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Replication profile of *PCDH11X* and *PCDH11Y*, a gene pair located in the non-pseudoautosomal homologous region Xq21.3/Yp11.2

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Abstract

In order to investigate the replication timing properties of *PCDH11X* and *PCDH11Y,* a pair of protocadherin genes located in the hominid-specific non-pseudoautosomal homologous region Xq21.3/Yp11.2, we conducted a FISH-based comparative study in different human and non-human primate (*Gorilla gorilla*) cell types. The replication profiles of three genes from different regions of chromosome X (*ZFX, XIST* and *ATRX*) were used as terms of reference. Particular emphasis was given to the evaluation of allelic replication asynchrony in relation to the inactivation status of each gene. The human cell types analysed include neuronal cells and ICF syndrome cells, considered to be a model system for the study of X inactivation. *PCDH11* appeared to be generally characterized by replication asynchrony in both male and female cells, and no significant differences were observed between human and gorilla, in which this gene lacks X-Y homologous status. However, in differentiated human neuroblastoma and cerebral cortical cells *PCDH11X* replication profile showed a significant shift towards allelic synchrony. Our data are relevant to the complex relationship between X-inactivation, as a chromosome-wide phenomenon, and asynchrony of replication and expression status of single genes on chromosome X.

Introduction

The non-pseudoautosomal X-Y homologous region (Xq21.3/Yp11.2) is the largest region (~4 Mb) shared by the human sex chromosomes. It was generated approximately 6 million years ago, close to the divergence of hominid and chimpanzee lineages, by a duplicative transposition from the X to the Y chromosome, followed by a paracentric inversion of the whole block and deletion of the DXS214 sub-interval on the Y chromosome (Schwartz *et al*. 1998, Ross *et al*. 2005, Williams *et al*. 2006). So far, only three genes have been described in this region: *TGIFLX/Y*, a testis-specific transcription factor from the homeobox gene family (Blanco-Arias *et al*. 2002); *PABPC5*, a poly-A binding protein, whose homologue has been lost from the Y chromosome during human evolution (Blanco *et al*. 2001); and *PCDH11X/Y*, a gene pair coding for protocadherin X and protocadherin Y, two cell-surface adhesion molecules expressed predominantly in brain (Yoshida & Sugano 1999, Blanco *et al*. 2000).

In female mammals most genes on one X chromosome are silenced as a result of X-chromosome inactivation, a silencing mechanism which evolved to restore equal gene expression between

males and females (Lyon 1961). However, pseudoautosomal genes and X-linked genes with Y homology or functional Y orthologues tend to escape X-inactivation and are usually expressed from both the active and inactive X chromosome (reviewed in (Disteche *et al*. 2002, Brown & Greally 2003).

Accordingly, it is reasonable to envisage that *PCDH11X* would escape from inactivation. The proposed role for protocadherin X and protocadherin Y in language development, handedness, and brain asymmetry (Crow 2002), combined with the lack of X-Y homologous status in non-human primates (Wilson *et al*. 2006), suggest the possibility of a sex-linked differential expression and gene dosage differences between human and primates. Indication of a possible escape of PCDH11X from X-inactivation was initially provided by results obtained with a human X chromosome-specific cDNA microarray showing elevated expression of *PCDH11X* in cells with multiple X chromosomes (Sudbrak *et al.* 2001). Recently Lopes and collaborators (2006) demonstrated that the CpG islands of *PCDH11X/Y* were not methylated in human and chimpanzee, and that there was a two-fold higher expression of *PCDH11X* in brain tissue in human females than in males. However, whether *PCDH11X* escapes from inactivation remains to be conclusively proved.

X-inactivation is associated with a series of epigenetic modifications (Reviewed in Chow *et al.* 2005). Besides undergoing changes in DNA methylation and histone modifications, the inactive X chromosome condenses into the Barr body and becomes late replicating in S phase. As a result, X chromosomes in female cells replicate asynchronously, with genes on the inactive X replicating later than their active counterparts.

Replication banding and fluorescence *in situ* hybridization (FISH) in its different guises have been repeatedly and successfully used as reliable cytological assays for the study of replication properties of chromosomes and genes (Dutrillaux *et al.* 1976, Vogel *et al.* 1989, Drouin *et al.* 1990, Haaf 1997, Takebayashi *et al.* 2005). In particular, the FISH-based 'dot counting' method (Selig *et al.* 1992), used in this investigation, allows one to visualize on cell nuclei the unreplicated DNA segments as single hybridization signals ('singlets') and the replicated loci as double signals ('doublets'). In a random cell population of an unsynchronized culture, genes which replicate early in the cell cycle will show a high percentage of doublets, while late-replicating genes will show mostly singlets.

The validity of this approach was initially tested by analysing genes from the cystic fibrosis (CF) region on human chromosome 7, the replication profiling of which had been previously determined in expressing and non-expressing cells by molecular methods. By 'dot-counting' on unsynchronized cell cultures the authors confirmed the feasibility of the method to infer replication timing properties of genes (Selig *et al*. 1992). In the same study the conclusion was reached that the replication timing of autosomal genes is normally highly synchronous, with 90% of cell nuclei showing an equivalent hybridization pattern on both homologues (singlet–singlet/SS or doublet–doublet/DD) and only 10% of nuclei showing an asynchronous pattern (singlet–doublet/SD).

However, subsequent studies on the replication timing patterns of murine chromosomal regions containing four imprinted genes (*Igf2*, *Igf2r*, *H19* and *Snrpn)* suggested that asynchronous replication – inferred from the high percentage of SD signals in a random population of unsynchronized cells – might be a characteristic of imprinted genetic regions (Kitsberg *et al.* 1993), consistent with earlier replication banding studies on the chromosomal region 15q11.2 (Izumikawa *et al*. 1991).

