Escape of X-linked miRNA genes from meiotic sex chromosome inactivation

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ABSTRACT
Past studies have indicated that transcription of all X-linked genes is repressed by meiotic sex chromosome inactivation (MSCI) during the meiotic phase of spermatogenesis in mammals. However, more recent studies have shown an increase in steady-state levels of certain X-linked miRNAs in pachytene spermatocytes, suggesting that either synthesis of these miRNAs increases or that degradation of these miRNAs decreases dramatically in these cells. To distinguish between these possibilities, we performed RNA-FISH to detect nascent transcripts from multiple miRNA genes in various spermatogenic cell types. Our results show definitively that Type I X-linked miRNA genes are subject to MSCI, as are all or most X-linked mRNA genes, whereas Type II and III X-linked miRNA genes escape MSCI by continuing ongoing, active transcription in primary spermatocytes. We corroborated these results by co-localization of RNA-FISH signals with both a corresponding DNA-FISH signal and an immunofluorescence signal for RNA polymerase II. We also found that X-linked miRNA genes that escape MSCI locate non-randomly to the periphery of the XY body, whereas genes that are subject to MSCI remain located within the XY body in pachytene spermatocytes, suggesting that the mechanism of escape of X-linked miRNA genes from MSCI involves their relocation to a position outside of the repressive chromatin domain associated with the XY body. The fact that Type II and III X-linked miRNA genes escape MSCI suggests an immediacy of function of the encoded miRNAs specifically required during the meiotic stages of spermatogenesis.

KEY WORDS: Germ cells, Spermatogenesis, Epigenetic regulation, X inactivation, Male fertility, Meiosis

INTRODUCTION
During spermatogenesis in a variety of Metazoan species, the sex chromosomes undergo a large-scale chromatin remodeling event unique to male germ cells, termed meiotic sex chromosome inactivation (MSCI) (Lifschytz and Lindsay, 1972; McKee and Handel, 1993; Kelly et al., 2002; Turner, 2007; Vibrationiski, 2014). During prophase of meiosis I in male mammals, the largely heterologous X and Y chromosomes synapse at the only homologous region shared by both, the pseudoautosomal region (PAR) (Koller and Darlington, 1934; Burgoyne, 1982; Chandley et al., 1984). Unsynapsed regions of the sex chromosomes outside the PAR are detected by sensor proteins, including BRCA1, that localize to unsynapsed chromosomal regions in spermatocytes and facilitate the recruitment of effector proteins that aid in the localization and enhancement of activity of ATR kinase, a member of the PI3K family (Turner, 2007; Royo et al., 2013). ATR is the primary kinase responsible for the phosphorylation of serine 139 of histone H2AX to form γ-H2AX (Royo et al., 2013). This post-translational modification of histone H2AX is believed to trigger a positive feedback loop between H2AX and MDC1 to promote the additional recruitment of epigenetic factors responsible for silencing the sex chromosomes during meiosis (Fernandez-Capetillo et al., 2003; Ichijima et al., 2011). It has been reported that molecular events leading to MSCI are initiated at the zygotene to pachytene transition during prophase I in spermatocytes, and that the actual gene silencing effects are first manifest at the early pachytene stage (Page et al., 2012; Royo et al., 2013).

Interestingly, it has been shown that an abundance of X-linked mRNA genes are expressed in spermatogonia (Wang et al., 2001), yet molecular evidence from microarray data describing transcriptional activity of the sex chromosomes in populations of spermatogonia, pachytene spermatocytes, and round spermatids indicates that all X- (and Y-) linked mRNA genes are silenced by MSCI in primary spermatocytes, and that a majority of these remain silenced in postmeiotic spermatids as well as a result of the effects of post meiotic sex chromatin (PMSC) (Namekawa et al., 2006; Turner et al., 2006). Thus, it appears that genes that are essential for the development of spermatogenic cells during premeiotic stages are over-represented on the X chromosome, whereas genes encoding products that function during meiosis are generally absent from the X chromosome. Additionally, genes expressed in postmeiotic spermatids are also predominantly autosomal, though certain sex-linked genes expressed at this stage are crucial to the process of spermiogenesis (Wang et al., 2001; Divina et al., 2005; Kihl and Camerini-Otero, 2005).

