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BRCA1 Does not Paint the Inactive X to Localize XIST RNA but may Contribute to Broad Changes in Cancer That Impact XIST and Xi Heterochromatin

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Abstract The BRCA1 tumor suppressor involved in breast and ovarian cancer is linked to several fundamental cell regulatory processes. Recently, it was reported that BRCA1 supports localization of XIST RNA to the inactive X chromosome (Xi) in women. The apparent cytological overlap between BRCA1 and XIST RNA across the Xi raised the possibility a direct role of BRCA1 in localizing XIST. We report here that BRCA1 does not paint the Xi or XIST territory, as do markers of Xi facultative heterochromatin. A smaller BRCA1 accumulation abuts Xi, although this is not exclusive to Xi. In BRCA1 depleted normal and tumor cells, or BRCA1 reconstituted cells, BRCA1 status does not closely correlate with XIST localization, however in a BRCA1 inducible system over-expression correlated strongly with enhanced XIST expression. We confirm frequent loss of an Xi in tumor cells. In addition to mitotic loss of Xi, we find XIST RNA expression or localization frequently become compromised in cultured breast cancer cells, suggesting Xi heterochromatin may not be fully maintained. We demonstrate that complex epigenetic differences between tumor cell subpopulations can have striking effects on XIST transcription, accumulation, and localization, but this does not strictly correlate with BRCA1. Although BRCA1 can have indirect effects that impact XIST, our results do not indicate a direct and specific role in XIST RNA regulation. Rather, regulatory factors such as BRCA1 that have broad effects on chromatin or gene regulation can impact XIST RNA and the Xi. We provide preliminary evidence that this may occur as part of a wider failure of heterochromatin maintenance in some cancers. J. Cell. Biochem. 100: 835–850, 2007.

Key words: BRCA-1; XIST; X-inactivation

BRCA1 is an essential tumor suppressor protein implicated in multiple functions, including DNA repair, transcriptional regulation, chromatin remodeling, ubiquitination, and more recently, mitotic checkpoint control [Bochar et al., 2000; Hashizume et al., 2001; Wang et al., 2004; Rosen et al., 2005]. However, the specific functions and mechanisms by which BRCA1 mutations predispose women to breast and ovarian cancer are not well understood. An important new role for BRCA1 was indicated by Ganesan et al. [2002] who reported that BRCA1 (−) tumors frequently lack an inactive X while retaining two X chromosomes, and BRCA1 supports localization of XIST RNA to the inactive X chromosome (Xi). A specific role for BRCA1 in directly localizing XIST RNA would implicate loss of BRCA1 in mis-regulation of X-linked genes in women. Thus, these unanticipated findings have potentially profound significance not only for understanding basic mechanisms in breast and ovarian cancer, but for understanding how the novel chromosomal RNA, XIST, localizes throughout and inactivates one X chromosome in females.

X chromosome dosage is stringently regulated through a multi-step process initiated in the early embryo, and is controlled by a large non-coding RNA from the XIST/Xist genes [Brown et al., 1992; Kay et al., 1993; Clemson et al., 1996]. All active genes, including XIST, produce a small focus of immature transcripts

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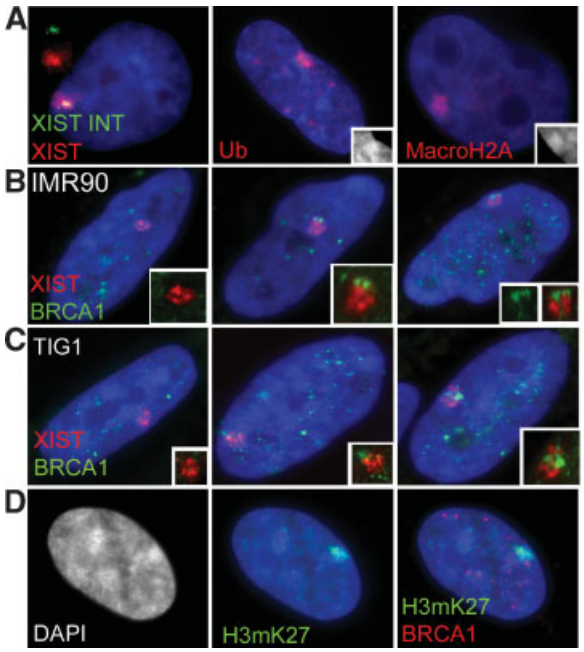

Fig. 1. Markers of X inactivation and BRCA1 relative to the inactive X. **A**: XIST RNA, Ubiquitin and MacroH2A paint the inactive X chromosome, which is visible also as the Dapi dense Barr body. **B** and **C**: BRCA1 shows some relationship but fails to paint it as seen by costaining with BRCA1 and XIST and also by staining of BRCA1 and other markers, including H3mK27 (**D**).

at their transcription site [e.g., Xing, 1993; Xing et al., 1995] (Fig. 1A). However, the XIST/Xist gene is unique in that a much larger accumulation of mature, spliced, and stable transcripts form an RNA "territory" that paints the Xi chromosome, coincident in size and shape with the interphase X chromosome DNA territory [Clemson et al., 1996, 2006] (Fig. 1A). In mouse ES cells the Xist gene becomes silenced by methylation on the active X chromosome while Xist RNA stably accumulates across the future Xi [Panning et al., 1997; Sheardown et al., 1997]. XIST RNA then triggers a series of chromosome-wide chromatin modifications, including hypoacetylation, ubiquitination and methylation of histones, which function synergistically with XIST to maintain a heritable, heterochromatic state (reviewed in Hall and Lawrence [2003] and Chang et al. [2006]). Loss of XIST RNA in somatic cells does not result in widespread reactivation of Xi [Brown and Willard, 1994] but contributes to slightly compromised or "leaky" maintenance of chromosome inactivation [Csankovszki et al., 2001]. Thus, the fact that XIST RNA continues to coat Xi in somatic cells [Clemson et al., 1996] reflects its contribution to the stringent fidelity with which X inactivation is maintained in adult cells. Indeed, even the stochastic reactivation of specific growth regulatory genes in just a very small fraction of cells could contribute to their abnormal growth and potential cancer progression.

BRCA1 has been clearly established to localize to the XY body and the unpaired X chromosome in pachytene mouse spermatocytes [Scully et al., 1997; Turner et al., 2004]. Although BRCA1 is involved in transient meiotic silencing of unpaired DNA via recruitment of ATR, this meiotic silencing is independent of XIST RNA and consistent with BRCA1's known role in DNA recombination and repair [Turner et al., 2004]. In contrast, Ganesan et al. [2002] reported that BRCA1 is involved in maintaining XIST RNA localization to the Xi in female *somatic* cells. Although the authors did not conclude this was a direct role of BRCA1 in a significant sub-fraction of cells BRCA1 appeared to co-localize extensively with XIST RNA across the Xi, similar to the morphological association of BRCA1 with the XY body in spermatocytes. Additionally, a $BRCA1^{-/-}$ tumor cell line, HCC1937, did not contain localized XIST RNA signals, whereas focal XIST RNA was restored upon reintroduction of BRCA1 in stable and in doxycycline-inducible transgenic cell lines. In addition, RNAi-mediated knockdown of BRCA1 resulted in loss of localized XIST staining. The overall conclusion that BRCA1 supports localization of XIST RNA to the Xi was further bolstered by the finding that the $BRCA1^{-/-}$ cells or tumors examined had lost localized XIST RNA, whereas the BRCA1 positive sporadic breast cancer samples studied had not. While Ganesan et al. [2002] did not extensively study X-linked gene expression nor conclude it was compromised, analysis of one Xi GFP-reporter transgene in cells following knockdown of BRCA1 showed increased expression in a very small fraction of cells.

In light of such an important report connecting BRCA1 to XIST RNA localization and X inactivation, we undertook to address distinct next questions that stem from these findings. Our studies used highly sensitive methods optimized for the detection of nuclear RNAs within individual cells, which have been shown to reliably detect nuclear transcripts from a wide assortment of genes expressed even at very

low levels [e.g., Lawrence et al., 1989; Smith et al., 1999]. We confirm that several breast cancer cell lines have lost Xi while retaining two Xs. Despite other similarities in results, key differences in molecular cytological observations or their interpretation clarify that the Ganesan et al. [2002] should not be interpreted to demonstrate a specific role of BRCA1 in XIST RNA localization. Our results demonstrate that BRCA1 does not extensively paint Xi to directly localize XIST RNA. Our findings are consistent with an effect of BRCA1 mis-regulation, particularly over-expression, on XIST transcription or abundance, however, this does not demonstrate a direct and specific role of BRCA1 in XIST regulation. Furthermore, we show that differences in XIST transcription, accumulation, or localization are often seen in cultured tumor cells independent of BRCA1 staining. Thus, broad cell regulators that affect the epigenetic or genetic state of a cell can impact XIST and Xi, which may be part of a broader loss of heterochromatin maintenance in cancer.