A 'dot-counting' comparison of the replication timing of genes on the human chromosome X that are known to escape inactivation (pseudoautosomal) with those that are inactivated (Boggs & Chinault 1994), as well as confirming the correlation between expression and relative time of replication of individual genes, established that genes subject to inactivation replicate asynchronously while genes that escape inactivation replicate synchronously, thus validating replication asynchrony as an indicator of an allele-specific mode of expression.

As part of a coordinated research effort aimed at evaluating the genetic and epigenetic status of *PCDH11X/Y* in human and non-human primates, we conducted a study on the replication timing of this gene pair carrying out a FISH-based comparative investigation in different human and gorilla (*Gorilla gorilla*) cell types, using as terms of reference three genes from chromosome X (*ZFX, XIST* and *ATRX*), previously assessed for their replication timing by means of similar and/or alternative analytical methods. In particular, in the attempt to infer information on *PCDH11X* possible escape from inactivation in

human cells, our analysis focused on comparing the allelic replication synchrony profile of this gene to those of the other X-linked genes under investigation, the inactivation status of which is known.

Materials and methods

Cell culture

Chromosome preparations were obtained from: (1) human male and female peripheral blood lymphocytes; (2) EBV-transformed human lymphoblastoid cell lines from male (COX, ECACC No. 85102902) and female (DO208915, ECACC No. 95010407); (3) EBV-transformed lymphoblastoid cell line from a female immunodeficiency, centromeric instability, facial anomalies (ICF) syndrome patient (GM08714, Coriell Cell Repositories); (4) EBV-transformed lymphoblastoid cell lines from female gorilla (*Gorilla gorilla*) (Machi cell line provide by Professor A. Rickinson, University of Birmingham); (5) human female neuroblastoma cell line SH-SY5Y (ECACC No. 94030304) before and after differentiation with retinoic acid; and (6) a cerebral cortical cell line developed from a female patient with Rasmussen encephalitis HCN-2 (ATCC No. CRL-10742). PHA-stimulated peripheral blood lymphocytes and B-lymphoblast cells were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) and 1% L-glutamine (Sigma). Prior to hypotonic treatment, one of the two cultures of the human female B-cell line (identified in the text as 'line 1'), was treated with thymidine (300 μg/ml) for 17 h and then washed and left in complete medium for 7 h, before adding colcemid (0.05 μg/ml). The cells were then treated with hypotonic solution (0.0075 M KCl) for 20 min at 37°C and fixed in three changes of Carnoy's fixative (3:1 methanol:acetic acid). Slides were prepared following standard procedures and stored at −20°C. The karyotype of each cell line (apart from the cortical cell line from which it had not been possible to obtain metaphase preparations due to the extremely slow growth rate) was checked for chromosome X numerical or structural abnormalities.

Neuroblastoma cell line differentiation

SH-SY5Y cells were grown in flasks in DMEM: Ham's F12 (1:1) complete medium. After trypsinization the cells were plated onto adherent substrate-coated (Collagen 1) dishes at low density ($3–5\times10^3$ cells/cm^2) and allowed to attach overnight. The following day *trans*-retinoic acid (RA) was added to the medium at a final concentration of 10 μM. After 5 days the medium was changed to NeuroBasal (NB) supplemented with B-27 (1×), Glutamax I (2 mM), KCl (20 mM), dibutyril cyclic AMP (2 mM) and rhBDNF (50 ng/ml). The cells were kept in this medium for 5 days before proceeding with the chromosome harvest protocol as above.

Probes

The following BAC probes were used in the *in situ* hybridization experiments: RP1-290C9 for *ZFX* (Sanger Institute), RP11-42M11 for *ATRX* (Sanger Institute), RP13-216E22 for *XIST* (Sanger Institute), and RP11-187C6 for *PCDH11* (provided by Dr Carole Sargent, University of Cambridge). BAC RP11-17E6 from chromosome 2q23.3 (Sanger Institute) was used as an autosomal control.

Fluorescence in-situ *Hybridization (FISH)*

The hybridization procedure followed a standard protocol. The probes were labelled with either Biotin-16-dUTP (Roche) or Digoxigenin-11-dUTP (Roche) by nick-translation. Following labelling, the probes were ethanol precipitated in a mix of salmon testis DNA (Gibco BRL), *Escherichia coli* tRNA (Boehringer) and 3 M sodium acetate. They were then dried on a heating block at 60°C with a 50× excess of human Cot-1 DNA and resuspended at 20 ng/μl in hybridization solution (50% formamide, 10% dextran sulphate, 2× SSC). The probes were denatured at 72°C for 5 min and pre-annealed at 37°C for 15 min, before being applied to the denatured slides. Prior to replication timing analysis on interphase nuclei, in order to detect the possible presence of numerical and/or structural abnormalities involving chromosome X and in particular the genes of interest, each BAC probe was co-hybridized with an FITC-directly labelled chromosome X paint (Cambio) and checked on metaphase chromosomes (in all cell lines, apart from the cortical cell line from which it had not been possible to obtain metaphase preparations due to the extremely slow growth rate). Before proceeding with the hybridization, the slides were denatured in 70% formamide at 70°C for 2 min, quenched in 2×SSC at

4°C and then dehydrated in an ethanol series. Following hybridization, the slides were washed in 50% formamide at 42°C for 10 min and 2×SSC at 42°C for 5 min. The biotinylated probes were detected with Texas red-conjugated streptavidin (Molecular Probes), followed by a layer of biotinylated anti-streptavidin (Vector Laboratories) and a final layer of Texas red-conjugated streptavidin. The digoxigenin probes were detected using mouse anti-digoxigenin antibody (Roche) and goat anti-mouse Alexa-488 (Molecular Probes). The slides were mounted with Vectashield (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI) for chromosome counterstaining.