It was surprising, therefore, that a study cataloguing testicular microRNAs (miRNAs) found that ~20% of miRNA genes expressed in the testis map to the X-chromosome and that 29 out of 77 X-linked miRNAs display testis-specific or testis-preferential expression (Song et al., 2009). The most surprising result was that steady-state levels of many of these X-linked miRNAs peaked in either primary spermatocytes or round spermatids, suggesting that, unlike all X-linked mRNAs examined to date, many X-linked miRNA genes might escape repression by MSCI and/or PMSC. Song et al. (2009) used qPCR methods to interrogate steady-state levels of X-linked miRNAs in purified populations of spermatogenic cell types including spermatogonia, pachytene spermatocytes and round spermatids to characterize expression of these miRNAs during premeiotic, meiotic and postmeiotic phases of spermatogenesis, respectively. Three types of X-linked miRNA genes were distinguished based on expression patterns during spermatogenesis: Type I are expressed in spermatogonia but repressed in spermatocytes and spermatids (similar to many X-linked mRNA genes); Type II are expressed in both spermatogonia and spermatocytes, but not in spermatids...
(suggesting escape from MSCI but not from PMSC); and Type III are expressed in spermatogonia, spermatocytes and spermatids (indicating escape from both MSCI and PMSC). However, steady state levels of RNA reflect a combination of de novo synthesis to generate new transcripts and RNA degradation to eliminate older transcripts. Thus, we sought to directly and specifically assess de novo transcription of X-linked miRNA genes to directly test the hypothesis that many of these genes truly escape MSCI and/or PMSC.

We used RNA-fluorescence in situ hybridization (RNA-FISH), DNA-FISH and immunofluorescence (IF) as described (Mahadevaiah et al., 2009; Namekawa and Lee, 2011) to detect nascent transcripts (pri-miRNAs) emanating from specific X-linked miRNA loci. Our results provide direct evidence for de novo transcription of Type II and III X-linked miRNA genes in pachytene spermatocytes, and also for de novo transcription of Type III X-linked miRNA genes in postmeiotic round spermatids, confirming that Type II miRNA genes escape MSCI and that Type III miRNA genes escape both MSCI and PMSC. In addition, our results reveal that whereas Type II miRNA genes and some Type III miRNA genes continue ongoing transcription during prophase of meiosis I, at least one Type III miRNA gene undergoes a brief, transient inactivation followed by reactivation of transcriptional activity. Finally, our results provide potential insight into the mechanism by which X-linked miRNA genes escape MSCI in pachytene spermatocytes, in that these genes consistently locate to a region at or beyond the periphery of the XY body, thus escaping the repressive chromatin domain normally associated with the XY body. Interestingly, this is similar to the suggested location relative to the repressive Barr body taken up by X-linked mRNA genes that escape X-chromosome inactivation in female somatic cells (Clemson et al., 2006; Chaumeil et al., 2006).

**RESULTS**

**RNA-FISH corroborates previously reported expression patterns of mRNA genes during spermatogenesis**

We first analyzed expression of autosomal and X-linked mRNA genes for which the expression patterns during spermatogenesis were previously known. In each case, we examined premeiotic spermatogonia from juvenile mice and pachytene spermatocytes, and postmeiotic round spermatids from adult mouse testes for gene-specific RNA-FISH signals (n=100 for each cell type) (Fig. 1).

The autosomal *Atr* gene is constitutively expressed throughout spermatogenesis (Keegan et al., 1996) and we detected positive RNA-FISH signals for this gene in each spermatogenic cell type. Appropriately for an actively expressed diploid gene, we detected up to two RNA-FISH signals for this gene in 2C premeiotic spermatogonia, up to four RNA-FISH signals in 4C pachytene spermatocytes, and up to one RNA-FISH signal in 1C round spermatids. Importantly, however, not all of the possible RNA-FISH signals for each expressed gene were visible in every cell examined. The percentages noted in the lower left of each panel in Fig. 1 indicate the proportion of 100 cells examined in which at least one RNA-FISH signal was detected.

The autosomal alphaA-crystallin (*Cryaa*) gene is normally expressed only in the lens of the eye (Robinson and Overbeek, 1996; Graw, 2009), so served as a negative control gene for our experiments in spermatogenic cells. The autosomal *Atr* gene is constitutively expressed throughout spermatogenesis (Keegan et al., 1996) and we detected positive RNA-FISH signals for this gene in each spermatogenic cell type. Appropriately for an actively expressed diploid gene, we detected up to two RNA-FISH signals for this gene in 2C premeiotic spermatogonia, up to four RNA-FISH signals in 4C pachytene spermatocytes, and up to one RNA-FISH signal in 1C round spermatids. Importantly, however, not all of the possible RNA-FISH signals for each expressed gene were visible in every cell examined. The percentages noted in the lower left of each panel in Fig. 1 indicate the proportion of 100 cells examined in which at least one RNA-FISH signal was detected.

