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Many X-linked microRNAs escape meiotic sex chromosome inactivation

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Abstract

Meiotic sex chromosome inactivation (MSCI) during spermatogenesis is characterized by transcriptional silencing of genes on both the X and Y chromosomes in mid to late pachytene spermatocytes1. MSCI is believed to result from meiotic silencing of unpaired DNA because the X and Y chromosomes remain largely unpaired throughout first meiotic prophase2. However, unlike X-chromosome inactivation in female embryonic cells, where 25–30% of X-linked structural genes have been reported to escape inactivation3, previous microarray4- and RT-PCR5-based studies of expression of >364 X-linked mRNA-encoding genes during spermatogenesis have failed to reveal any X-linked gene that escapes the silencing effects of MSCI in primary spermatocytes. Here we show that many X-linked miRNAs are transcribed and processed in pachytene spermatocytes. This unprecedented escape from MSCI by these X-linked miRNAs suggests that they may participate in a critical function at this stage of spermatogenesis, including the possibility that they contribute to the process of MSCI itself, and/or that they may be essential for post-transcriptional regulation of autosomal mRNAs during the late meiotic and early postmeiotic stages of spermatogenesis.

Keywords

spermatogenesis; meiosis; RNA interference; sex chromosomes; X inactivation

Micro RNAs (miRNAs) are 22–24- nucleotide (nt) non-coding RNAs encoded by genes located on all mammalian chromosomes except the Y6. miRNAs mainly function post-transcriptionally as regulators of mRNA stability and translational efficiency7, and thus play essential roles in normal development and physiological function8–11. Our recent

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AUTHOR CONTRIBUTIONS

R.S., S.R., J.M. and C.P. performed the experiments. W.Y. and J.R.M. designed the study and wrote the manuscript.

expression profiling of testicular miRNAs identified several X-linked miRNAs that display elevated levels in pachytene spermatocytes12, in which MSCI is ongoing. This led us to wonder if X-linked miRNA genes escape MSCI. To test this hypothesis, we examined the expression of 77 X-linked miRNAs in 12 mouse organs using a novel PCR-based method12–15. 29 out of 77 (~38%) X-linked miRNAs were expressed in a testis-preferential (26 miRNAs) or testis-specific (3 miRNAs) pattern (Fig. 1 and Supplementary Table 1), suggesting that these X-linked miRNAs have functional roles during spermatogenesis. Mapping studies revealed 11 X-linked miRNA gene clusters (≥2 miRNA genes/10kb/cluster), that together encode 58 miRNAs (Supplementary Table 2). The remaining 19 X-linked miRNAs appear to be encoded by genes that do not belong to clusters. Five of the 77 X-linked miRNAs (*Mirn19b*, *Mirn92a*, *Mirn1198*, *Mirn680* and *Mirnlet-7f*) also have one or more indistinguishable autosomal copies (Supplementary Table 2) and thus were not further analyzed in the following expression assays.

We employed a SYBER green-based real-time quantitative PCR (qPCR) method13 to determine expression levels of 72 X-linked miRNAs in developing testes at postnatal days 7 (P7), P14, P21 and adulthood, and in purified populations of 9 different spermatogenic cell types encompassing all stages of spermatogenesis, including premeiotic spermatogonia, meiotic spermatocytes and postmeiotic spermatids. We translated qPCR data into a number scale ranging from 0 (undetectable levels) to 4.0 (highest levels) (Fig. 2 and Supplementary Table 3). Examination of age-specific testes (first 4 columns in Fig. 2) corroborated the expression patterns indicated by our cell-type specific studies because the composition of spermatogenic cell types represented in each of the developing stages of the mouse testis is known16,17. We grouped the 72 X-linked miRNAs into three different “types” reflecting their expression patterns during spermatogenesis (Fig. 3 & Supplementary Table 3). “Type I” X-linked miRNAs (10 miRNAs, 14%) displayed a pattern similar to that previously observed for many X-linked mRNAs5,18,19, showing higher levels of expression in premeiotic spermatogonia followed by diminished levels in spermatocytes and spermatids. These X-linked miRNA genes are subject to MSCI as well as to postmeiotic sex chromatin (PMSC) that normally represses expression of most, but not all X-linked mRNAgenes in spermatids4. “Type II” X-linked miRNAs (16 miRNAs, 22%) were down-regulated in type B spermatogonia, then up-regulated in one or more types of primary spermatocytes, and then decreased again in postmeiotic spermatids. These miRNAs appear to escape MSCI but not PMSC. “Type III” X-linked miRNAs (46 miRNAs, 64%) were up-regulated in primary spermatocytes (relative to spermatogonia) and remained up-regulated in round spermatids, indicating that they escape both MSCI and PMSC. We did not find any X-linked miRNAs that were suppressed by MSCI and then escaped PMSC. Importantly, although ~13% of X-linked mRNA genes have been previously shown to escape PMSC4, we are aware of no previous reports of any X-linked gene escaping MSCI. Together, type II and type III miRNAs make up 86% of the 72 X-linked miRNA genes studied and all appear to escape MSCI.