MATERIALS AND METHODS

Cell Lines

HCC1937 cells were obtained from ATCC (CRL-2336). BRCA1-reconstituted derivatives of HCC1937, HCC1937+vector and HCC1937+BRCA1; [Scully et al., 1999], were provided by S. Ganesan and D. Livingston. Cells were grown in RPMI medium +HEPES and supplemented with 10% FBS, 2 mM L-glutamine, 1% pen/strep. HCC1937 cells with a doxycycline-inducible BRCA1 allele HCC1937-BRCA1 Clone 5 were kindly provided by J. Feunteun and additional samples provided by S. Ganesan and D. Livingston. Cells were grown for several weeks in DMEM supplemented with 10% FBS (doxycycline free), 1% pen/strep, 2 mM L-glutamine, 1% nonessential amino acids, 100 μg/ml zeocin, and 5 μg/ml blasticidin. To induce BRCA1 expression, cells were treated with 2 μg/ml doxycycline for 24–72 h. Human diploid fibroblast WI-38 cells (CCL-75) and MDA-MB-436 cells (HTB-130) were obtained from ATCC, and TIG-1 cells (AG06173) were obtained from Coriell Cell Repositories. G3 is a subclone of F2-6, an HT1080 fibrosarcoma cell line that carries a stably integrated XIST transgene on an autosome [Hall et al., 2002]. WI38, TIG-1, and G3 cells were grown in MEM supplemented with 10% FBS and 1% pen/strep. MDA-MB-436 cells were grown in Leibowitz's L-15 Medium with 2 mM L-glutamine supplemented with 10 ng/ml insulin, 10 ig/ml glutathione and 10% FBS. Samples of the MCF10A series of breast mammary epithelia were kindly provided by Jeff Nickerson. SUM149PT cells were a gift of A. Mercurio, UMMS.

Antibodies

The MS110 monoclonal antibody was obtained from S. Ganesan. In addition, a polyclonal antibody to BRCA1 (KAPST0201, Stressgen) was used at a dilution of 1:500 (Figure S2G; S3C-D), and direct comparison of this with the MS110 antibody in two colors revealed fully coincident staining patterns. A monoclonal antibody to polyubiquitinated proteins (UbFk2, Affiniti) was used for immunofluorescence. Polyclonal antibodies to BRCA1 (KAPST0201, Stressgen), histone H3-3mK27 (Upstate), and macroH2A (Upstate) were also used for IF.

Fluorescence In Situ Hybridization

Cells were processed for DNA or RNA FISH according to previously established protocols [Clemson et al., 1996; reviewed in Tam et al., 2004]. *XIST* was detected with a 10 kb plasmid (pG1A) spanning intron 4 to the 3′ end of XIST or plasmid (pXISTHb-B) containing intron 1 (Hunt Willard and Carolyn Brown, described in Clemson et al. [1996]). Probes were nick-translated using biotin-11-dUTP or digoxigenin-16-dUTP (Boehringer Mannheim). Hybridization was detected with either anti-digoxigenin antibody (Boehringer Mannheim) coupled with rhodamine or fluorescein or, for biotin detection, avidin conjugated to Alexa-streptavidin 594 (red) or fluorescein-conjugated avidin (Boehringer Mannheim). For combined immunostaining and RNA FISH (Figs. 2E–G and S2G), cells were processed as described above with RNAs in added to antibody incubations. After detection and washing, cells were refixed in 4% paraformaldehyde in PBS for 10 min at 25°C and then processed for RNA FISH as described above.

Digital imaging analysis was performed using an Axiovert 200 or an Axiophot Zeiss microscope equipped with a 100× PlanApo objective (NA 1.4) and Chroma 83000 multi-bandpass dichroic and emission filter sets

(Brattleboro, VT), set up in a wheel to prevent optical shift. Images were captured with an Orca-ER camera (Hamamatsu, NJ) or a cooled charge-coupled device (CCD) camera (200 series, Photometrics). Where rhodamine was used for detection in red, a narrow band-pass fluorescein filter was inserted to correct for any bleed-through of rhodamine fluorescence into the fluorescein channel.

Immunofluorescence

For immunostaining we tested two fixation methods: paraformaldehyde fixation followed by triton extraction, essentially as described by Ganesan et al. [2002] or brief triton extraction prior to fixation as described previously [Clemson et al., 1996; Tam et al., 2004]. For extraction, cells were rinsed in CSK buffer followed by extraction in CSK buffer with 0.5% Triton X-100 and 2 mM vanadyl adenosine for 5 min. Cells were then fixed in 4% paraformaldehyde in 1× PBS for 10 min, incubated with primary antibodies in 1× PBS/1% BSA for 1 h at 37°C, and then rinsed successively in 1× PBS, 1× PBS + 0.1% Triton X-100, and 1× PBS for 10 min. Detection was with secondary antibodies tagged with fluorescein, rhodamine or Texas Red (Jackson ImmunoResearch, West Grove, PA) diluted 1:500 in 1× PBS/1% BSA.

RNA Interference

G3 cells were plated and transfected with small interfering RNAs specific for BRCA1 (siRNA SMARTpool® BRCA1; Dharmacon) or a control siRNA (Dharmacon) using RNAifect (Qiagen) or Oligofectamine (Invitrogen) as described by the manufacturers. The media was changed 24 h after transfection. Cells were fixed either 48 or 72 h after transfection as previously described. They were then processed for immunofluorescence or RNA FISH as described above. Knockdown of detectable BRCA1 in most cells was confirmed by immuno staining.

RESULTS

BRCA1 Does not Substantially Overlap With XIST RNA to Coat Xi Facultative Heterochromatin but in Some Cells Abuts or Slightly Overlaps it

We first examined the relationship of BRCA1 to the Xi and the XIST RNA territory. Ganesan et al. [2002] reported that BRCA1 "colocalizes" with the inactive X (XIST RNA) in a significant fraction (5–10% with XIST and 4–12% for MacroH2A) of cells, with the images presented showing complete overlap of BRCA1 protein and the XIST RNA territory. This observation provides a key underpinning for the strong possibility that BRCA1 directly and specifically supports XIST RNA localization across the Xi. A complete overlap would not occur by random chance and is typical for other factors functionally involved with Xi facultative heterochromatin, such as H3mK27, macroH2A and ubiquitin (Fig. 1A,D). After exhaustive analysis of BRCA1 and XIST RNA staining in several female human and mouse cell types, testing different protocols and antibodies in dozens of experiments, we report that BRCA1 does not extensively coincide with XIST RNA to "paint" the inactive X chromosome (Table I), as would be in keeping with a direct role in XIST RNA localization.

Our findings did show some more limited association of BRCA1 foci with the inactive X (Table I). A focus of BRCA1 abutted or partially overlapped XIST RNA in 3–5% of cells. When a somewhat larger BRCA1 focus or cluster appeared closely associated, BRCA1 often appeared to occupy a "gap" in the XIST RNA territory (Fig. 1B,C). This was confirmed by optical sectioning and 3-D rendering in a related study examining BRCA1's broader relationship to heterochromatin [Pageau and Lawrence, in press]. We also more frequently observed one of the 5–20 small BRCA1 foci was immediately *adjacent* to the inactive X in 13% of TIG1 cells. Results using H3mK27, a marker Xi facultative heterochromatin, were similar (Fig. 1D).

TABLE I. Relationship of BRCA1 to the Inactive X Chromosome as Measured by Overlap of BRCA1 With XIST RNA

Cell type	% slight overlap with XIST	% adjacent to XIST
TIG1	3	13
WI38	4	10
X3 mouse[a]	7	28
IMR90	5	15

BRCA1 was considered to be adjacent to XIST/Xi if the signals appeared to be physically touching but not overlapping.
[a]These cells have two inactive X chromosomes and association with either inactive Xs was noted. Overlap with both Xi's was never noted.

Although the frequency of BRCA1 association with Xi increases during its replication [Pageau and Lawrence, in press; Chadwick and Lane, 2005], full overlap with XIST RNA was also not seen during Xi replication.

In some experiments we used ubiquitin staining to highlight Xi [de Napoles et al., 2004; Fang et al., 2004; Smith et al., 2004] and saw some overlap of one or a few dots of BRCA1 with a large ubiquitinated structure in 13–17% of cells (not shown). However, further analysis suggested that a ubiquitinated structure other than the DAPI-dense Barr Body showed this association with BRCA1. This highlights the importance of using Xi definitive markers (XIST RNA) to examine this relationship, which bears on other recent studies (see Discussion).