**Table:**

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<td><em>Cryaa</em></td>
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<td><em>Scml2</em></td>
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Fig. 1. RNA-FISH and IF staining of spermatogenic cell spreads to detect de novo transcription during specific stages of spermatogenesis. Autosomal mRNA, X-linked mRNA and three of each type of X-linked miRNA genes were examined for expression in each cell type. DAPI (blue) stains DNA. An antibody against γ-H2AX (red) localizes to the XY body in pachytene spermatocytes. White arrows point to positive RNA-FISH signals seen as green foci to indicate sites of active transcription. Percentages of cells expressing RNA-FISH signals are found in the bottom left corner. In the bottom right corner a green ‘+’ indicates that the gene is predominantly expressed whereas a red ‘−’ indicates that the gene is predominantly repressed in the cell type shown. n=100 for each cell type investigated.
positive RNA-FISH signals for these genes in spermatogonia, but not at all (for *Pgk1*) or only very rarely (for *Scml2*) in spermatocytes or spermatids (Fig. 1). Finally, the autosomal *Pgk2* mRNA gene is known to activate transcription in primary spermatocytes and to continue to be actively transcribed in postmeiotic spermatids (McCarrey et al., 1992; Kumari et al., 1996), and this was corroborated by our detection of only very rare RNA-FISH signals for this gene in spermatogonia, but a significantly greater incidence of positive RNA-FISH signals in spermatocytes and spermatids (Fig. 1).

**RNA-FISH reveals de novo transcription patterns of Type I, II and III X-linked miRNA genes during spermatogenesis**

We next investigated the prevalence of RNA-FISH signals representing expression of X-linked miRNA genes during spermatogenesis. This included three examples each of Type I, II and III X-linked miRNA genes. Our analysis of three Type I miRNA genes, *mir-105, mir-322* and *mir-542*, revealed positive RNA-FISH signals in an abundance of spermatogonia, but very few or no such signals in spermatocytes or spermatids (Fig. 1), thus corroborating the report by Song et al. (2009) that these X-linked miRNA genes are indeed subject to both MSCI and PMSC.

Next, we examined RNA-FISH signals for three Type II X-linked miRNA genes including *mir-448, mir-547* and *mir-764*. All three of these genes displayed positive RNA-FISH signals in both spermatogonia and spermatocytes, but not in round spermatids (Fig. 1), consistent with the suggestion that Type II X-linked miRNA genes escape the repressive effects of MSCI but not PMSC (Song et al., 2009). Finally we assayed three Type III X-linked miRNA genes, *mir-421, mir-465* and *mir-742* and found abundant RNA-FISH signals for all three of these genes during all three phases of spermatogenesis (Fig. 1). Note that for an actively expressed X-linked gene, RNA-FISH signals are expected in only half of the postmeiotic round spermatids because only half of those cells contain an X chromosome, and whereas intercellular exchange of mRNAs or mature miRNAs might be expected via intercellular bridges that connect round spermatids emanating from a common spermatocyte (Braun et al., 1989), nascent transcripts should only be detectable in the cell of origin.

**RNA-FISH signals represent nascent transcripts**

We confirmed that our RNA-FISH signals are, indeed, representative of nascent transcripts produced by ongoing de novo transcription in multiple ways. First, we performed DNA-FISH on the same slides on which we had previously performed RNA-FISH to validate that the RNA-FISH signals seen in pachytene spermatocytes specifically co-localized with the relevant gene locus (Fig. 2A). We used an antibody against γ-H2AX to delineate the XY body (blue color in Fig. 2) and then performed RNA-FISH (green signals in Fig. 2A), followed by DNA-FISH (red signals in Fig. 2A). Proportions of cells in which co-localization of RNA- and DNA-FISH signals (yellow signals in Fig. 2A) was observed are noted for each gene in Fig. 2A. As a positive control, the autosomal gene *Atr* was examined and showed positive co-localization between RNA- and DNA-FISH signals in >96% of cells observed. As expected, we detected very few positive RNA-FISH signals for either the X-linked mRNA control gene (*Pgk1*) (~3-6%) or for either of the two Type I X-linked miRNA genes (*mir-105* and *mir-322*) examined (~3-6% for both). Consequently, co-localization between RNA- and DNA-FISH signals for these genes was very rare because active transcription of these genes was rare. However, on investigation of Type II X-linked miRNA genes *mir-764* and *mir-547*, and Type III X-linked miRNA genes *mir-742* and *mir-465*, all of which escape MSCI, we detected positive RNA-FISH signals near the XY body that co-localized with positive DNA-FISH signals in 75-90% of the pachytene spermatocytes examined (n=30) (Fig. 2A).

We next performed RNA-FISH to localize nascent transcripts in pachytene spermatocytes, in combination with IF staining for γ-H2AX, to delineate the XY body, and RNA polymerase II (RNAPII), to detect foci of active transcription (Fig. 2B). Staining with an antibody against the Ser2P-RNAPII form of RNAPII specifically associated with the elongation phase of transcription reveals a dappled pattern of foci of active transcription in the nuclei of pachytene spermatocytes (see Fig. S1), which is indicative of discrete RNAPII transcription foci or ‘factories’ (Osborne et al., 2004, 2007). However, no RNAPII signal was detectable within the repressed XY body chromatin domain (Fig. S1), in line with previous reports showing an absence of transcription markers in the repressed XY chromatin domain of pachytene spermatocytes (Khalil et al., 2004; Turner et al., 2004, 2005).