Using digoxigenin-labeled locked nucleic acid (LNA) probes, we performed *in situ* hybridization to localize mir-718-3p (type I), mir-883a-3p (type III) and mir-883a-5p (type III) in adult mouse testes. The most intensive hybridization signals for mir-718-3p were

detected in spermatogonia (Fig. 4a–d), which is consistent with the qPCR data showing that mir-718-3p is a type I X-linked miRNA. Signals for mir-883a-3p were most abundant in pachytene spermatocytes (Fig. 4e–i), whereas signals for mir-883a-5p were clearly visible in pachytene spermatocytes and increased further in spermatids (Fig. 4j–n). These results confirm that while type I X-linked miRNAs are subject to MSCI, type III X-linked miRNAs are up-regulated in spermatocytes (relative to spermatogonia), confirming that these X-linked miRNAs escape MSCI, and type III miRNAs are also up-regulated in spermatids indicating they also escape PMSC.

Most miRNA genes (~80%) are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (RNA pol II) before being further processed to precursor and mature miRNAs 20,21. Because the timing and site of production of mature miRNAs can be distinct from those of their pri-miRNAs, we chronicled expression of X-linked pri-miRNAs during spermatogenesis. We analyzed levels of pri-miRNAs for 33 miRNA genes (4 type II and 29 type III) in 8 purified spermatogenic cell types using a semi-qPCR method22. All 33 pri-miRNAs were undetectable or expressed at very low levels in spermatogonia, but were detectable at higher levels in spermatocytes and spermatids (Table 1). To validate these results, we performed Northern blot analyses on two exemplary X-linked pri-miRNAs (*Mirn883a* and *Mirn106a*) (Supplementary Figure 1). These results indicate that the increased levels of types II and III X-linked miRNAs that we observed in spermatocytes and/or spermatids result from *de novo* transcription in these cell types.

To further confirm *de novo* transcription of X-linked miRNA genes in spermatocytes and spermatids, we performed chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays to detect binding of RNA pol II to miRNA genes in developing testes (Fig. 5). P7 testes contain only spermatogonia, whereas P10 testes contain spermatogonia plus early meiotic spermatocytes, and P14 testes include the first appearance of pachytene spermatocytes in mice16,17. Thus, changes in levels of RNA pol II-bound miRNA gene fragments in ChIP products from testes at different ages can be used to estimate RNA pol II activity at specific loci in different spermatogenic cell types. *Pgk1* is an X-linked mRNA gene known to be subject to MSCI, while *Pgk2* is its autosomal homolog that is actively transcribed in meiotic spermatocytes and therefore unaffected by MSCI5,25. In our ChIP-qPCR assays, association of pol II with *Pgk1* was most abundant in P7 (spermatogonia only) and P10 (spermatogonia plus early meiotic spermatocytes) testes, but then decreased in P14 testes coincident with the first appearance of pachytene spermatocytes, and thereafter (Fig. 5b). Conversely, association of pol II with *Pgk2* was first detected at low levels in P10 and P14 testes, and then increased significantly in P17, P21 and adult testes. Thus, these opposite patterns were diagnostic of genes that are or are not regulated by MSCI, respectively.

We used this approach to examine association of RNA pol II with three type III miRNA genes, *Mirn743a*, *Mirn883a* and *Mirn106a*. Significant levels of *Mirn743a* were first detected in RNA pol II ChIP products from P10 testes and higher levels were found in products from P14 and P21 testes, correlating with the initial up-regulation of *Mirn743a* pri-miRNA in preleptotene spermatocytes followed by a gradual increase in leptotene+zygotene spermatocytes, pachytene spermatocytes and round spermatids as shown in Table 1.

Association of RNA pol II with *Mirn883a* was first detected in P17 testes, correlating with the later initial up-regulation of this pri-miRNA in pachytene spermatocytes as (Table 1). Finally, association of RNA pol II with *Mirn106a* was initially high in P7 and P10 testes correlating with expression of this pri-miRNA in type A spermatogonia, then decreased in P14 testes correlating with diminished levels of the *Mirn106a* pri-miRNA in type B spermatogonia and early primary spermatocytes, then elevated again in P17 and P21 testes correlating with up-regulation of this pri-miRNA in pachytene spermatocytes, and decreased in adult testes reflecting diminished expression in spermatids (Fig. 5b and Table 1). These data demonstrate that association of RNA pol II with miRNA genes correlates well with changes in levels of X-linked pri-miRNAs during spermatogenesis, further supporting our contention that increased levels of type II and III X-linked miRNAs in spermatocytes and round spermatids are a result of *de novo* transcription of the corresponding miRNA genes by RNA pol II in these cell types, and that these X-linked genes must, therefore, escape MSCI or MSCI+PMSC.