The distinction shown here, that BRCA1 does not "paint" the inactive X with XIST RNA but shows a spot adjacent to or abutting it, is a critical one, as it does not fit with BRCA1 directly supporting localization of XIST RNA to Xi and does not necessarily indicate that BRCA1 has a *specific* relationship to the Xi that is distinct from other chromosomes. In fact, other work from our lab indicates that BRCA1 has a broader relationship to replicating constitutive heterochromatin on many chromosomes, and thus associates with both Xi and Xa [Pageau and Lawrence, in press]. We do not rule out a slightly greater association with Xi due to its more heterochromatic nature.

$BRCA1^{-/-}$ HCC1937 Cells do not Contain Mislocalized XIST RNA Because Most Cells do not Transcribe XIST

Ganesan et al. [2002] reported $BRCA1^{-/-}$ HCC1937 cells lacked localized XIST RNA signals, but retain two X DNA signals, as our results confirm. However, Ganesan et al. [2002] had surmised that XIST RNA was likely mislocalized because they were unable to detect focal XIST RNA despite the suggested presence of low levels of XIST RNA by RT-PCR. Our lab has previously demonstrated the ability to detect dispersed XIST RNA in the nucleoplasm (Supplemental Figure S1) [Clemson et al., 1998; Hall et al., 2002] and to sensitively detect a small concentration of RNA at the transcription site of a variety of genes, including XIST (Fig. 2A), [e.g., Smith et al., 1999] allowing investigation of whether XIST is being transcribed in individual cells. If XIST were transcribed and mislocalized, it should thus be detectable as dispersed signal through the nucleoplasm as well as a small intense transcription focus of RNA (as illustrated in Fig. 2A). In numerous RNA FISH experiments, we consistently found no evidence of mislocalized XIST RNA (Fig. 2B,E), when it was strongly detected in parallel control samples (Fig. 2A). Hybridization to XIST DNA showed two loci present (Fig. 2E), yet in repeated experiments, the typical transcription focus was also clearly

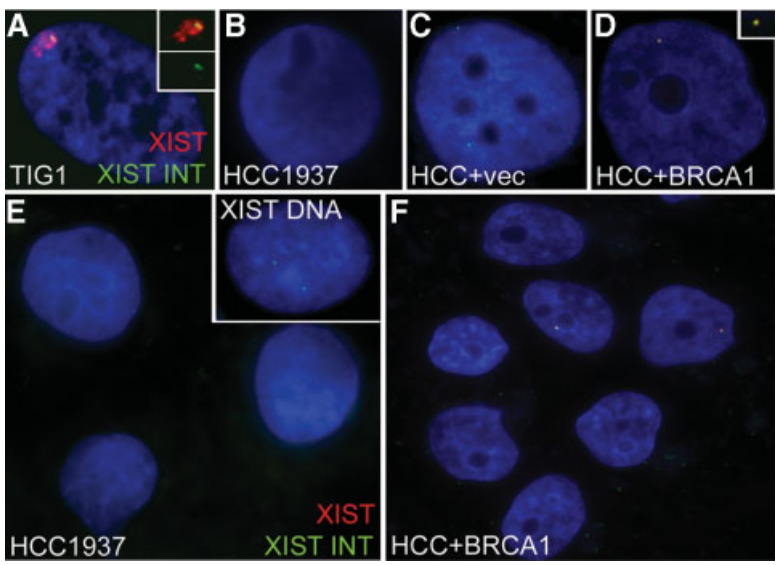

Fig. 2. (**A**) XIST RNA in TIG1 fibroblast controls. XIST intron is shown in green and the mature XIST transcript is in red. XIST RNA in HCC1937 cells (**B**) and HCC1937 cells reconstituted with either vector (**C**) or BRCA1 (**D**). **E** and **F** show a field of cells with XIST RNA (E and F) or DNA (E, inset).

lacking in 97% of cells (Fig. 2B,E), with just 3% (14/500) having a small transcription spot. Two-color XIST hybridization using both a cDNA probe and an XIST intron probe confirmed this small signal was just a transcription focus. Thus, XIST RNA is neither mislocalized nor transcribed in the majority of these HCC1937 BRCA1-mutant cells, despite two X chromosomes in over 80% of cells.

We found that other markers of X-inactivation, H3mK27 and ubiquitin, were also absent in the majority of these cells. MacroH2A bodies were seen in ~2–3% of HCC1937 cells (not shown). However, these did not correspond to DAPI-dense Barr Bodies but appeared to reflect other macroH2A-rich nuclear and perinuclear structures, as has been reported [e.g., Zhang et al., 2005].

HCC1937 Cells Stably Reconstituted With BRCA1 do not Localize Mature XIST RNA Across Xi but Many Cells Transcribe XIST

We next examined $BRCA1^{-/-}$ cells (HCC1937) stably reconstituted with BRCA1 (kindly provided by S. Ganesan). In the previous study, the term "focal XIST staining" was used to described any localized XIST RNA signal, so that a larger XIST RNA territory functionally associated with Xi was not explicitly discriminated from a smaller RNA spot indicative of just transcription. It was reported that BRCA1 reconstituted cells exhibited reappearance of "focal XIST RNA" [Ganesan et al., 2002]. While many cells in the BRCA1 reconstituted line showed no XIST transcription, we found most (57–62%) display a small bright XIST RNA spot (Fig. 2D,F) consistent with a transcription focus. This confirms some difference from the parental HCC1937 cultures studied, as reported by Ganesan. However, further analysis shows that, the XIST signals were clearly not the typical large territory of XIST RNA that paints the X chromosome, even in cells clearly staining positively for BRCA1. Simultaneous hybridization with an intron-specific probe confirmed that these signals are primarily newly synthesized immature transcripts at the gene, not the much larger accumulation of spliced, stable XIST RNA that inactivates an entire chromosome [Clemson et al., 1996] (Fig. 2A v. 2F). Repeated experiments in cells from two different sources confirmed these findings. Additionally, in parallel samples stained for H3mK27 or macroH2A, we did not observe a Barr body or similarly large structure stained for hallmarks of Xi in the vast majority of cells.

The appearance of XIST transcription foci in the BRCA1 reconstituted cells may reflect an effect on transcription, consistent with a previous report that XIST is among a subset of genes whose transcription is increased by BRCA1 [Welcsh et al., 2002]. However, since ~9% of HCC1937 cells reconstituted with vector alone expressed XIST transcription foci (Fig. 2C), these differences could reflect epigenetic changes within different subclones or cell populations. To further investigate this, a second, independently derived aliquot of HCC1937 cells reconstituted with BRCA1 (kindly provided by J.J. Chen) was analyzed. Repeated RNA FISH confirmed that these cells, clearly positive for BRCA1, did not contain any focal XIST RNA. However, further DNA FISH analysis indicated these cells have only one X-chromosome (not shown). This is consistent with other evidence [Sirchia et al., 2005; Richardson et al., 2006] that mitotic loss of Xi that arise in subpopulations of breast tumor cells can account for loss of XIST RNA signals.

Cells Over-Expressing BRCA1 Show Enhanced XIST Expression but Variations in XIST Also Can Occur Independently of BRCA1

Given the difficulty of examining unstable tumor cell populations that can evolve differences due to subcloning, sorting, or propagation, it was important to examine a BRCA1-inducible system. Therefore, we focused efforts on HCC1937 cells in which BRCA1 can be induced by doxycycline (kindly provided by J. Feunteun). Using these same HCC1937 clone 5 cells, Ganesan et al. [2002] reported that in ~10% of cells induced to stain brightly for BRCA1, 95% had "focal XIST RNA" accumulations (whereas no change in XIST RNA was noted by RT-PCR).