We then examined co-localization between gene-specific RNA-FISH signals and foci of Ser2P-RNAPII. For the same genes for which we observed abundant RNA-FISH signals that co-localized with positive DNA-FISH signals in pachytene spermatocytes in Fig. 2A (*Atr, mir-764, mir-547, mir-742* and *mir-465*), we observed similarly abundant co-localization between an RNA-FISH signal and a focus of Ser2P-RNAPII as shown in Fig. 2B, indicating ongoing de novo transcription at these sites. The extent of this co-localization is best viewed in three dimensions in an animated movie (Movie 1). Finally, we used DNA-FISH to examine co-localization between the corresponding gene loci encoding each specific mRNA or miRNA expressed in pachytene spermatocytes and the elongation-phase specific form of RNAPII as shown in Fig. 2C, and also observed significant concordance between these signals. Interestingly, for those genes that are actively transcribed in pachytene spermatocytes, the frequency of co-localization of RNA-FISH and RNAPII signals was consistently, though only slightly, higher than the frequency of co-localization of DNA-FISH and RNAPII signals (see Fig. S2). We assume this reflects the fact that even actively expressed genes might not always be caught in the act of active transcription because there might be a short period between completion of one round of transcription and initiation of the next round. Taken together, these data provide substantial evidence that our RNA-FISH signals are, indeed, representative of sites of active, de novo transcription producing nascent pre-mRNA or pri-miRNA transcripts at the corresponding loci.

Interestingly, we also examined the intensity and distribution of RNAPII foci in multiple types of primary spermatocytes (see Fig. S3), and found that foci of transcription appear less prevalent in earlier primary spermatocytes (preleptotene, leptotene and zygotene) than in later (pachytene) primary spermatocytes. This includes a stage in leptotene spermatocytes where there appears to be a transient, genome-wide reduction of transcriptional activity similar to that previously noted by Turner et al. (2005).

**The timing and pattern of silencing by, or escape from, MSCI varies among X-linked genes**

We staged primary spermatocytes using meiosis-specific markers for SCP3 (a protein essential for synopsis of homologous chromosomes) and γ-H2AX, and we used RNA-FISH to examine the dynamics of cessation, or the lack thereof, of active transcription of different types of X-linked genes. For two X-linked mRNA genes known to be subject to MSCI, we observed transcriptional silencing either at the zygotene stage (for *Scml2*) or the leptotene stage (for *Pgk2*).
Pgk1) of first meiotic prophase (Fig. 3). Two Type I X-linked miRNA genes, (mir-105 and mir-542), showed a similar cessation of transcription at the leptotene stage (Fig. 3, mir-542 not shown), supporting the notion that these genes are subject to MSCI coincidentally with X-linked mRNA genes. By contrast, two Type II X-linked miRNA genes (mir-764 and mir-448) showed detectable transcripts in all types of primary spermatocytes (Fig. 3, mir-448 not shown), indicating they escape MSCI by avoiding silencing at any stage of first meiotic prophase. We observed this same pattern for one of the two Type III X-linked miRNA genes we investigated (mir-421), but for a second Type III X-linked miRNA gene (mir-742), we observed transient inactivation at the zygotene stage, followed by robust reactivation by the early pachytene stage (Fig. 3). These results suggest that X-linked miRNA genes can escape from MSCI by either continuing transcriptional activity throughout first meiotic prophase, or by undergoing transient inactivation at a stage (leptotene) when most other X-linked genes become silenced by MSCI, but then reactivating de novo transcription shortly thereafter in early pachytene spermatocytes.

**Escape from MSCI is associated with localization of gene loci outside the repressive chromatin domain of the XY body**

Finally, to begin to examine the mechanism by which genes encoding Type II and III X-linked miRNAs escape MSCI, we used IF staining for γ-H2AX to delineate the XY body, and DNA-FISH to localize specific gene loci in pachytene spermatocytes. We first investigated the location of the constitutively expressed autosomal gene, Atr, relative to the XY body in pachytene spermatocytes. Because this gene is autosomal and only the sex chromosomes are contained within the XY body, the DNA-FISH signal we detected for the Atr gene was, as expected, located well outside of the XY body (Fig. 4). We next investigated the locations of two X-linked mRNA genes, Scml2 and Pgk1, both of which are subject to MSCI as shown in Fig. 1. Both of these loci were detected within the...
repressive chromatin domain of the XY body in pachytene spermatocytes (Fig. 4). Similarly, three Type I X-linked miRNA genes—mir-105, mir-322 and mir-542—which are also subject to MSCI, were also found located within the XY body domain (Fig. 4). However, three Type II X-linked miRNA genes—mir-448, mir-547 and mir-764—as well as three Type III X-linked miRNA genes—mir-421, mir-465 and mir-742—all of which escape MSCI, were all found to be located at or beyond the periphery of the XY body (Fig. 4).