BrdU pulse-labelling

The cells were incubated at 37°C with 10 μM 5′-Bromo-2′-deoxyuridine (BrdU) (Sigma) for 30 min before cell fixation. Prior to FISH, the BrdU pulse-labelled cells were pretreated by permeabilization in 0.5% Triton-X for 10 min and then transferred to 0.1 M HCl for 10 min at room temperature. The slides were then washed in 2×SSC for 5 min and equilibrated in 50% formamide/2×SSC for at least 15 min before denaturation. After hybridization the BrdU labelling was detected using a mouse anti-BrdU antibody (Roche) followed by a goat anti-mouse Alexa 488 antibody (Invitrogen).

Microscopy analysis

The slides were examined using an Olympus BX-51 epifluorescence microscope coupled to a Sensys charge-coupled device (CCD) camera (Photometrics). A minimum of 100 nuclei were analysed for each hybridization experiment. Texas red, Alexa-488 and DAPI fluorescence images were taken as separate grey-scale images using specific filter combinations and then pseudocoloured and merged using the software package Genus (Applied Imaging International).

Statistical analysis

The replication pattern of an experiment was measured as the counts of SS, SD and DD nuclei, and summarized as the percentage of SD counts (%SD). Statistical significance of differences between experiments was tested using the *R* statistical analysis package version 2.3.1 [R Development Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.] We used the loglm() function to perform contingency table analysis, testing for differences between genes within cell lines and between cell lines within genes. We compared individual differences between pairs of genes as well as testing for no difference between all genes within a cell type.

RT-PCR

Detection of gene expression was performed using RT-PCR. RNA samples were extracted from four cell lines (female B-cell line DO208915, female ICF B-cell line GM08714, SH-SY5Y female neuroblastoma cell line before and after differentiation with retinoic acid) and a female brain control using QIAGEN RNeasy Mini Kit according to the manufacturer's protocol. Samples were treated with DNAseI prior to reverse transcription according to the manufacturer's the protocol (Invitrogen), with 1 μg of RNA treated with DNAseI. cDNA was prepared using SuperScriptIII (Invitrogen) according to standard procedure. PCR reactions used the following primers:

Xist, product size 80 bp:
Forward: 5′-AACCAGGAAAGAGCTAGTATGAGGAA-3′;
Reverse: 5′-TTCGCAAGCTGAATTAAGTG-3′.

ATRX, product size 79 bp:
Forward: 5′-CATCATCTAGTTGAACTTTGGCATTT-3′;
Reverse: 5′-CAATAAACGGCCAGAATTTCCA-3′.

ZFX, product size 80 bp:
Forward: 5′-CGAATATAGGTCTGCAGACTC-3′;
Reverse: 5′-CACACTTGAACGGCATCTCT-3′.

PCDH11, product size 133 bp:
Forward: 5′-CCCTTTCGTTTACCTCTTCATTC-3′;
Reverse: 5′-CACAAGATTACAAAGTTGAGAGCAG-3′.

RT-PCR reactions were performed using RedTaq (Sigma) according to the manufacturer's protocol, using a 2.5 mM final concentration of $MgCl_2$. Cycling parameters were as follows: 94°C–4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final 5 min at 72°C. Products

were resolved on a 2% agarose gel using HyperLadderV (Bioline).

Quantitative RT-PCR

Quantitation of PCDH11 expression was performed by real-time RT-PCR using the above primers. The results were normalized to the control gene RPII, product size 267 bp: forward: 5′-GCAC CACGTCCAATGACAT-3′; reverse 5′-GTGCGGC TGCTTCCATAA-3′. Quantitation was performed using SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's protocol, on an iCyler real-time PCR machine (BioRad). Analysis was performed using the standard curve method and normalization to expression of the RPII control, using the iCyler software.

Results

We compared the replication timing properties of *PCDH11X* to its homologue on the Y chromosome (*PCDH11Y),* and three other genes from chromosome X (*ZFX, XIST* and *ATRX*) by conducting a FISH-based 'dot-counting' assay on a range of different human cell types and a gorilla (*Gorilla gorilla*) lymphoblastoid cell line.

The percentage of different replication patterns – single–single (SS), double–double (DD) and single –double (SD) fluorescent signals – for each probe in interphase was determined by randomly scoring at least 100 nuclei for each hybridization experiment (examples in Figure 1). In agreement with the analytical parameters most recently applied in similar assays (Singh *et al*. 2003, Gribnau *et al*. 2005, Gimelbrant & Chess 2006), a value of %SD ≥ 30% was considered indicative of replication asynchrony. As single signals in interphase can occur as a result of inefficient hybridization, the efficiency of hybridization was checked in parallel on metaphase chromosomes on the same slide. Experiments in which the hybridization efficiency was considered not satisfactory were omitted from the analysis.

The replication profile or frequency of different replication patterns for *PCDH11X* was initially assessed by hybridizing and analysing chromosome preparations obtained from a human female peripheral blood culture and from two different cultures of a human female B-lymphoblastoid cell line, which