Two aspects of results with this system provide insight into the basis for the observations made by Ganesan et al. [2002]. First, we confirm that after 24–72 h of induction the very high BRCA1 over-expressing cells have an increased probability of expressing XIST RNA (typically larger accumulations of XIST RNA) (Fig. 3B). In Ganesan et al. [2002], the highly XIST-positive cell fraction noted was the subset induced to over-express BRCA1, as indicated by

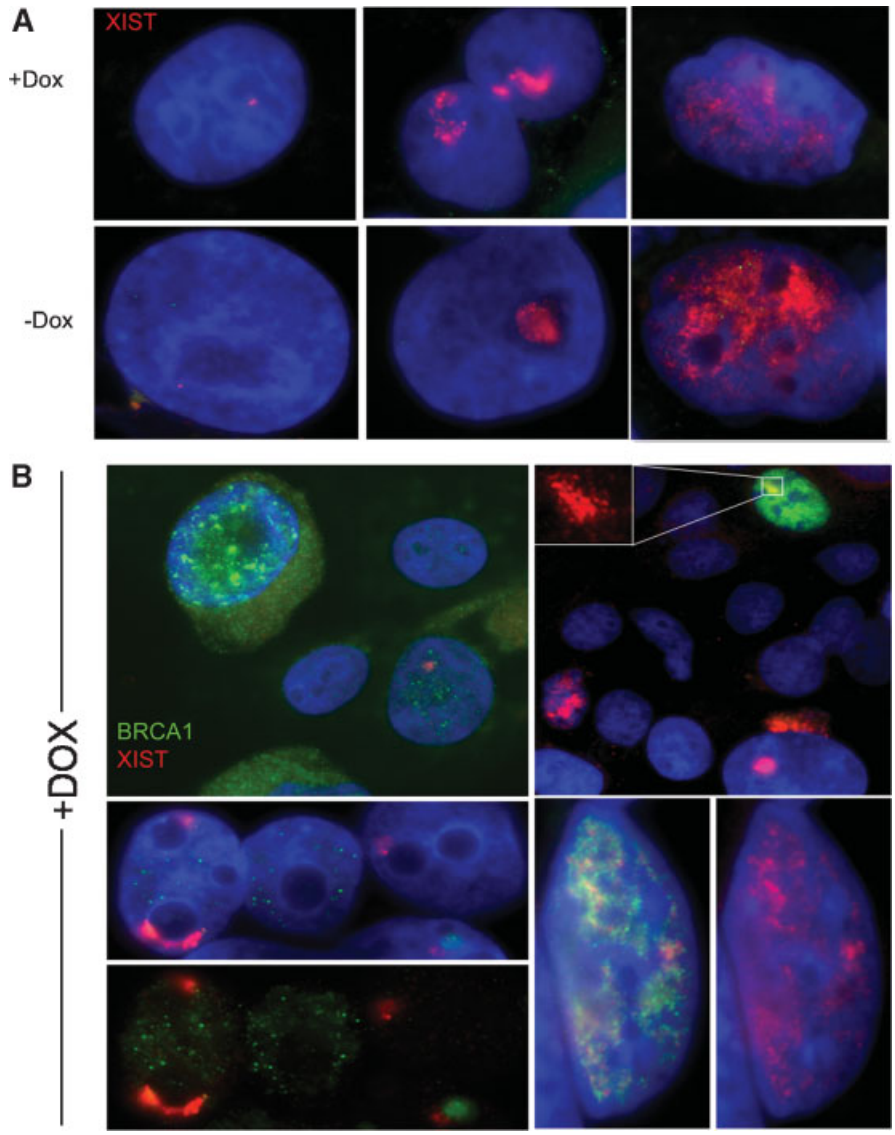

Fig. 3. XIST RNA and BRCA1 in cells with a doxycycline-inducible BRCA1 gene. (**A**) These HCC1937 cells were either induced to express BRCA1 (+DOX) or not (−DOX). BRCA1 is shown in green and XIST RNA in red. (**B**) All cells were treated with DOX. Note that some cells without BRCA1 have localized XIST and some with BRCA1 have mislocalized XIST.

extremely bright, diffuse BRCA1 stain throughout the nucleus. In our experiments, these cells, ~1% of the population, had a ~5–8× higher frequency (~75–90%) of XIST RNA signals (typically abundant XIST RNA) (e.g., Fig. 3B). Thus, we concur that cells over-expressing BRCA1 have substantially increased transcription and accumulation of XIST RNA.

Second, this analysis clearly demonstrated the striking differences in XIST RNA phenotype that can occur independently of BRCA1 staining. A significant cell fraction in dox-treated cultures express XIST, but, surprisingly, such cells were also frequently seen in the non-induced cultures (e.g., 11.5% in dox-treated cultures vs. 8% in non-treated, Table II and Fig. 3A). Repeated experiments with two different samples (provided from the original source by J. Feunteun and D. Livingston) revealed similar results. Simultaneous detection of BRCA1 and XIST RNA repeatedly showed a substantial number of XIST RNA positive cells prior to induction, ranging from large often abnormal XIST RNA signals (11–14%) to very

TABLE II. XIST RNA Phenotypes in BRCA1 Inducible System

Experiment	Doxycycline	% XIST territory	% with dispersed XIST	% XIST transcription focus	Total % XIST positive	% of XIST positive cells without BRCA1 staining
1	+	11	2	18	31	92
	−	8	4	18	30	100
2	+	12	2	9	23	87
	−	8	3	11	23	100
Ave	+	11.5	2	13.5	27	89.5
	−	8	3.5	14.5	26.5	100

Experiments 1 and 2 were performed using cells grown for extended periods in FBS guaranteed to be doxycycline-free. Cells were induced with doxycycline for 24–48 h prior to fixation (Dox) or left uninduced (No Dox).

small transcription foci (9–18%) (Fig. 3A). Most cells expressing focal XIST RNA had no significant BRCA1 signal, as quantified in Table II. We interpret the striking variability of XIST phenotypes common in cancer to demonstrate that intercellular differences and complex epigenetic effects impact XIST expression and localization, thus this complicates the analysis of any effects of BRCA1.

The strong correlation between high BRCA1 expressors and abundant XIST RNA signal suggests some effect of BRCA1 on XIST transcription and possibly stability, consistent with other evidence that inducible BRCA1 can upregulate XIST [Welcsh et al., 2002]. We cannot exclude any possibility that cells that over-expressed BRCA1 upon dox-treatment happened to reflect a cell subset that had XIST RNA prior to induction. Irrespective of an effect on transcription, in four experiments XIST localization did not vary significantly between the induced and uninduced cells (Fig. 3A and Table II). Many of the XIST signals were atypically large and irregular in shape, and in a subfraction of cells the RNA dispersed widely throughout the nucleoplasm. The frequently abnormal XIST RNA rarely coincided with a DAPI-dense region suggestive of a Barr body, but often was in a DAPI-dim region in or around the nucleolus. Hybridization to hnRNA using a Cot-1 DNA probe (Methods) showed some XIST RNA signals overlapped regions depleted of hnRNA, whereas many did not. In one experiment we determined whether the very bright BRCA1 cells had a macroH2A body, (frequently used as a marker for the Xi by Ganesan et al. [2002]), however, only 5% of the BRCA1- overexpressing cells had a macroH2A body, similar to the rest of the population (data not shown).

Overall, these results demonstrate that BRCA1 does not consistently promote normal XIST RNA localization, but BRCA1 overexpression appears to enhance XIST RNA transcription.

Maintenance of XIST RNA Localization and X Inactivation After RNAi-Mediated BRCA1 Knockdown

We used Dharmacon BRCA1 siRNA smartpool (Methods) to examine the impact of RNAi-mediated knockdown on XIST RNA localization and other hallmarks of Xi. Of several knockdown experiments in three cell types, we focused our quantitative analysis on two experiments in HT1080 G3 cells (see Methods, [Hall et al., 2002]) because these had the most cells showing BRCA1 depletion, as assayed by strong immunofluorescence to experimental and control RNAi samples. In cells treated with RNAi to BRCA1 for 48–72 h, despite a significant decrease in BRCA1 staining (Fig. 4A and Table III), no effect was seen on XIST RNA localization (Fig. 4B), nor was an effect on intensity noted. We also studied other Xi hallmarks, with the potential impact on ubiquitination of particular interest (Fig. 4C–E). Ubiquitin is enriched on the Xi (or the inactivated transgenic autosome in G3) [de Napoles et al., 2004; Smith et al., 2004], BRCA1 is known to act as an ubiquitin ligase [Hashizume et al., 2001], and a previous study reported that BRCA1 knockdown diminishes ubiquitin nuclear foci [Morris and Solomon, 2004]. We found no reduction in conjugated ubiquitin (detected with the UbFk2 antibody) associated with the Barr body, indicating that marked depletion of BRCA1 did not perturb Xi ubiquitination (Fig. 4D). We also saw no reduction in H3mK27 or macroH2A (Fig. 4E,C).