Because two-dimensional images do not always accurately depict the full extent of interactions between two entities within a three-dimensional context, we used Imaris software to reconstruct confocal images of pachytene spermatocyte nuclei, in which the XY body was stained for \( \gamma \)-H2AX and specific X-linked gene loci were localized by DNA-FISH, into animations that can be rotated through three dimensions to clearly reveal the relative locations of these entities (Movies 2-13). These experiments confirmed the expected location within the XY body of X-linked mRNA genes and Type I X-linked miRNA genes known to be subject to transcriptional repression by MSCI. By contrast, our three-dimensional depictions revealed a consistent peripheral location of X-linked Type II and III miRNA relative to the XY body in 100% of cells examined for four of six genes and in >93% of cells examined for the remaining two genes.

**DISCUSSION**

The function of MSCI during spermatogenesis remains a point of speculation. It has been demonstrated that aberrant expression of certain Y-linked genes that are normally suppressed by MSCI results in spermatogenic failure (Royo et al., 2010), but it is not clear how the need to suppress a small number of specific sex-linked genes would lead to the evolution of a pan-chromosomal repression mechanism such as MSCI. It has been suggested that MSCI helps prevent the occurrence of potentially deleterious genetic recombination events during meiosis that might otherwise disrupt the mammalian sex determination mechanism that is based on the function of specific sex-linked genes (McKee and Handel, 1993). Yet another suggested contribution of MSCI is that it sequesters the poorly paired XY bivalent from the mechanism that might otherwise induce programmed cell death of spermatocytes carrying mispaired homologues (McCarrey, 2001; Yan and McCarrey, 2009). Whatever the biological function(s) of MSCI might be, it appears to be a crucial mechanism that is absolutely required for normal spermatogenesis, and, hence, for normal male fertility in mammals (Turner et al., 2006; Royo et al., 2010). In addition, MSCI appears to be a very thorough and comprehensive mechanism of pan-chromosomal repression, leading to the transcriptional silencing of all sex-linked mRNA genes examined to date (Namekawa et al., 2006). Thus, whereas numerous X-linked mRNA genes have been shown to escape X chromosome inactivation in female somatic cells, no X-linked mRNA gene has yet been shown unequivocally to escape MSCI in primary spermatocytes. Nevertheless, we (Song et al., 2009) and others (Marcon et al., 2008; Buchold et al., 2010; Modzelewski et al., 2012; Liu et al., 2015) have reported that several X-linked miRNAs show an increase in steady-state levels during first meiotic prophase, including in pachytene spermatocytes.

Inasmuch as an increase in steady-state levels of RNA can result from either increased synthesis or decreased degradation of the RNA, or both, we set out to distinguish between these possibilities by assessing the extent to which X-linked miRNA genes undergo active, de novo transcription in spermatocytes in which MSCI has otherwise maximally repressed transcription of all other sex-linked genes. We used a combination of DNA- and RNA-FISH methods to detect specific gene loci and their nascent transcripts, respectively, in
conjunction with IF staining to delineate the repressive chromatin domain of the XY body and to localize foci of RNA polymerase II in nuclei of spermatogenic cells. Our data directly demonstrate nascent transcripts from Type II and III miRNA genes in primary spermatocytes at stages that have previously been characterized as periods of pan-chromosomal inactivation of transcription from either sex chromosome as a result of MSCI, and, in the case of Type III miRNA genes, in round spermatids as well at stages where PMSC normally suppresses many, though not all, other X-linked genes. This result for Type II and III X-linked miRNA genes stands in contrast to that for control X-linked mRNA genes, Pgo1 and Scml2, as well as for Type I X-linked miRNA genes, which are all subject to repression by both MSCI and PMSC and for which positive RNA-FISH signals were not detected in pachytene spermatocytes. Importantly, our RNA-FISH signals are representative of primary miRNA (pri-miRNA) transcripts, and not pre-miRNA transcripts or mature miRNA transcripts. pri-miRNA transcripts represent the nascent transcripts that would be expected to be found in association with the gene locus from which they are transcribed and the corresponding DNA-FISH signal with the elongation phase-specific form of RNAP II foci. Third, our DNA-FISH plus RNA-FISH co-localization studies showed that Type II and III X-linked miRNA (and autosomal control mRNA) gene loci at which we detected positive RNA-FISH signals were also occupied by RNAPII in pachytene spermatocytes, whereas Type I X-linked miRNA gene loci as well as X-linked mRNA gene loci which showed no RNA-FISH signals were not occupied by RNAPII. Taken together, and in combination with data previously reported by us (Song et al., 2009) and others (Marcon et al., 2008; Buchold et al., 2010; Modzelewski et al., 2012), there is now compelling evidence that Type II and III X-linked miRNA genes do indeed escape MSCI and continue to undergo active, de novo transcription throughout first meiotic prophase. As such, Type II and III X-linked miRNA genes stand as the only X-linked genes shown to date to escape the otherwise pan-chromosomal effects of MSCI.