The transcriptionally inactive status of the sex chromosomes in the XY body was previously established by observations of a lack of ^{3}H-uridine incorporation over the sex chromosomes in primary spermatocytes26,27, exclusion of RNA Pol II from this region, and the presence of heterochromatin-related proteins (e.g. HP1B, γH2Ax and H3K9) associated with these chromosomes1. The transcriptional silencing effects of MSCI have been confirmed by numerous studies showing diminished levels of X-linked mRNA transcripts in primary spermatocytes5,18,19. However, while these assays demonstrate that the sex chromosomes in spermatocytes are indeed transcriptionally repressed in general, they cannot exclude the possibility that certain specific sub-regions of either sex chromosome may remain euchromatic so as to allow transcription of small numbers of genes in these regions to remain or become active.

In summary, we have shown that, unlike all X-linked mRNA genes studied to date, an abundance of X-linked genes encoding miRNAs escape MSCI. This, in turn, suggests that the function(s) performed by these X-linked miRNAs are sufficiently critical to have favored selection to maintain ongoing transcription of the loci encoding these miRNAs despite the repressive effects of MSCI on all other X-linked genes.

METHODS

Preparation of purified spermatogenic cell populations

Populations of cells highly enriched for specific spermatogenic cell types were prepared from CD-1 mice (Charles River Laboratories) using the Sta Put method based on sedimentation velocity at unit gravity16,17. Sertoli cells and primitive type A spermatogonia were isolated from 6 day-old mice. Type A spermatogonia and type B spermatogonia were isolated from 8 day-old mice, while preleptotene spermatocytes, leptotene + zygotene spermatocytes, and early (juvenile) pachytene spermatocytes were purified from 18-day old mice. Adult mice (60–70 day-old) were used for isolation of adult pachytene spermatocytes, round spermatids, elongated spermatids (containing residual bodies) and spermatozoa. Cellular morphology was assessed using phase contrast microscopy to determine the purity of the cells16,17. Pachytene spermatocytes, round spermatids, elongated spermatids +

residual bodies, and spermatozoa with≥95% purity and other spermatogenic cell types with ≥85% purity were used in this study. The purity of adult pachytene spermatocytes was further verified by immunostaining of γH2AX (marker for the XY body in pachytene spermatocytes) (Supplementary Fig. 2a). The purity of the spermatogenic cells purified was also validated by examining levels of X-linked mRNAs that are known to be subjected to MSCI (*Usp26, Tktl1* and *Tex11*) and autosomal mRNAs that are known not to be expressed in spermatocytes including *Pgk2, Scyp2, Tuba3, Piwil2, Actb* and *Gapdh*5 (Supplementary Fig. 2b). Primer sequences for these mRNAs are listed in Supplementary Table 4.

Preparation of small RNA cDNAs and quantitative PCR analyses for X-linked miRNAs

Isolation of small RNAs from multiple mouse tissues, developing testes, and purified spermatogenic cell populations was performed as described previously12. Small RNA cDNAs (srcDNAs) were prepared as reported13 and used for both the SYBER green-based quantitative PCR (qPCR) and semi-qPCR analyses. Levels of 77 X-linked miRNAs in multiple mouse tissues were determined using a semi-qPCR method13. A SYBR green-base real-time qPCR13 was employed for determining levels of miRNAs in developing testes and 9 purified spermatogenic cell populations. Heat maps representing relative levels of miRNAs expression were generated as described12. Gene symbols, miRNA IDs, primer sequences, expression levels, and expression categories are summarized in Supplementary Tables 1 (multi-tissue expression profiling) and 3 (expression in developing testes and purified spermatogenic cells).

Northern blot analysis

Total RNA was isolated from developing testes at postnatal day7 (P7), P10, P14, P17, P21, and adult (12 weeks of age) stages as described24. DNA probes specific to miRNA genes were amplified using PCR (Supplementary Table 4) and were used for hybridization. Northern blot analyses were performed as described24 to detect two control mRNAs, *Pgk1* (X-linked gene subject to MSCI) and *Pgk2* (autosomal gene, not subject to MSCI), and two exemplary X-linked pri-miRNAs (*Mirn883a* and *Mirn106a*).