In other BRCA1 knock-down experiments from our lab and others [Starita et al., 2004; Pageau and Lawrence, in press], it has been

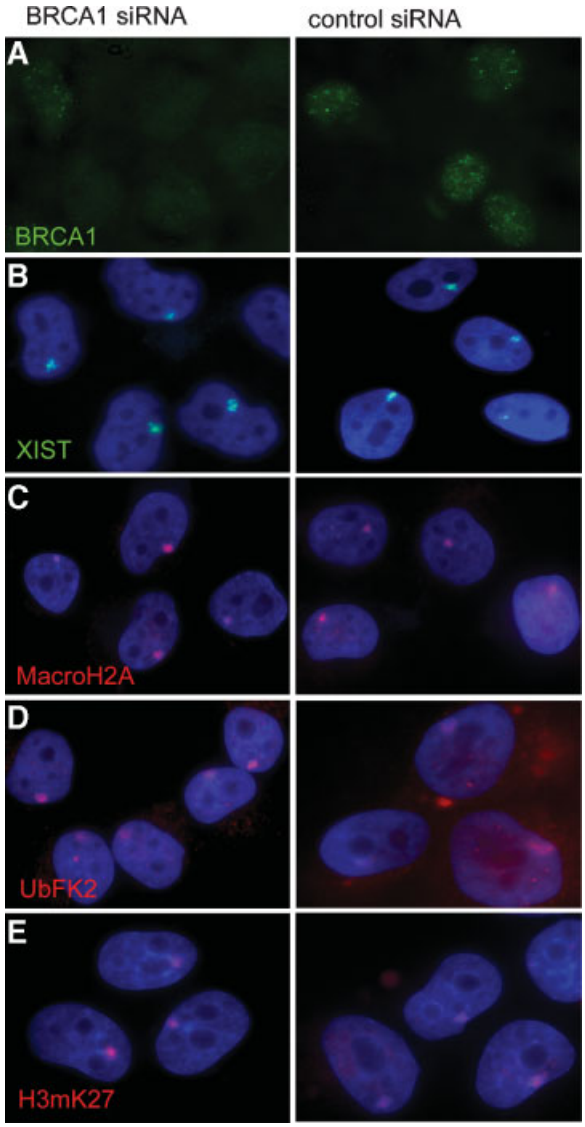

Fig. 4. RNAi to BRCA1 in G3 cells. Despite knockdown of BRCA1 by siRNA (**A**, 4 cells/field shown), other hallmarks of X inactivation, including XIST RNA, MacroH2A, UbFK2, and H3mK27 are maintained (**B–E**). A comparison with control siRNA-treated cells is shown (right column).

TABLE III. BRCA1 siRNA Results

SiRNA	% BRCA1 positive	% XIST RNA positive
Control siRNA	44	97
BRCA1 siRNA	6	98

The % of BRCA1-Foci positive G3 cells is shown for cells treated with a control siRNA v. a BRCA1-specific siRNA. In the right-hand column, the % of XIST RNA positive cells is shown for each group. A 7× reduction in Brca1 staining was seen in this experiment.

established that acute short-term BRCA1 loss often results in cell cycle arrest. Whether this occurs may depend upon the degree of BRCA1 knockdown or the cell type. The lack of change in macroH2A in the experiments shown (Fig. 4C) provides evidence that these cells are likely still cycling, as macroH2A is mostly seen in S phase [Chadwick and Willard, 2002]. Therefore, in this experiment the marked reduction of BRCA1 staining reflects bona fide BRCA1 depletion, rather than failure of cells to enter S-phase. While it is not clear why we did not see loss of XIST RNA localization as reported by Ganesan et al., our findings indicate that short-term depletion of BRCA1 does not consistently result in compromised XIST RNA or other Xi hallmarks.

Compromised XIST RNA is Frequently Seen in Cultured Breast Cancer Cells, Unlike Normal Diploid Cells

We examined XIST RNA in a number of breast cancer tumor cell samples to address a few specific questions: (1) Is there direct evidence that compromised XIST RNA localization (suggestive of Xi reactivation) does occur in some cancer cells? (2) If so, does this occur in BRCA1(+) and/or BRCA1(−) samples? (3) Are there BRCA1 mutant tumor cells that show normal XIST RNA? In the earlier study by Ganesan et al., only cells from the BRCA1-dependent breast cancers had lost XIST RNA, but the sample examined was not large, and two recent studies, one from the same authors, indicate that this may occur in the presence of BRCA1. Richardson et al. [2006] report that a significant fraction of BRCA1 + basal-like (BLC) also lack XIST RNA, most frequently as a result of mitotic loss of the Xi. However, the question remains as to whether, when Xi is retained, does this heterochromatin become compromised as indicated by mislocalization or reduced accumulation of XIST RNA.

As summarized in Table IV, we examined several BRCA1-positive breast cancer lines (MCF7, MDA-MB-231) including a breast cancer model series of progressive transformation (MCF10A, MCF10ANeoT, and MCF10ACA1a). We also looked at two newly characterized BRCA1(−) cell lines, SUM149PT and MDA-MB-436 [Elstrodt et al., 2006]. For comparison, we also scored IMR-90 fibroblasts, which are primary fibroblasts that have been cultured for

TABLE IV. XIST RNA Localization in Various Breast Cancer Cell Lines

Cell type	Description/BRCA1 status	Localized XIST RNA (%)	Loose/mislocalized XIST (%)	XIST transcription focus (%)	No XIST (%)
HCC1937	Breast cancer line/BRCA1$^{-/-}$	0	0	3	97
HCC 4.9	Reconstituted HCC1937 BRCA1+	0	0	62	38
HCC 4.12	Vector-reconstituted HCC1937 BRCA1-	0	0	9	91
MCF7[a]	Primary breast cancer BRCA1$^{+/+}$	52	14	9	26
MDA-MB-436	Breast cancer BRCA1$^{-/-}$	43	39	8	10
MDA-MB-231	Metastatic breast cancer BRCA1+	0	0	0	100
MCF10A[b]	Breast epithelium, immortalized BRCA1+	88	6	5	1
MCF10AneoT[b]	MCF10A + Ras (pre-malignant) BRCA1+	83	13	3	1
MCF10ACA1a[b]	MCF1O1NeoT, malignant BRCA1+	83	9	6	2
IMR90	Fibroblast (control) BRCA1+	98–100	0–2	0	0

For each cell line, the BRCA1 status is shown in addition to the % of cells showing each XIST RNA pattern.
[a]The statistics for MCF7 cells included in this table are for cells that were the earliest passage examined in our laboratory.
[b]These cells represent a breast cancer model described previously in Santner et al. [2001]. The presence of 2 X chromosomes in the majority of each culture was confirmed by DNA FISH.

many passages (bottom Table IV); these are representative of many primary lines that show essentially stable XIST RNA expression and localization in culture. Results of the MCF10A series illustrate that some malignant cells retain apparently normal XIST RNA and Xi hallmarks, as all three lines in this model of progressive transformation demonstrated clear XIST territories in the vast majority of cells (Table IV and Fig. 5C). Analysis of other hallmarks in this series, including H3mK27, depletion of hnRNA staining by Cot1 hybridization, and appearance of a Barr body with DAPI, confirmed that this was well correlated with the presence of an inactive X chromosome (data not shown).

In contrast, analysis of other breast cancer tumor cells exhibit a range of aberrant XIST RNA/X-inactivation phenotypes, ranging from complete loss of XIST signals to mislocalization of XIST RNA. The MDA-MB-231 (BRCA1+) cells, derived from a breast cancer metastasis, had no XIST RNA signals, despite two X chromosomes in 75% of cells (shown by DNA

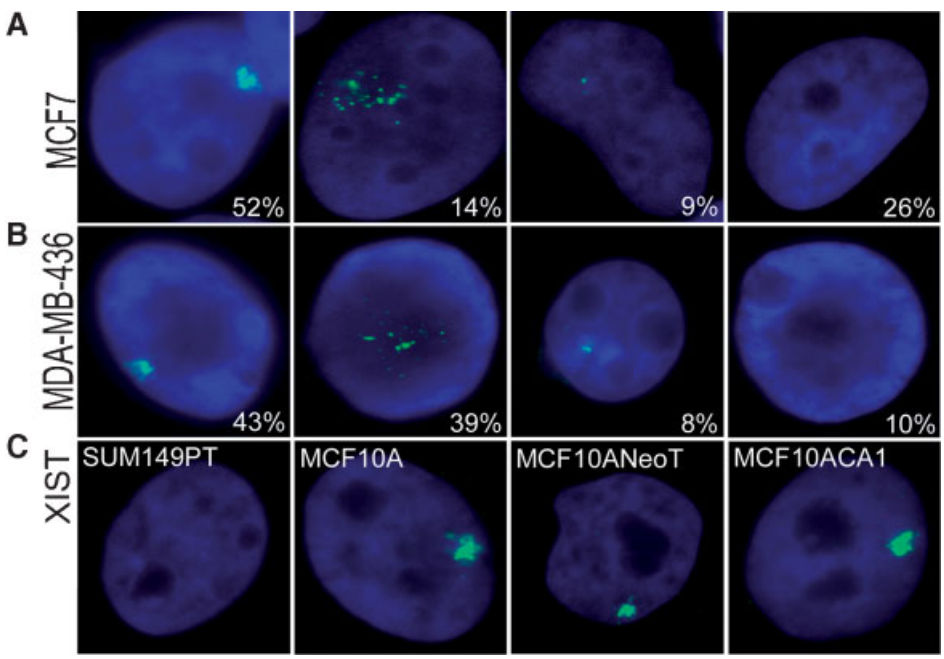

Fig. 5. XIST RNA in various tumor lines including MCF7 (**A**), MDA-MB-436 (**B**), SUM149PT (**C**, left) and the MCF10A breast series (C, right). Numbers indicate the percentage of cells of each type exhibiting the following patterns: localized XIST, mislocalized XIST, and XIST transcription focus, and no XIST RNA.