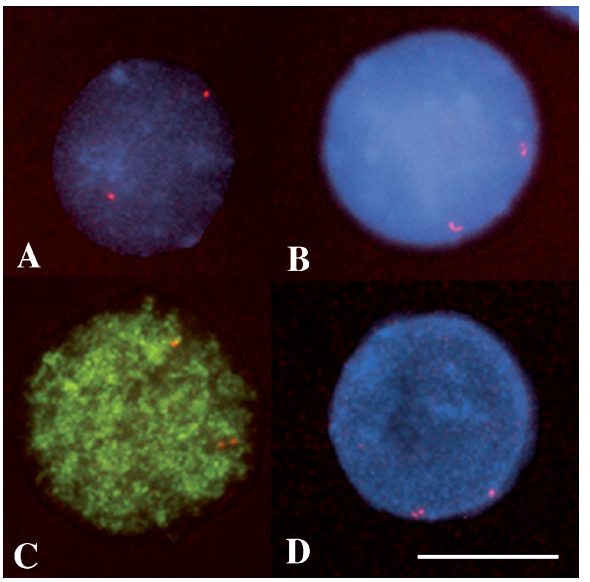

Figure 1. Fluorescence *in situ* hybridization (FISH) allows visualization of replicated and unreplicated DNA fragments in the cell nuclear context. Examples of interphase nuclei showing in red: (**A**) a single–single (SS) hybridization pattern (both DNA segments have not replicated yet); (**B**) a double–double (DD) hybridization pattern (both DNA segments have already replicated); (**C**) and (**D**) single–double (SD) hybridization patterns (one fragment has replicated, but its homologue has not replicated yet). In **A**, **B** and **D** the nuclei are counterstained with Dapi (blue). In **C** hybridization was performed on BrdU pulse-labelled cells. The anti-BrdU antibody (green) identifies nuclei in S phase. (Scale bar = 10 μm.).

hereafter will be referred to as line 1 and line 2. The replication profile of *PCDH11X* was also analysed in a B-lymphoblastoid cell line obtained from a female ICF patient. As shown in Table 1, replication asynchrony between the two alleles was observed for the *PCDH11X* locus in all of these cell types, including the ICF cells, with the percentage of hemizygous replication patterns or %SD (one singlet and one doublet in the same nucleus) consistently within a range of 36–39%, and no statistically significant difference observed when comparing the *PCDH11X* replication profile between any of cell types analysed (chi-squared test of no differences between blood, line 1, line 2 and ICF cell line $p < 0.172$). A hemizygous pattern of replication – with a %SD value (37%) within the same range observed above – was also observed in gorilla female cells. When the replication profile of PCDH11X was compared between human and gorilla B cells there was no statistically significant difference between gorilla and line 2, and only a marginal difference

between gorilla and line 1 ($p = 0.03$) was observed, with the difference between line 1 and 2 probably due to the slightly different culture conditions.

In order to compare *PCDH11X* to its homologue on chromosome Y, the 'dot-counting' assay with the *PCDH11* probe was then carried out on chromosome preparations obtained from a human male peripheral blood culture and a human male B-lymphoblastoid cell line. Findings of SD values equivalent to 30% in male blood and 41% in male B cells confirmed that – similarly to the two alleles on the X chromosomes in female cells – in male cells the X and Y homologue genes replicate asynchronously. Statistical comparison of the gene pair replication profile in male vs female blood or male vs female B cells also showed no difference.

We then compared *PCDH11X* to *ATRX* (Xq13.3) and *XIST* (Xq13.2), two X-linked genes known to undergo inactivation (reviewed in Carrel & Willard 2005). The range of SD pattern frequency in the different human and gorilla cell types was 35–42% for *ATRX* and 31–40% for *XIST*, in both cases indicative of allelic asynchrony. There were no significant differences between *PCDH11X* and *ATRX* replication profiles in line 1, line 2, blood and gorilla. There was a significant difference between *PCDH11X* and *XIST* in blood cells ($p < 0.06$) and in line 2 ($p < 0.02$) but not in line 1 ($p < 0.68$). However, when account is taken of the number of tests performed in this analysis the significance is only marginal. As well as comparable levels of asynchrony, no significant changes in the replication timing profile of *PCDH11X* and *ATRX* were also observed in the ICF cells, with the percentages of SS (single–single) and DD (double–double), as well as the total percentages of 'singlets' and 'doublets', being very similar in ICF and controls for both genes.

Comparisons were then carried out between *PCDH11X* and *ZFX* (Xp22.2-p21.3), a gene known to escape inactivation and previously reported by FISH analysis to replicate synchronously (Boggs & Chinault 1994). Although a relatively lower percentage of hemizygous replication patterns was consistently observed for *ZFX* in each of the cell types analysed, no statistically significant differences were found when the replication profiles for *PCDH11X* and *ZFX* were compared in either blood, line 1 or gorilla, and only a marginally significant difference was observed in line 2 ($p < 0.02$).

As a further control we compared the replication asynchrony of the four X-linked genes with that of a randomly chosen autosomal probe, mapping on chromosome 2. The percentages of hemizygous patterns observed when hybridizing this autosomal probe to chromosome preparations from male (%SD = 24%) and female (%SD = 22%) peripheral blood cells were significantly lower than those observed for all the other genes in male and female blood ($p < 0.0001$), consistent with a synchronous replication pattern expected for autosomal genes.

Since the percentage of SD can sometimes be overestimated because of doublet artefacts arising from the separation of denatured chromatin strands in G1 and G2, we carried out a further set of experiments in which bromodeoxyuridine (BrdU) was incorporated during cell culture in order to identify and assay cells in S phase exclusively. Observations conducted on the human female B-lymphoblastoid cell line after treatment with BrdU and hybridization showed – as expected – an increase in the percentage of SD signals for *PCDH11X* (50%) and similarly raised values for the other three X-linked genes used as controls (*ZFX* = 48%, *XIST* = 44% and *ATRX* = 49%), further confirming the finding of an asynchronous replication pattern for all the X-linked genes analysed – irrespective of their inactivation status – as previously shown by our experiments on unsynchronized B cells. Unexpectedly, a substantial increase in replication asynchrony after BrdU treatment was also observed for the autosomal control. We suspect that its distinctive late-replicating nature when compared to the other loci – suggested by the relative percentages of total singlets and doublets (Table 1) – might have slightly biased the analysis in synchronized conditions.