Unexpectedly, we observed rare incidences of positive RNA-FISH signals in various spermatogenic cell types for genes that are normally repressed in these cell types. These included rare positive signals in spermatogonia and spermatocytes for a tissue-specific autosomal mRNA gene (Cryaa) normally expressed only in the eye, and rare signals in spermatocytes and spermatids for an X-linked mRNA gene (Scml2) and Type I X-linked miRNA genes (mir-105, mir-322, mir-542) normally subject to both MSCI and PMSC. We ascribe these incidences to leaky expression of these genes in cells in which they would otherwise be repressed, indicating that neither the mechanisms regulating tissue-specific repression of certain autosomal genes nor the mechanisms regulating MSCI and PMSC are completely effective, though the incidence of such leaky expression never exceeded 6% in our studies. Importantly, even in these rare instances of positive RNA-FISH signals for Type I X-linked miRNA genes in pachytene spermatocytes, we observed co-localization with RNAPII, further confirming that our RNA-FISH signals are consistently indicative of nascent transcripts.

We also provide data to confirm that Type III X-linked miRNA genes escape PMSC and continue to undergo active transcription in postmeiotic round spermatids. However, unlike MSCI, PMSC was already known to be an incompletely penetrant mechanism of suppression of X-linked gene expression in postmeiotic spermatids, as several X-linked mRNA genes have previously been shown to be actively transcribed in these cells (Wang et al., 2005; Namekawa et al., 2006). Our results show that Type III X-linked miRNA genes also escape PMSC and that these genes are therefore distinct from Type I and II X-linked miRNA genes in this regard, as the latter two are repressed in spermatids and thus subject to PMSC.

Importantly, our RNA-FISH signals are representative of primary miRNA (pri-miRNA) transcripts, and not pre-miRNA transcripts or mature miRNA transcripts. pri-miRNA transcripts represent the nascent transcripts that would be expected to be found in association with both the gene locus from which they are transcribed and the RNA polymerase II complex responsible for that transcription. Were we detecting either pre-miRNAs or mature miRNAs, we would have expected to see (1) many more positive RNA-FISH signals in each cell, and (2) many more RNA-FISH signals that did not co-localize with either RNA polymerase II foci or the corresponding DNA-FISH signal.

We observed some variance in the timing of transcriptional inactivation of X-linked genes subject to MSCI. Previous studies have reported that MSCI leads to silencing of X-linked genes between the zygotene to pachytene stages of first meiotic prophase (Monesi, 1965; Turner et al., 2006). We investigated only two...
X-linked mRNA genes, *Pgk1* and *Scml2*, but found a discrepancy in the timing of silencing of these genes, with the *Pgk1* gene becoming silenced at the leptotene stage whereas the *Scml2* gene did not become silenced until the zygotene stage of first meiotic prophase. Nevertheless, our data are consistent with the notion that all X-linked genes subject to MSCI are fully silenced by the pachytene stage.

Many X-linked mRNA genes are known to escape X-chromosome inactivation (XCI) in female somatic cells in both mice and humans (Carrel and Willard, 2005; Berletch et al., 2011). However, it has been shown that these loci actually undergo a transient silencing coincident with the inactivation of all other X-linked genes by XCI, and then become transcriptionally reactivated shortly thereafter (Lingenfelter et al., 1998). Thus, we wondered if escape of Type II and III X-linked miRNA genes from MSCI might also follow a transient period of inactivation. For all Type II and all but one Type III X-linked miRNA genes from MSCI might also follow a transient period of inactivation. However, for at least one Type III X-linked miRNA gene (*mir-742*), there was a transient period of inactivation during the zygotene stage of first meiotic prophase, followed by reactivation by the early pachytene stage and thereafter. Interestingly, this coincides with a period of apparent reduction in the overall level of RNA polymerase II in primary spermatocyte nuclei that spans the leptotene to zygotene stages (see Fig. S3). Nevertheless, this suggests that for the most part, Type II and III X-linked miRNA genes escape MSCI by avoiding the repressive characteristics associated with MSCI (Namekawa et al., 2006; Turner, 2007).