FISH) (data not shown). Results for the MCF7 (BRCA1+) and MDA-MB-436 (BRCA1-mutant) cells were of particular interest because these exhibited four distinct XIST RNA phenotypes (Table IV and Fig. 5A,B), suggesting that Xi heterochromatin was not well maintained. Some cells had a well-defined XIST RNA territory whereas others lacked detectable XIST RNA, despite the presence of two X chromosomes. A third population showed loss of proper XIST RNA localization, with substantial RNA adrift in the nucleoplasm, and finally a small sub-fraction had only an XIST transcription focus. Thus some compromise of Xi heterochromatin does occur in these cultured cancer samples.

Of particular interest was analysis of two additional BRCA1 mutant tumor cell samples. In the MDA-MB-436 ($BRCA1^{53}96^{+1G>A}$; [Elstrodt et al., 2006]), which lack functional BRCA1, 43% of cells showed significant localized XIST RNA, whereas the rest exhibit similar patterns of mislocalized XIST RNA or just transcription foci, similar to the MCF7 cells (Fig. 5B). These cells are thus an example of a BRCA1-cell line that maintains some localized XIST RNA on Xi in a significant fraction of cells, suggesting that loss of normal BRCA1 does not necessarily result in complete loss of XIST RNA. In contrast, the SUM149PT ($BRCA1^{2288delT}$; [Elstrodt et al., 2006]) cells lack an inactive X or localized XIST RNA (Fig. 5C) but the vast majority of cells did not contain 2 X chromosomes, suggesting mitotic loss of Xi. Therefore, while our results point to alterations in X chromosome dosage, this is not strictly associated with BRCA1 loss.

Changes in XIST or Xi May Occur in the Context of Broader Changes to Heterochromatin in Cancer

Finally, we note that it is important to consider whether compromised Xi heterochromatin might reflect a broader degradation of heterochromatin maintenance, which can be evaluated using an assay to delineate the heterochromatic compartment of nuclei. As previously shown, the silenced Xi and the peripheral heterochromatic compartment can be assessed by hybridization to hnRNA with a Cot-1 DNA probe [Hall et al., 2002; Tam et al., 2004]. As exemplified in Figure 6A,B normal primary fibroblasts and the non-malignant MCF10A (mammary epithelial cells) show a well-defined thick rim of peripheral heterochromatin, in addition to other heterochromatic blocks in the nucleoplasm. This peripheral heterochromatic rim has long been known to be characteristic of most normal cell types. In contrast, the HCC1937 cells shown illustrate an unusually thin hnRNA depleted rim of DAPI staining at the periphery, and in these cells there is actually a suggestion of more Cot-RNA expression in peripheral regions (Fig. 6C). While not the focus of our study here, this comparison serves to emphasize that the significance of defects in XIST RNA or Xi heterochromatin will ultimately need to be understood in the context of whether there is broader failure in heterochromatin maintenance throughout the genome.

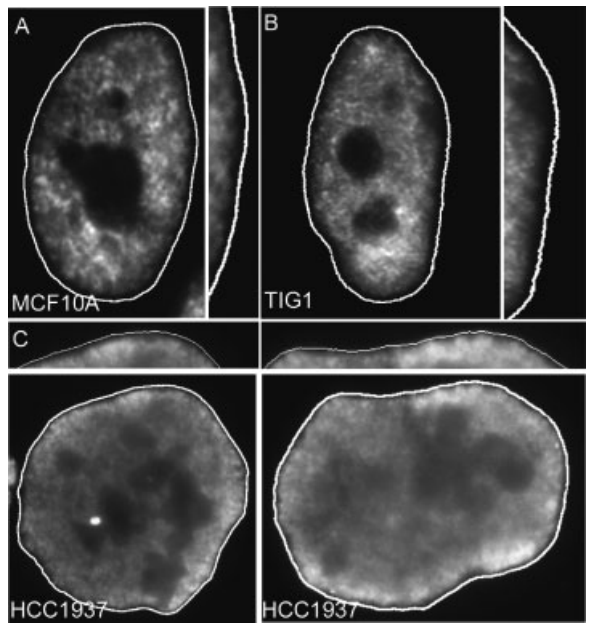

Fig. 6. A compromised heterochromatic compartment is seen in HCC1937 cells compared to control. The heterochromatic compartment is visualized using the Cot1 assay for hnRNA. **A** and **B**: In control breast epithelia (MCF10A) or fibroblast (TIG1) cells a rim of heterochromatin devoid of transcription is clearly seen, in addition to the Cot1-depleted Barr body. HCC1937 cells have a much thinner rim by comparison (**C**). A portion of the heterochromatic compartment is enlarged for each cell type.

DISCUSSION

The reported relationship of BRCA1 to XIST RNA localization has far-reaching implications for research into both breast cancer and X chromosome dosage, thus it is critical that any questions about the nature of this relationship be further examined. Recent studies agree that

mitotic segregation errors were the most commonly seen mechanism for the presence of two active X chromosomes in breast cancer. Aspects of our findings are also in keeping with more recent studies from the Miozzo [Sirchia et al., 2005] and Livingston labs [Richardson et al., 2006] that indicate that increased X-chromosome abnormalities seen in breast cancer most frequently may arise due to mitotic loss of the Xi and duplication of Xa. However, the question remains as to whether Xi *reactivation*, possibly through mis-regulation of XIST RNA, also contributes to the increased X-linked gene expression often seen [Jazaeri et al., 2002; Spatz et al., 2004]. In depth molecular cytological analysis indicates that BRCA1 does not have a direct role in localizing XIST RNA across Xi facultative heterochromatin. Despite some similarities in results, our findings indicate that any correlation between BRCA1 and "focal XIST RNA" reflects a difference in transcription, not localization of XIST RNA. While Ganesan et al. [2002] conclude that "BRCA1 supports localization of XIST RNA", the authors state twice that they cannot rule out the effect is on transcription. Moreover, findings here demonstrate interesting but complex epigenetic effects that can impact XIST transcription, abundance and localization, even between cells in the same culture. It is known that treatment of cells with demethylating agents can induce XIST expression [Clemson et al., 1998], and in other recent studies we find that specific changes that broadly affect chromatin can impact XIST RNA (Hall and Lawrence, in prep). Findings here are suggestive that BRCA1 may be among the regulatory factors that can impact XIST RNA phenotype; however, even if so, there is no clear evidence that this reflects a specific role of BRCA1 in XIST regulation, rather than broader effects of BRCA1 on the epigenetic or regulatory state of the cell which could impact heterochromatin (e.g., via chromatin remodeling or misregulation of subsets of genes) [Bochar et al., 2000; Welcsh et al., 2002; Rosen et al., 2005].

Several experimental findings in our lab were similar to those reported by Ganesan et al. [2002], using cell sources, antibodies and other reagents as close to the original study as possible (kindly facilitated by several authors of the Ganesan study). For example, we observe a spatial "association" of BRCA1 and the XIST RNA territory at about the same frequency reported by Ganesan (5–12%); the parental HCC1937 cultures examined had two XIST loci but no inactive X or XIST RNA signal; over half of cells stably reconstituted with BRCA1 showed "focal XIST RNA signal" unlike the parental cultures examined in parallel; in the BRCA1 doxycycline-inducible system, those cells that clearly over-expressed BRCA1 had a much higher frequency of XIST RNA accumulations than cells which did not stain brightly for BRCA1. However, when examined more closely, we can further clarify the implications of such observations based on detailed molecular cytology of XIST RNA and X inactivation, as discussed below.