Next we investigated the replication timing properties of *PCDH11X* in human neuronal cells, carrying out fluorescent *in situ* hybridization experiments on chromosome preparations obtained from the SH-SY5Y neuroblastoma cell line, before and after differentiation with retinoic acid, and from the HCN-2 cerebral cortical cell line. The latter are cells of neuroepithelial origin and express neuronal markers even in the undifferentiated state. In both cases a statistically significant ($p<0.02$) reduction in the asynchrony of replication was observed for *PCDH11X* with a 29% of SD patterns in HCN-2, and 28% of SD patterns in the differentiated SH-SY5Y, with the same neuroblastoma cell line showing a higher percentage of asynchrony (38%) before differentiation. Corresponding values for

Table 1. Summary of the 'dot-counting' replication timing assay carried out on five different genes (*PCDH11*, *ATRX*, *XIST*, *ZFX* and autosomal control probe) in different human and gorilla cell types

	SS	DD	SD	SD%	N	Singlets	Doublets
PCDH							
♀ blood	48	16	37	36%	101	66%	34%
♀ line 1	58	11	40	37%	109	72%	28%
♀ line 2	40	25	39	37%	104	57%	45%
♀ line ICF	45	16	39	39%	100	65%	35%
♀ Gorilla	41	23	38	37%	102	59%	41%
♀ neurobl.	40	26	40	38%	106	57%	43%
♀ neurobl.dif.	93	21	44	28%	158	73%	27%
♀ cortical	61	11	30	29%	102	75%	25%
♂ blood	66	19	36	30%	121	69%	31%
♂ line	51	10	43	41%	104	70%	30%
ATRX							
♀ blood	50	11	45	42%	106	68%	32%
♀ line 1	62	20	48	37%	130	66%	34%
♀ line 2	52	19	38	35%	109	65%	35%
♀ line ICF	51	15	35	35%	101	68%	32%
♀ Gorilla	49	14	39	38%	102	67%	33%
♀ neurobl.	62	12	46	38%	120	71%	29%
♀ neurobl.dif.	48	17	44	40%	109	64%	36%
XIST							
♀ blood	67	8	34	31%	109	77%	23%
♀ line 1	49	13	41	40%	103	67%	33%
♀ line 2	61	14	34	31%	109	72%	28%
♀ Gorilla	53	10	43	40%	106	70%	30%
♀ neurobl.	66	20	39	31%	125	68%	32%
♀ neurobl.dif.	63	15	49	38%	127	69%	31%
ZFX							
♀ blood	55	19	37	33%	111	66%	34%
♀ line 1	56	21	35	31%	112	66%	34%
♀ line 2	61	13	40	35%	114	71%	29%
♀ Gorilla	48	21	36	34%	105	63%	37%
♀ neurobl.	66	21	34	28%	121	69%	31%
♀ neurobl.dif.	77	13	30	25%	120	77%	23%
Autosom.							
♀ blood	84	6	25	22%	115	84%	16%
♀ neurobl.dif.	77	7	33	28%	117	80%	20%
♂ blood	86	2	28	24%	116	86%	14%
BrdU-Lab.							
PCDH	47	8	54	50%	109	n.a.	n.a.
ATRX	40	12	51	49%	103	n.a.	n.a.
Xist	41	18	47	44%	106	n.a.	n.a.
ZFX	73	36	100	48%	209	n.a.	n.a.
autosom.	44	19	48	43%	111	n.a.	n.a.

'SS' is the number of single–single hybridization patterns observed in each experiment, 'DD' is the number of double–double hybridization patterns observed in each experiment and 'SD' is the number of single–double hybridization patterns observed in each experiment (also shown as a percentage), while 'N' is the total number of nuclei analysed for each experiment. 'BrdU-labelled' indicates the observations carried out on BrdU-positive nuclei. Percentages of total number of single signals ('Singlets') and double signals ('Doublets') are also included.

ATRX and *XIST* in the neuroblastoma cells both before and after differentiation were asynchronous, while *ZFX* in both non-differentiated and differentiated neuroblastoma cells presented with a more synchronous pattern. In the differentiated neuroblastoma cells the %SD value for *PCDH11X* was found to be significantly different from *ATRX* ($p < 0.05$), marginally different from *XIST* ($p = 0.05$), but not significantly different from *ZFX* and the autosomal control.

Finally, we tested the hypothesis of no differences in replication profile between all four X-linked genes in females within each cell type for blood, line 1, line 2, differentiated neuroblastoma and gorilla (Figure 2). Only differentiated neuroblastoma cells showed significant differences between the genes ($p = 0.01$ when the SD values were compared to SS and DD values pooled together and $p = 0.02$ when the SS and DD values were not pooled together).

Expression of *PCDH11* was not detected by RT-PCR in any of the cell lines analysed, but only in female brain cells used as control (Figure 3). On the contrary, expression of *ATRX*, *XIST* and *ZFX* was detected in all samples. Since the lack of expression