Previous studies have suggested that X-linked mRNA genes that escape XCI in female somatic cells might do so by localizing to the periphery of the repressive chromatin domain of the Barr body (Chaumeil et al., 2006; Clemson et al., 2006). Our DNA-FISH experiments revealed a similar non-random distinction in the location of X-linked genes that are (X-linked mRNA and Type I miRNA genes) or are not (Type II and III miRNA genes) subject to MSCI, respectively. Thus, X-linked genes that are subject to MSCI are consistently located within the repressive XY body chromatin domain demarcated by IF detection of γ-H2AX, whereas X-linked genes that escape MSCI are consistently found at or beyond the periphery of the XY body. This suggests that escape from MSCI involves looping out of chromatin beyond the repressive γ-H2AX domain, allowing these loci the opportunity to interact with foci of RNA polymerase II located adjacent to the XY body. It is intriguing to note that whereas the molecular mechanisms responsible for achieving the pan-chromosomal silencing associated with XCI in female somatic cells and MSCI in primary spermatocytes are distinct (McCarrey et al., 2002; Turner et al., 2002), the mechanisms by which either X-linked mRNA genes escape XCI or X-linked miRNA genes escape MSCI appear to be similar, with both involving localization of the escaping loci to a region outside of the repressive chromatin domain of the Barr body or XY body, respectively.

A previous report from Mueller et al. (2008) suggested that multicopy X-linked mRNA genes might preferentially escape PMSC. We therefore wondered if the Type II and III X-linked miRNA genes that escape MSCI might map within or adjacent to multicopy mRNA genes on the mouse X chromosome. However, analysis of map positions of these genes revealed no such physical overlap or proximity. We also investigated the distribution of miRNA genes among regions of the mouse X chromosome reported to be most highly conserved (the XCR region) (Graves et al., 1995) or more recently added during evolution (the XAR and New regions) (Graves et al., 1995; Soh et al., 2014). We found that all three types of miRNA loci are located predominantly in the more recently added regions of the X chromosome, with a preponderance (≈80%) of Type I miRNA genes mapping within the XAR region, whereas Type II and III miRNA genes were equally distributed among the XAR and New regions.

Recent reports from studies of X-linked mRNA genes that escape XCI indicate that CTCF might facilitate or stabilize regions containing escaping genes (Berletch et al., 2015), and a similar mechanism could be involved in stabilizing the formation and/or sequestration of euchromatic loops containing Type II or III X-linked miRNA genes at the periphery of the XY body in primary spermatocytes. Indeed, CTCF has been implicated in establishing and maintaining euchromatic chromatin loop domains or ‘insulated neighborhoods’ in autosomes (Filipowicz et al., 2005; Sun et al., 2013; Dowen et al., 2014), and in providing an insulator function to partition euchromatic and heterochromatic regions of chromosomes (Wendt et al., 2008).

Taken together, our data provide compelling evidence that Type II and III X-linked miRNA genes escape MSCI and, in the case of Type III genes, PMSC as well, and maintain ongoing, *de novo* transcription in spermatocytes and spermatids. The function(s) of the miRNAs encoded by these escaping genes remain(s) to be determined. However, it would appear that they serve one or more crucially important functions which favored selection of a mechanism by which these genes are able to escape the otherwise pan-chromosomal effects of MSCI and, to a lesser extent, PMSC.

**MATERIALS AND METHODS**

**Cells**

Adult mouse germ cells (spermatocytes and spermatids) were isolated from the testes of CD-1 adult male mice (>60 days old) after the mice were euthanized using methods approved in advance by the UTSA Institutional Animal Care and Use Committee (IACUC). Testes were decapsulated and the seminiferous tubules were physically dispersed and minced. The resulting testes cell suspension was allowed to settle on ice for 5 min and cells from the top layer of the suspension were recovered. Spermatogonia were isolated from testes of male mice at postnatal day (P) 6 to 8, and early primary spermatocytes were purified from testes of male mice at P18. In both cases the desired spermatogenic cell type(s) were purified using a Staphylococcus aureus Gradient as described (McCarrrey et al., 1992). Populations of type A+B spermatogonia, and preleptotene, leptotene/zygotene and early pachytene spermatocytes were >85% pure and were further confirmed by characteristic morphological features and/or by staining for SCP3 and γ-H2AX.