BRCA1's Association With Part of Xi is Consistent With a Relationship to Heterochromatin on Many Chromosomes

While we find a spot or cluster of BRCA1 associates with the Xi in a small but significant subset of cells, this more limited relationship has a different functional implication than if BRCA1 more fully painted the XIST-RNA associated heterochromatin. A key part of the evidence that XIST RNA had a functional role in Xi silencing was its almost complete overlap with the whole Xi DNA territory [Clemson et al., 1996]. The apparent full overlap of BRCA1 with the XIST RNA territory shown was the underpinning for the possibility raised by the Ganesan et al. [2002] study that BRCA1 might be directly involved in localizing XIST RNA. Instead, this and other work from our lab indicates that BRCA1 foci have a broader relationship to constitutive heterochromatin associated with centromeres, including both Xa and Xi, and that this is temporally linked to replication [Pageau and Lawrence, in press]. This broader association with heterochromatin may appeared to suggest a specific relationship to XIST RNA across the Xi.

Two more recent studies appeared to confirm the reported relationship of BRCA1 to the XIST RNA-coated facultative heterochromatin of Xi, however the markers used did not definitively detect XIST RNA [Chadwick and Lane, 2005; Ouyang et al., 2005]. Here we show that studies using ubiquitin or macroH2A as a marker indicate that these do not always faithfully show the XIST RNA/Xi, but can identify some other structure with which BRCA1 can associate. Also, although Chadwick and Lane conclude that BRCA1 co-localizes with XIST RNA

coated facultative heterochromatin, B. Chadwick concurs that when viewed in 3-D the BRCA1 may closely appose but not overlap most of the XIST RNA or the markers used (B. Chadwick, personal communication). Interestingly, Ganesan et al., report that ChIP with BRCA1 antibodies bring down XIST RNA, which may occur because BRCA1 closely associates with a small part of the XIST RNA territory in some cells, particularly during replication of Xi [Pageau and Lawrence, in press].

Over-expression of BRCA1 was Closely Correlated With Enhanced XIST Expression

In normal somatic cells, the XIST RNA accumulation on Xi consists of two populations: a small spot of immature new transcripts from the XIST locus and a larger accumulation of mature, spliced, and stable RNA that functionally interacts with the X chromosome [Clemson et al., 1996]. In our analysis we discriminated between XIST transcription foci, the larger localized XIST RNA territory, or mislocalized XIST RNA dispersed in the nucleoplasm. We found no evidence of XIST transcription in over 95% of BRCA1$^{-/-}$ HCC1937 parental cells. On the other hand, most BRCA1 stably reconstituted cells transcribed XIST, but did not accumulate mature XIST RNA across the Xi. To avoid studying cell populations cultured separately, analysis of the BRCA1 dox-inducible system used by Ganesan et al. [2002] became important. We confirm that the brightest BRCA1 staining cells (which grossly overexpress BRCA1) are highly likely to have XIST RNA. However, these XIST territories were highly unusual and often not properly localized. In addition, cells lacking any BRCA1 expression often contained localized XIST RNA territories. This all suggests that any effect that BRCA1 might have would be on XIST RNA transcription or stability, not on XIST RNA localization. It is unclear that the BRCA1 over-expression results indicate physiological relevance, and the XIST positive cells lacking obvious BRCA1 in the inducible cell system further complicated this analysis.

Complex Epigenetic Differences Between Subpopulations of Cultured Tumor Cells Impact XIST Transcription, Abundance, and Localization

Several aspects of our results underscore the complex XIST phenotypes seen in cultured cells derived from tumors (and not seen in cultured, non-senescent primary cells). We interpret these to reflect multiple epigenetic (or genetic) differences which can impact chromatin status and XIST (e.g., DNA or histone methylation, acetylation, phosphorylation, etc.) [e.g., Clemson et al., 1996; discussed in Hall and Lawrence, 2003 and LLH and JBL, unpublished results]. A subtle example is the that there is consistently just one XIST RNA transcription focus in the BRCA1 stably reconstituted HCC1937 line although we show two XIST loci, so why does only one express? Some recent evidence [Sirchia et al., 2005] indicates that some HCC1937 cultures contain two identical active X chromosomes (and have lost the Xi due to segregation), which would make it even more puzzling why only one allele expresses. Our results on cultured breast cancer tumor cells also show variable changes to XIST RNA expression and localization can evolve in these cultures (discussed below). However the most dramatic misregulation of XIST we have seen is in cells of the BRCA1 dox-inducible system, including the "non-induced" cultures grown in dox free serum for at least 4 weeks. These cultures show an extreme variation of XIST expression, abundance, and localization, within cells of the same culture, with 10–20% of cells showing XIST RNA with no obvious BRCA1 staining. This may be a property of this particular subclone of HCC1937 cells. In any case, other observations indicate that changes in XIST can be induced by changes to DNA methylation [Clemson et al., 1998; Tinker and Brown, 1998] or specific perturbations to chromatin generally [Hall et al., in prep], therefore, we do not interpret our findings to indicate a specific role of BRCA1 on regulation of XIST expression, but BRCA1 may be one of several basic cell regulators that can impact XIST through broader changes to chromatin or cell function.

X Chromosome Abnormalities in Certain Cancers May Arise by Multiple Mechanisms

Results from BRCA1(+) breast tumor samples show that loss of XIST RNA or Xi can occur independent of BRCA1, consistent with recent studies [Kawakami et al., 2004; Sirchia et al., 2005; Richardson et al., 2006]. One BRCA1 mutant line studied here lacked any XIST RNA expression but also had one or more X chromosomes, consistent with recent studies pointing

to prevalent mitotic segregation errors in BRCA1 dependent and Basal-like cancers, which may be biologically similar [Richardson et al., 2006]. Of particular interest was one BRCA1 mutant tumor cell line that retained significant localized XIST RNA on Xi in many cells. These cells have essentially undergone long term knockdown of normal BRCA1, bolstering evidence from our RNAi experiments that lack of normal BRCA1 does not necessarily lead to abrogation of localized XIST RNA. We cannot rule out that compromised XIST localization or expression among many cells of this tumor sample is somehow related to BRCA1 loss, but normal BRCA1 is not directly required for XIST RNA localization. Importantly results from MCF7 cells would suggest that compromised XIST RNA occurs even in the presence of BRCA1 as these have BRCA1 and show similar XIST patterns.

Our results do not dispute that X-inactivation is lost or frequently compromised in breast and some other cancer types, as first reported decades ago [Savino and Koss, 1971; Perry, 1972]. Other studies implicate X-linked genes in breast cancer, particularly certain sub-types. Interestingly, males with Klinefelter (XXY) syndrome have a markedly increased risk of breast cancer [Swerdlow et al., 2001], and gene expression profiles have revealed a disproportionate increase in X-linked gene expression in BRCA1-associated ovarian tumors [Jazaeri et al., 2002]. Reconstitution of BRCA1-mutant breast cancer cells with BRCA1 is reported to repress several X chromosome transcripts [Jazaeri et al., 2004]. More recent studies suggest that changes in X chromosome dosage and X-linked gene expression are particularly common in BRCA1(−) and BLC breast cancer, both phenotypically similar and aggressive tumor types. While this commonly occurs through isodisomy of Xa, in some cases there is retention and apparent reactivation of the Xi [Kawakami et al., 2004; Sirchia et al., 2005; Richardson et al., 2006]. Importantly, alterations in two other chromosomes are also noted in these tumors [Richardson et al., 2006].

Whether BRCA1 dependent cancers (or certain similar sub-types, such as BLC tumors) show significantly more failure in the *maintenance* of the heterochromatic state on Xi would best be addressed by analysis of larger numbers of primary tumors of different types. In addition, whether BRCA1 has some role in Xi heterochromatin necessary for proper X-linked gene expression would be more definitively addressed by analysis of BRCA1 mutations that cause embryonic lethality. Since failure of X-inactivation is an embryonic lethal as are full pathogenic BRCA1 mutations, it would be important to determine whether such embryonic lethal mutants interfere with normal dosage compensation of X-linked genes, which would show more profound changes of X-gene expression than in somatic cells. Finally, as our preliminary analysis of peripheral heterochromatin illustrates, it should be considered that effects on heterochromatin may be broader than just the Xi, particularly since we find that BRCA1 interacts broadly with pericentric heterochromatin linked to its replication [Pageau and Lawrence, in press].

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REFERENCES

Bochar DA, Wang L, Beniya H, Kinev A, Xue Y, Lane WS, Wang W, Kashanchi F, Shiekhattar R. 2000. BRCA1 is associated with a human SWI/SNF-related complex: Linking chromatin remodeling to breast cancer. Cell 102:257–265.

Brown CJ, Willard HF. 1994. The human X inactivation center is not required for maintenance of X chromosome inactivation. Nature 368:154–156.

Brown CJ, Hendrich BD, Rupert JL, Lafreniere RG, Xing Y, Lawrence JB, Willard HF. 1992. The human XIST gene: Analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell 71:527–542.