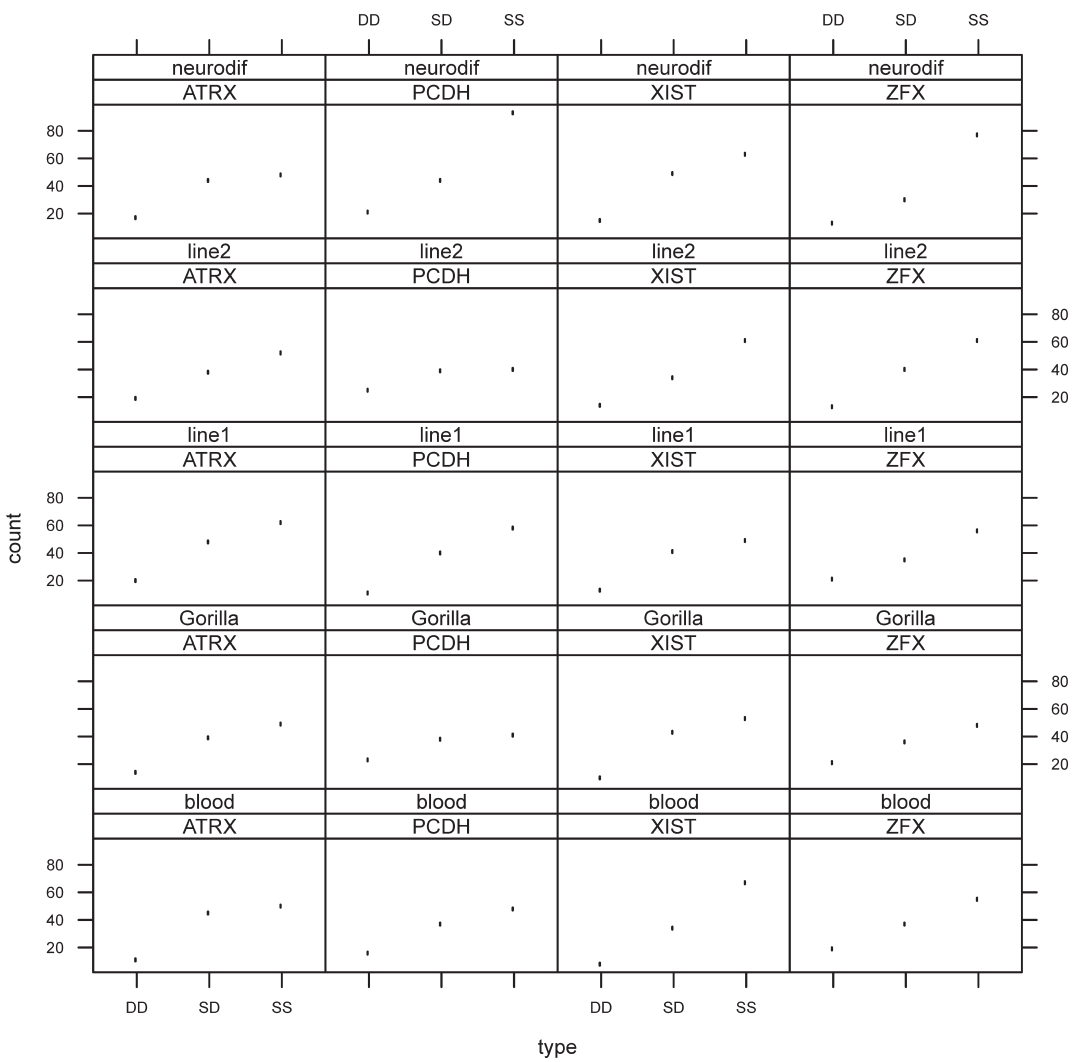

Figure 2. Graphic representation of the test carried out on the hypothesis of no differences in replication profile between all four X-linked genes (*ATRX*, P*CDH11X*, *XIST* and *ZFX*) in female human blood cells, female human B cells (line 1 and line 2), female gorilla B cells and female human differentiated neuroblastoma cells. Only differentiated neuroblastoma cells showed significant differences between the genes.

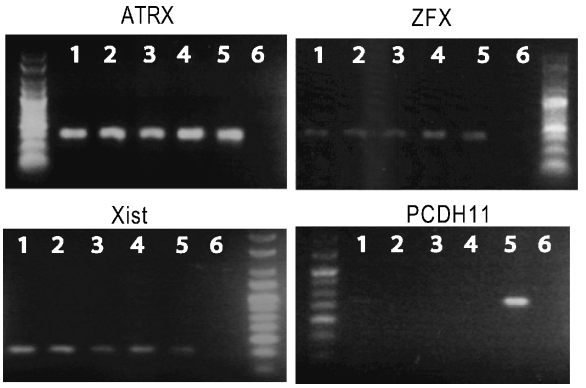

Figure 3. Expression of X-linked genes in cell lines. Samples are as follows: 1: DO208915 (female B-cell line), 2: GM08714 (female ICF B-cell line), 3: SH–SY5Y (female neuroblastoma cell line), 4: differentiated SH–SY5Y, 5: female brain control, 6: negative control. Molecular weight marker is HyperLadderV (bioline). Expression of control genes can be observed in all samples and all products are appropriate size. Expression of PCDH11 is observed only in female brain control sample.

of *PCDH11* in cells expressing neuronal markers was unexpected, we decided to conduct further investigations. To confirm that there was no low-level expression of *PCDH11* in the cell lines analysed we performed quantitative RT-PCR using SYBR green. The results in Table 2 reveal that the level of PCDH11 expression in the neuroblastoma samples before and after differentiation is negligible.

Discussion

This is the first investigation on the replication timing properties of a gene pair (*PCDH11X/Y*) belonging to the non-pseudoautosomal X-Y homologous block (Xq21.3/Yp11.2), the largest shared region among sex chromosomes generated by a duplicative transposition from the X to the Y chromosome after the divergence of hominid and chimpanzee lineages.

By means of a comprehensive and statistically validated FISH assay we show that *PCDH11* replicates asynchronously in both male and female human cells, as well as gorilla female cells. Replication asynchrony is usually associated with an allele-specific mode of expression, normally brought about by imprinting, X chromosome inactivation, or other mechanisms leading to monoallelic expression. Previous investigations on the replication timing of the human X chromosome, although leading in few circumstances to apparent discrepancies, have generally agreed in concluding that genes known to escape X-inactivation – either because pseudoautosomal or X-linked with Y homology or functional Y orthologues – replicate synchronously, while genes known to be inactivated replicate asynchronously (Boggs & Chinault 1994, Torchia *et al*. 1994, Hansen *et al*. 1995, 1996, Xiong *et al*. 1998, Yeshaya *et al*. 1999). Our observations showing homologous and allelic replication asynchrony for *PCDH11* seem not to support the prediction, based on the X/Y homologous status of this gene in human, that *PCDH11X* might escape inactivation.