**RNA fluorescence in situ hybridization**

The RNA fluorescence *in situ* hybridization (RNA-FISH) technique was adapted from established protocols (Mahaidevaiah et al., 2009; Namekawa and Lee, 2011). Germ cells resuspended in ice cold RPMI buffer+L-glutamine were dropped onto slides and CSK buffer (100 mM NaCl, 300 mM MgCl₂, 10 mM PIPES) with supplements (0.5% Triton X-100, 1 mM EGTA, 2 mM vanadyl ribonucleoside) was added and allowed to incubate for 15 min to permeabilize the cells. Cells were then fixed with 4% paraformaldehyde (PFA) for 10 min, washed with ice cold PBS, then dehydrated through an ice-cold ethanol series and air dried. Probes were denatured for 10 min at 80°C, and hybridization buffer (50% formamide, 10% dextran, 2× SSC, 0.1% BSA, 200 mM vanadyl ribonucleoside) was added. BAC or cosmid probes were incubated for 30 min at 37°C, then added to the slides and allowed to hybridize overnight at 37°C. The next day the slides were run through a series of stringency washes: (1) solution A (50% formamide, 4× SSC) preheated to 42°C, three times for 5 min each; (2) solution B (2× SCC), preheated to 42°C, three times for 5 min each; (3) solution C (4× SSC, 0.1% Tween-20), three times for 5 min each at room temperature. The slides were then blocked with blocking buffer (4× SCC, 4 mg/ml BSA, 0.1% Tween-20) for 30 min at 37°C. Finally, anti-digoxigenenin (DIG)-fluorescein (Millipore) antibody was diluted in...
Slides were then washed and mounted with Vectashield mounting medium. For timing and RNAPII experiments, slides were incubated with 0.1% Tween-20, then incubated in a 1:100 dilution of Alexa Fluor 594 goat anti-mouse antibody (Life Technologies, A11005) for 30 min at room temperature. For timing and RNAPII experiments, slides were incubated with both secondary antibodies at a 1:100 dilution of Alexa Fluor 594 goat anti-mouse antibody (Life Technologies) and Alexa Fluor 488 goat anti-rabbit antibody (Life Technologies, A11008) for 30 min at room temperature. Slides were then washed and mounted with Vectashield mounting medium with or without DAPI (Vector Laboratories), and stored at −20°C.

**BACs/fosmids used for RNA-FISH**

Bacterial artificial chromosomes and fosmids were purchased from the BACPAC Resource Center (BPRC, Children’s Hospital Oakland Research Institute, Oakland, CA, USA). BACs/fosmids used for this study are listed in Table S1. All sequences (genomic, BAC or fosmid) were determined using genome information from assembly MGSCv37 (mm9). BACs/fosmids were labeled using the DIG-Nick Translation Kit (Roche) and DNA-FISH was performed to examine co-localization of the gene of interest. Images of RNA-FISH signals were recorded from 30 cells per gene, then analyzed using Imaris software Slice and Easy 3D modes. Finally, the images were transformed into maximum intensity projections using Imaris software, and processed files were viewed in Imaris and saved as jpeg files. 3D movie files of pachytene spermatocytes were generated using up to 50 optical sections with the aid of Imaris software Slice and Easy 3D modes. Finally, the images were cropped and merged using Photoshop CS4 software (Adobe).

**DNA fluorescence in situ hybridization**

Slides previously processed for RNA-FISH were subsequently subjected to DNA-FISH using methods adapted from established protocols (Mahadevaiah et al., 2009; Namekawa and Lee, 2011). Coordinates of images of RNA-FISH signals were recorded from 30 cells per gene, then DNA-FISH was performed to examine co-localization of the gene of interest. Briefly, after RNA-FISH, coverslips were carefully removed and slides were rinsed with PBS and twice with 2× SSC, incubated in RNase A (Qiagen) for 30 min at 37°C, PBS for 5 min at room temperature, and 0.2 N HCl with Trition X-100 for 10 min on ice, then in denaturing solution (70% formamide, 2× SSC) preheated to 80°C for 5 min. Slides were then subjected to a dehydration ethanol series and air dried prior to addition of probes. Probes were denatured at 80°C for 10 min, then hybridization buffer (4× SSC, 1 mg/ml BSA, 0.1% Tween-20) to a concentration of 1:10 and added to the slides and incubated for 1 h at 37°C, to detect DIG-labeled probes hybridized to their target RNAs. RNA-FISH was performed using DNA probes that covered each target mRNA gene. It should be noted that some of the RNase-FISH probes covered more than one gene locus, including certain mRNA loci in addition to the targeted mRNA gene (Table S1). However, we are aware of no validated case of any X-linked mRNA gene escaping MSCI to be actively expressed in pachytene spermatocytes, so the potential for confounding expression signals emanating from any X-linked mRNA genes is assumed to be very low in these cells.

**Microscopy**

Slides were analyzed using a Zeiss 510 Meta confocal microscope. Diode 405 nm at 25 mW, the tunable argon 458/477/488/514 nm, and the helium-neon 543 nm lasers were used to detect DAPI, Alexa Fluor 488, and Alexa Fluor 594, respectively. Fifty optical slices were taken with a 100× Plan-Apo 1.4 NA Oil objective with DIC of representative cells, with at least 100 cells examined per gene. Three-dimensional (3D) images were processed using the 3D deconvolution algorithm of AutoQuant software (Media Cybernetics) to reduce out-of-focus light. AutoQuant-processed files were saved as IDS files, and viewed using Imaris software (Bitplane). Processed files were viewed in Imaris and snapshots of 3D images, which had been transformed into maximum intensity projections using Imaris software, were taken and saved as jpeg files. 3D movie files of pachytene spermatocytes were generated using up to 50 optical sections with the aid of Imaris software Slice and Easy 3D modes. Finally, the images were cropped and merged using Photoshop CS4 software (Adobe).