Chadwick BP, Lane TF. 2005. BRCA1 associates with the inactive X chromosome in late S-phase, coupled with

transient H2AX phosphorylation. Chromosoma 114:432–439.
Chadwick BP, Willard HF. 2002. Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome. J Cell Biol 157:1113–1123.
Chang SC, Tucker T, Thorogood NP, Brown CJ. 2006. Mechanisms of X-chromosome inactivation. Front Biosci 11:852–866.
Clemson CM, McNeil JA, Willard HF, Lawrence JB. 1996. XIST RNA paints the inactive X chromosome at interphase: Evidence for a novel RNA involved in nuclear/chromosome structure. J Cell Biol 132:259–275.
Clemson CM, Chow JC, Brown CJ, Lawrence JB. 1998. Stabilization and localization of Xist RNA are controlled by separate mechanisms and are not sufficient for X inactivation. J Cell Biol 142:13–23.
Clemson CM, Hall LL, Byron M, McNeil J, Lawrence JB. 2006. The X chromosome is organized into a gene-rich outer rim and an internal core containing silenced nongenic sequences. Proc Natl Acad Sci USA 103:7688–7693.
Csankovszki G, Nagy A, Jaenisch R. 2001. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J of Cell Biol 153:773–783.
de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, Koseki H, Brockdorff N. 2004. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell 7:663–676.
Elstrodt F, Hollestelle A, Nagel JH, Gorin M, Wasielewski M, van den Ouweland A, Merajver SD, Ethier SP, Schutte M. 2006. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. Cancer Res 66:41–45.
Fang J, Chen T, Chadwick B, Li E, Zhang Y. 2004. Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. J Biol Chem 279:52812–52815.
Ganesan S, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, Mok SC, Randrianarison V, Brodie S, Salstrom J, Rasmussen TP, Klimke A, Marrese C, Marahrens Y, Deng CX, Feunteun J, Livingston DM. 2002. BRCA1 supports XIST RNA concentration on the inactive X chromosome. Cell 111:393–405.
Hall LL, Lawrence JB. 2003. The cell biology of a novel chromosomal RNA: Chromosome painting by XIST/Xist RNA initiates a remodeling cascade. Semin Cell Dev Biol 14(6):369–378.
Hall LL, Byron M, Sakai K, Carrel L, Willard HF, Lawrence JB. 2002. An ectopic human XIST gene can induce chromosome inactivation in postdifferentiation human HT-1080 cells. Proc Natl Acad Sci USA 99:8677–8682.
Hashizume R, Fukuda M, Maeda I, Nishikawa H, Oyake D, Yabuki Y, Ogata H, Ohta T. 2001. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. J Biol Chem 276:14537–14540.
Jazaeri AA, Yee CJ, Sotiriou C, Brantley KR, Boyd J, Liu ET. 2002. Gene expression profiles of BRCA1- linked, BRCA2-linked, and sporadic ovarian cancers. J Natl Cancer Inst 94:990–1000.
Jazaeri AA, Chandramouli GV, Aprelikova O, Nuber UA, Sotiriou C, Liu ET, Ropers HH, Yee CJ, Boyd J, Barrett JC. 2004. BRCA1-mediated repression of select X chromosome genes. J Transl Med 2:32.
Kawakami T, Zhang C, Taniguchi T, Kim CJ, Okada Y, Sugihara H, Hattori T, Reeve AE, Ogawa O, Okamoto K. 2004. Characterization of loss-of-inactive X in Klinefelter syndrome and female-derived cancer cells. Oncogene 23:6163–6169.
Kay GF, Penny GD, Patel D, Ashworth A, Brockdorff N, Rastan S. 1993. Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. Cell 72:171–182.
Lawrence JB, Singer RH, Marselle LM. 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. Cell 57:493–502.
Morris JR, Solomon E. 2004. BRCA1: BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. Hum Mol Genet 13:807–817.
Ouyang Y, Salstrom J, Diaz-Perez S, Nahas S, Matsuno Y, Dawson D, Teitell MA, Horvath S, Riggs AD, Gatti RA, Marahrens Y. 2005. Inhibition of Atm and/or Atr disrupts gene silencing on the inactive X chromosome. Biochem Biophys Res Commun 337:875–880.
Pageau G, Lawrence B. 2006. BRCA1 foci in normal S-phase nuclei are linked to interphase centromeres and replication of pericentromeric heterochromatin. J Cell Biology, in press.
Panning B, Dausman J, Jaenisch R. 1997. X chromosome inactivation is mediated by XIST RNA stabilization. Cell 90:907–916.
Perry M. 1972. Evaluation of breast tumour sex chromatin (Barr body) as an index of survival and response to pituitary ablation. Br J Surg 59:731–734.
Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. 2006. X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell 9:121–132.
Rosen EM, Fan S, Ma Y. 2006. BRCA1 regulation of transcription. Cancer Lett 236:175–185.
Santner SJ, Dawson PJ, Tait L, Soule HD, Eliason J, Mohamed AN, Wolman SR, Heppner GH, Miller FR. 2001. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat 65:101–110.
Savino A, Koss LG. 1971. The evaluation of sex chromatin as a prognostic factor in carcinoma of the breast. A preliminary report. Acta Cytol 15:372–374.
Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T, Livingston DM. 1997. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell 88:265–275.
Scully R, Ganesan S, Vlasakova K, Chen J, Socolovsky M, Livingston DM. 1999. Genetic analysis of BRCA1 function in a defined tumor cell line. Mol Cell 4:1093–1099.
Sheardown SA, Duthie SM, Johnston CM, Newall AET, Formstone EJ, Arkell RM, Nesterova TB, Alghisi GC, Rastan S, Brockdorff N. 1997. Stabilization of Xist RNA mediates initiation of X chromosome inactivation. Cell 91:99–107.
Sirchia SM, Ramoscelli L, Grati FR, Barbera F, Coradini D, Rossella F, Porta G, Lesma E, Ruggeri A, Radice P, Simoni G, Miozzo M. 2005. Loss of the inactive X

chromosome and replication of the active X in BRCA 1-defective and wild-type breast cancer cells. Cancer Res 65:2139–2146.

Smith KP, Moen PT, Wydner KL, Coleman JR, Lawrence JB. 1999. Processing of endogenous pre- mRNAs in association with SC-35 domains is gene specific. J Cell Biol 144:617–629.

Smith KP, Byron M, Clemson CM, Lawrence JB. 2004. Ubiquitinated proteins including uH2A on the human and mouse inactive X chromosome: Enrichment in gene rich bands. Chromosoma 113:324–335.

Spatz A, Borg C, Feunteun J. 2004. X-chromosome genetics and human cancer. Nat Rev Cancer 4:617–629.

Starita LM, Machida Y, Sankaran S, Elias JE, Griffin K, Schlegel BP, Gygi SP, Parvin JD. 2004. BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number. Mol Cell Biol 24:8457–8466.

Swerdlow AJ, Hermon C, Jacobs PA, Alberman E, Beral V, Daker M, Fordyce A, Youings S. 2001. Mortality and cancer incidence in persons with numerical sex chromosome abnormalities: A cohort study. Ann Hum Genet 65:177–188.

Tam R, Smith KP, Lawrence JB. 2004. The 4q subtelomere harboring the FSHD locus is specifically anchored with peripheral heterochromatic unlike most human telomeres. J Cell Biol 167:269–279.

Tinker AV, Brown CJ. 1998. Induction of XIST expression from the human active X chromosome in mouse/human somatic cell hybrids by DNA demethylation. Nucleic Acids Res 26:2935–2940.

Turner JM, Aprelikova O, Xu X, Wang R, Kim S, Chandramouli GV, Barrett JC, Burgoyne PS, Deng CX. 2004. BRCA1, histone H2AX phosphorylation, and male meiotic sex chromosome inactivation. Curr Biol 14:2135–2142.

Wang RH, Yu H, Deng CX. 2004. A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. Proc Natl Acad Sci USA 101:17108–17113.

Welcsh PL, Lee MK, Gonzalez-Hernandez RM, Black DJ, Mahadevappa M, Swisher EM, Warrington JA, King MC. 2002. BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. Proc Natl Acad Sci USA 99:7560–7565.

Xing Y. 1993. Nuclear Structure Studied by Fluorescence Hybridization: Visualization of Individual Gene Transcription and RNA Splicing. Thesis.

Xing Y, Johnson CV, Moen PT, McNeil JA, Lawrence JB. 1995. Nonrandom gene organization: Structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. J Cell Biol 131:1635–1647.

Zhang R, Poustovoitov MV, Ye X, Santos HA, Chen W, Daganzo SM, Erzberger JP, Serebriiskii IG, Canutescu AA, Dunbrack RL, Pehrson JR, Berger JM, Kaufman PD, Adams PD. 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell 8:19–30.