current models to study regulation of human XIST.

Conclusions
DNase I hypersensitivity mapping around the human XIST gene has identified fewer candidate regulatory regions than are observed flanking mouse Xist. In
particular, 3' to \textit{XIST} only a single, developmental-specific DHS site was observed in undifferentiated mouse ES cells with an integration of the human \textit{XIST} domain. 5' to \textit{XIST} a DHS site was identified in a region of sequence conservation amongst non-rodent eutheria. This region showed bi-directional promoter and enhancer activity. The lack of conservation of regulatory elements for \textit{XIST} lends support to previous conjectures that human \textit{TSIX} is no longer a functional regulator of \textit{XIST}.

\section*{Methods}

\subsection*{Tissue Culture & Cell Lines}

ES-10 cells, a derivative of J1 male mouse embryonic stem cells with a 480-kb human \textit{XIC} transgene, were generously provided by Dr. B. Migeon and maintained as described [22]. The L1.10.1 transgenic derivative of human male fibrosarcoma cell line HT-1080 was grown as described [19]. Mouse-human somatic cell hybrids 11t-4Az5 (containing a human Xi as well as six human autosomes in addition to mouse chromosomes) and 160-12 (containing a human Xa) were maintained as described [52].

\subsection*{DNase I Hypersensitivity Mapping}

The preparation of nuclei and the DNase I digest were as described [53]. Briefly, cells were harvested and washed twice in ice-cold PBS, then resuspended at 1 × 10^7 cells/ml in 10 ml ice cold sucrose-triton, swelled on ice for 15 minutes, and then homogenized 10 times in a Dounce homogenizer with a pestle (7 ml, Wheaton). Homogenized cells were transferred to a 15-ml falcon tube and spun at 1,200 rpm for 15 min at 4°C to recover nuclei which were then resuspended in 1.5 ml of ice cold buffer (50 mM Tris-Cl pH7.9, 100 mM NaCl, 3 mM MgCl_2, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and aliquoted into seven 1.5 ml eppendorf tubes (200 μl each). Nuclei were digested with an increasing amount of DNase I (10 U/μl, RNase free, Roche) (i.e. 1/128, 1/64, 1/32, 1/16, 1/8, 1/4 U/μl) at 37°C for 20 minutes. The digestions were stopped and DNA extracted by adding 1 ml DNazol (Invitrogen) following the manufacturer’s protocol. DNase I treated DNA was digested with restriction enzymes according to the manufacturer’s recommendations and analyzed by Southern blotting with random-primed P^32-labelled probes generated by PCR with primers listed in Table 1 [53]. All analyses were repeated at least twice. As the cell lines contain variable proportions of the human genome they showed differences in cross-hybridization. In order to be identified as a DHS site a band could not be visible in the undigested lane and needed to be replicated.

\subsection*{Sequence Comparisons, Plasmid Construction and Luciferase Assay}

For the dot-plot analyses, we used Mulan (http://mulan.dcode.org/[54]). Five DNA fragments of interest were generated by PCR amplification of human genomic DNA from GM01416 for 35 cycles using primers listed in Table 2. PCR fragments were first cloned into pGEM-T Easy vector (Promega) via TA cloning prior to Table 1 Primers for probe generation

| Symbol | Size | Name                          | Sequence (5' to 3') |
|--------|------|-------------------------------|---------------------|
| 36     | 921 bp | IP368F | TCTGCTCACCAATTGACCTGTAAG |
|        |       | IP359R | GAGGACGTGTCAGAGACACTAGG |
| 45     | 874 bp | IP453F | CATGGGAAAGCAGCAGACTTCT |
|        |       | IP444R | GGCGCTGAATGTGAGCAATAGAT |
| 101    | 1190 bp | IP1011F | GAATAGCTCAATGGCAATGTTACT |
|        |       | IP1000R | GTGCTTCAATGTCCTTTAACAGG |
| 86     | 1041 bp | U862F | TGGACTGCAGTCGCTGTGCT |
|        |       | U873R | ATAATCTGCTACTGAGGGGCT |
| 105    | 1209 bp | U1056F | TGCTGACCTGCTGAAAGGTCG |
|        |       | U1068R | CTATACATACCTCTGTGATTCAGTC |
| 117    | 1140 bp | U1179F | CTCTGTGACTGCTGAAAGGTCG |
|        |       | U1190R | TCTGTGACTGCTGAAAGGTCG |

* underlined is genomic sequence from NCBI; not underlined is adaptor (i.e. SacI & KpnI sites)

\section*{Table 2 Primers for cloning}

| Fragment Content | Name | Sequence (5' to 3') |
|------------------|------|---------------------|
| XIST promoter, positive control | 0 F | TCGAGCTCTTTGTACCAATTGACCTGTAAG |
|                  | 0 R | CGGGTACCAGGGAGCTGTCAGAGAACACTAGG |
| 1 kb upstream of XIST promoter | -1 kb F | TCGAGCTCTTTGTCACCAATTGACCTGTAAG |
|                  | -1 kb R | CGGGTACCAGGGAGCTGTCAGAGAACACTAGG |
| 2 kb upstream of XIST promoter | -2 kb F | TCGAGCTCTTTGTCACCAATTGACCTGTAAG |
|                  | -2 kb R | CGGGTACCAGGGAGCTGTCAGAGAACACTAGG |
| 3 kb upstream of XIST promoter | -3 kb F | TCGAGCTCTTTGTCACCAATTGACCTGTAAG |
|                  | -3 kb R | CGGGTACCAGGGAGCTGTCAGAGAACACTAGG |
| 65 kb upstream of XIST, covers HS 101 | -65 kb F | TCGAGCTCTTTGTCACCAATTGACCTGTAAG |
|                  | -65 kb R | CGGGTACCAGGGAGCTGTCAGAGAACACTAGG |
to insertion into pGL4.10 and pGL4.23 reporter vectors (Promega) upstream of the firefly luciferase gene. The identities of pGL4 clones were confirmed by restriction enzyme digestion and partial sequence analysis. Transient transfection was performed in 24-well plates with 80% confluent HT1080 cells. 0.8 μg of firefly luciferase plasmid (pGL4, Promega) and 80 ng of the Renilla luciferase plasmid (Promega) were co-transfected into cells using 2 μl Lipofectamine™ 2000 (Invitrogen). For each transfection assay, the pGL4.13 vector, which contains a promoter/enhancer element, was used as positive control and the pGL4.10 vector, which contains neither promoter nor enhancer, was used as negative control. Transfection of the pGL4.23 vector with a basal promoter/enhancer, was used as negative control. After 24 h, cell lysates were prepared from each transfected culture and were analyzed in a 96-well plate luminometer (Perkin Elmer Wallace) according to manufacturer’s protocol in the Dual Luciferase Kit (Promega). Each fragment was tested in triplicate, and each experiment was repeated at least three times. To control for transfection efficiency, the ratio of firefly luciferase signal to Renilla luciferase signal was calculated for each transfected sample.

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Authors’ contributions

SC carried out the molecular studies and drafted the manuscript. CB participated in the design of the study and revised the manuscript. Both authors read and approved the final manuscript.

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