One possible explanation is that in fact, against the expectations, in human – as well as non-human primates – *PCDH11X* does not escape inactivation. Interestingly, autosomal monoalleleic expression has been reported for three other Protocadherin gene families (*Pcdha*, *Pcdhb* and *Pcdhc*), which reside in a single large cluster located on chromosome 5q, with their stochastic regulation believed to help specifying neuronal cell identity (Esumi *et al*. 2005).

It has recently become clear that escape from inactivation is a phenomenon more common than initially envisaged. A comprehensive X-inactivation study has shown that more than 15% of X-linked genes escape inactivation to some degree, with the proportion of genes escaping inactivation differing between different regions of the X chromosome, this occurrence reflecting the evolutionary history of the sex chromosomes (Carrel & Willard 2005). However, *PCDH11X* would not be the first case of an X-linked gene expected to escape that does not in fact conform. For instance, unlike other genes present in the Xq28 pseudoautosomal region (PAR2), both *SYBL1*, a synaptobrevin-like gene, and *SPRY3*, a human homologue of *sprouty* in *Drosophila*, have been reported to undergo inactivation on both X and Y (D'Esposito *et al*. 1996, Matarazzo *et al*. 2002, De Bonis *et al*. 2006).

Table 2. Results of quantitative RT-PCR on cell line samples relative to female brain control; the expression level is negligible in the cell lines tested

Sample	Relative expression
Neuroblastoma	
Differentiated neuroblastoma	0.00001776
Undifferentiated female brain	0.00001874
	1.00000000

An alternative interpretation of our results is that, notwithstanding its asynchronous replication profile, *PCDH11X* does escape inactivation, as recently published expression data would also suggest (Sudbrak *et al*. 2001, Lopes *et al*. 2006). Replication asynchrony for genes known to escape inactivation has been reported earlier. In particular, female cell lines with or without *TIMP1* expression from the inactive X showed a similar extent of replication asynchrony for the two alleles of this gene (Anderson & Brown 2005). Also, in a study on the role of replication timing in X inactivation choice, all loci tested in undifferentiated ES cells replicated asynchronously, including the *Smcx* gene, which is known to escape X inactivation (Gribnau *et al*. 2005). Asynchronous replication of an X-linked pseudoautosomal locus was also reported by (Vorsanova *et al*. 2001).

A comprehensive study on the replication timing of the human genome carried out by microarray analysis has confirmed the existence of a significant positive correlation between the probability of gene expression and replication timing (Woodfine *et al*. 2004). However, from a cytological perspective the correspondence between expression of individual genes or alleles and replication timing, especially on chromosome X, appears less straightforward than generally believed, with different aspects of chromatin structure and intranuclear organization emerging, from a series of original investigations, closely intertwined with replication timing. First, the initial prediction that asynchronous replication might represent a general characteristic of imprinted genetic regions (Kitsberg *et al*. 1993) was subsequently challenged by Kawame *et al*. (1995), who, by using a bromodeoxyuridine method to detect replicated DNA by flow cytometry, studied the allele-specific replication for several sites within the human Prader-Willi/Angelman and IGF2/H19 imprinted regions in mouse, and showed that no obvious allele-specific differences in time of replication were detected at most loci previously reported to replicate asynchronously in the same cell type as determined by a FISH-based replication assay (Knoll *et al*. 1994, LaSalle & Lalande 1995). The authors suggested that FISH data implying replication asynchrony in non-expressing cells reflect structural differences between the maternal and paternal alleles rather than differences in replication timing. Similar conclusions on replication imprinting reflecting differences in chromatin structure between homologues were also reached by Bickmore & Carothers (1995). More recently, asynchronous replication patterns of imprinted loci were found to be consistent with their differential localization to nuclear compartments with different replication characteristics (Gribnau *et al*. 2003).

Particularly significant evidence pointing at chromosome-pair non-equivalence and coordination of replication at the whole chromosome level was provided by a study by Ensminger & Chess (2004) on the replication timing of mono-allelically expressed genes along human autosomes and on chromosome X, confirming uniform late replication of loci on the inactive X. Gartler *et al*. (1999) had previously demonstrated that the silent *XIST* gene on the active chromosome X does replicate before the expressed allele on the inactive X, their results advocating for the first time the view that the replication timing of a gene is determined by the multi-replicon domain in which the gene resides, and not necessarily its expression status. It was recently demonstrated that asynchrony is present before initiation of X-inactivation and replication timing appears to be coordinated along the X chromosome over relatively large distances around the *Xist* locus (Gribnau *et al*. 2005).

Azuara *et al*. (2003) also conducted a study on leuckocyte-specific genes aimed at examining the relationship between gene silencing, chromatin structure and DNA replication. Their observations on integration of transgenes within heterochromatin provide further evidence for the importance of local chromosome environment in determining the timing of replication, and show that retarded sister-chromatid resolution is a feature of inactive chromatin.

Our findings on *PCDH11X/Y* and other X-linked genes are consistent with a model according to which the replication timing properties of X-linked genes are related principally to the epigenetic characteristics of the chromosomal environment in which the alleles reside. The four genes analysed in this study – two of them known to undergo inactivation (*ATRX* and *XIST*), one (*ZFX*) known to escape inactivation and *PCDH11*, the gene at the centre of our investigation, the inactivation status of which remains to be conclusively proved – show no significant differences when their replication profiles are compared in the majority of cell types analysed, and they consistently show similar levels of replication

asynchrony in human and gorilla female cells. In our opinion this is to be attributed primarily to the 'non-equivalence' and temporal shift in replication between the active and inactive X chromosome, rather than to the expression status of each single locus, with the slightly variable levels of asynchrony between the genes reflecting uneven epigenetic changes along the inactive X. This conclusion seems to be substantiated by our observations that similar levels of asynchrony were observed when comparing the replication profile of *PCDH11X* to *PCDH11Y* – its homologue on chromosome Y – in human male cells, and also when carrying out the replication assay for *PCDH11X* in gorilla, in which this gene lacks X-Y homologous status and consequently is very likely to be inactivated. Our results also reconcile with observations by Gribnau *et al.* (2005) who – in their paper on the replication timing of the X chromosome before the onset of X inactivation – reported all the X-linked genes tested to replicate asynchronously.

The discrepancy between our observations on ZFX (SD > 30% in non-neuronal cells) and the results (SD < 20%) in a previous study on the same and other X-linked genes carried out by Boggs & Chinault (1994) is puzzling, but most probably due to slight differences in the protocols and parameters for the interpretation of the FISH results. Worth mention is that the original set of 'dot-counting' assays carried out by different research groups in the early 1990s seem to be all generally characterized by relatively low counts of single–double (SD) hybridization patterns. At the time the conclusion was reached that most of the homologous alleles replicate highly synchronously, with normally less than 10% of nuclei showing an SD pattern, this generally believed to be the result of sub-optimal hybridization conditions rather than an indication of replication asynchrony (Selig *et al.* 1992, Kitsberg *et al.* 1993, Boggs & Chinault 1994, Amiel *et al.* 1997). However, more recently, the perception of allele-specific mode of expression and replication has substantially changed, and it has become clear that asynchronous replication is a more common occurrence than expected, found in association not only with imprinting, but also with random mono-allelic expression, pathologies, tandem duplications as well as encountered in random assays (Chess *et al.* 1994, Amiel *et al.* 1998a,b, 2001, Smith & Higgs 1999, Mostoslavsky *et al.* 2001, Reish *et al.* 2002, 2003, Gimelbrant *et al.* 2005, Gimelbrant & Chess 2006). In conclusion, asynchronous replication – as a biological phenomenon – might have been initially underestimated, and relevant experimental evidence misinterpreted and over-cautiously discarded. This is also reflected by the fact that through the course of the years the percentage of SD generally perceived as the threshold for asynchrony has increased from the initial '>10%' to the current '≥30%' (Selig *et al.* 1992, Kitsberg *et al.* 1993, Goren & Cedar 2003, Gimelbrant & Chess 2006), with the distinction between asynchronous and synchronous replication appearing much less defined than previously thought.

Our investigations in neuronal markers expressing cell types – the retinoic acid differentiated neuroblastoma cells and the cerebral cortical cells – show an intriguing shift in the replication pattern of *PCDH11X* towards allelic synchrony, with a statistically significant difference between the SD value for *PCDH11X* in the neuroblastoma cell line before and after differentiation with retinoic acid, and also between the undifferentiated neuroblastoma cells and the cortical cells. This appears to be independent from the expression status of *PCDH*X in these cells, as shown by our real-time RT-PCR analysis. A statistically significant reduction in asynchrony was also observed when comparing the SD value for *PCDH11X* in differentiated neuroblastoma and cortical cells to all the other cell types analysed. Replication asynchrony of PCDH11X in differentiated neuroblastoma was significantly different from ATRX and XIST, but not different from ZFX and the autosomal control. Taken all together, these data seem to indicate a relaxation in the temporal control of allelic replication in neuroblastoma differentiated and cortical cells, probably due to tissue-specific epigenetic adjustments along the inactive X chromosome, although another explanation could be that, upon differentiation, global changes at the cell-cycle level could affect the replication profile of some genes. Also there seems to be no obvious link between replication properties of these X-linked genes and alleles, as inferred cytologically by FISH, and their expression status.

Finally, we observed no significant changes in the replication timing profiles of *PCDH11X* and *ATRX* in ICF cells, as demonstrated by similar percentages of SS, SD and DD patterns for both genes in ICF cells and controls. The ICF syndrome was previously reported to be characterized by significant disturbances in the inactive X methylation and by a shift in

replication timing of some of the X-linked genes, and deemed to provide an important model system for the study of X chromosome inactivation (reviewed in Gartler & Hansen 2002). We found for *PCDH11X* and *ATRX* no cytological evidence of advanced replication timing as that previously shown by molecular methods for other X-linked genes in ICF (Hansen *et al*. 2000, Tao *et al*. 2002), and considered the major determinant of escape from inactivation in this syndrome. This is not necessarily unexpected as the phenotypic similarity of affected ICF males and females seems to suggest this to be a significant, but limited, phenomenon, not necessarily affecting all genes on the inactive X. However, it should also be pointed out that discrepancies between cytological and molecular investigations on the replication timing properties of the inactive X in ICF syndrome have been reported earlier (Bourc'his *et al*. 1999, Hansen *et al*. 2000).

In conclusion, our study provides novel and controversial data on the replication timing properties of a genomic region of evolutionary interest and aims to contribute to the debate on the complex relationship between X-inactivation, as a chromosome-wide phenomenon, and asynchrony of replication and expression status of single genes and alleles. Our investigations, combined with a critical review of the relevant literature, have allowed us to reassess the validity of the 'dot-counting' method as a cytological assay for replication profiling. Whilst confirming the soundness of this FISH-based approach – specifically when supported by a robust statistical analysis – for a cell-by-cell assessment of replication properties of genes and alleles, our results highlight the potential risks of non-contextual and non-comparative 'dot-counting'. Finally, by proving the unsuitability of this method to infer the inactivation status of X-linked genes, our analysis reaffirms the need for a combined molecular and cytological approach to reach definitive conclusions on interrelated replication and expression issues.

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