RT-PCR detection of primary miRNAs

Because of the potential for secondary structures to be formed within pri-miRNAs, it was necessary to use a different method to detect these than was used to detect mature miRNAs. cDNAs were generated using total RNA isolated from 8 spermatogenic cell types including type A, and type B spermatogonia, preleptotene, leptotene + zygotene, early pachytene, and mid to late pachytene spermatocytes, round spermatids and elongated spermatids plus residual bodies. Reverse transcription(RT) was performed using random hexamers and Thermoscript (a thermostable reverse transcriptase from Invitrogen) at an elevated temperature to denature secondary structures in regions containing mature miRNAs, as described22. Elimination of contaminating genomic DNA was confirmed by 1) amplifying *Klhl10* and *Catsper3* cDNAs encompassing small introns as reported previously23,24, and 2) examining no-RT control samples (data not shown). Primer sequences and expected sizes of PCR products are included in Supplementary Table 4. Due to the very low abundance of these pri-miRNAs, the exponential range of amplification during RT-PCR was between 30

and 40 cycles. We performed test PCRs for varying numbers of cycles on each pri-miRNA to determine the exponential range for each. Consequently, this method yielded results that were semi-quantitative.

Immunofluorescent staining

γH2AX is expressed from late spermatogonia through pachytene spermatocytes28 and its staining shifts from autosomes to the incompletely synapsed XY body in pachytene spermatocytes. MIWI is known to be expressed in the cytoplasm of spermatocytes and round spermatids. MIWI is concentrated in chromatoid bodies in round spermatids29,30. Therefore, we used two proteins as markers for spermatocytes and round spermatids. Immunofluroscence staining was performed as described24. Affinity-purified rabbit anti-MIWI polyclonal antibodies were prepared by GenScript using a synthetic peptide (SQPKRRRGPGGTLP, corresponding to a.a 365–378 of the MIWI protein). A mouse monoclonal anti-γH2AX antibody was purchased from Abcam (Cat#ab22551). Alexa Fluor 488 (green) anti-mouse IgM or anti-rabbit IgG was used as the secondary antibody. Cell nuclei were counterstained using propidium iodide (PI). Immunofluroscent images were captured using a fluorescence microscope (Carl Zeiss) equipped with a digital camera and image analysis software (Carl Zeiss).

miRNA *in situ* hybridization using digoxigenin-labeled locked nucleic acid (LNA) probes

All miRCURY LNA™ microRNA detection probes were purchased from Exiqon, and have high affinity and discrimination, enabling specific and sensitive detection of microRNAs (mmu-mir-883a-3p probe: atactgagagctgttgcagtta; mmu-mir-883a-5p probe: gtaactgctacttctctcagca, mmu-mir-718 probe: cgacacccggccgggcggaag). The LNA probes were labeled with digoxingenin using a DIG oligonucleotide tailing kit (Roche) following the manufacturer's instructions. An *in situ* hybridization kit from Biochain was used which provided all the reagents, including hybridization solution, AP-conjugated anti-digoxingenin antibody, NBT and BCIP. Hybridization was performed according to the kit manufacturer's instructions. Adult mouse testes were dissected, fixed with acetone and embedded in O.C.T. compound. Cryosections of 10 μm were cut using a cryostat and stored in −80°C until use. Slides were fixed in 4% paraformaldehyde at room temperature (RT) for 20 minutes followed by two washes in PBS. Fixed sections were then pre-hybridized for 3 hours at 50°C. Hybridization was carried out at 45°C overnight. After washing, the slides were incubated in a blocking solution for 1 hour at RT followed by incubation with AP-conjugated anti-digoxingenin antibody and γH2AX or MIWI antibody at 4°C overnight. After PBS and Alkaline Phosphatase buffer washes, the slides were incubated in NBT/BCIP in the dark until the desired intensity of staining was reached. The slides were then washed with PBS and then incubated with a secondary antibody for immunofluorescence labeling, as described above. Two types of controls were included: 1) digoxigenin-labeled probes competed with excessive amounts (100× more) of unlabeled probes, and 2) hybridization without probes. Both types of controls showed no hybridization signals (Supplementary Figure 3).

Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) assay

RNA pol II ChIP was performed using the ChIP-IT Express Enzymatic kit (Cat# 39097, Active Motif) as described in the manufacturer's protocol with modifications (see Supplementary Methods for a detailed protocol). The RNA polII monoclonal antibody (mAb) provided in this kit was raised against a synthetic peptide "YSPTSpPS" corresponding to human RNA pol II. This mAb recognizes the C-terminal repeat of the largest subunit of phosphorylated (Ser 5p) RNA polymerase II and is reactive to mouse RNA pol II.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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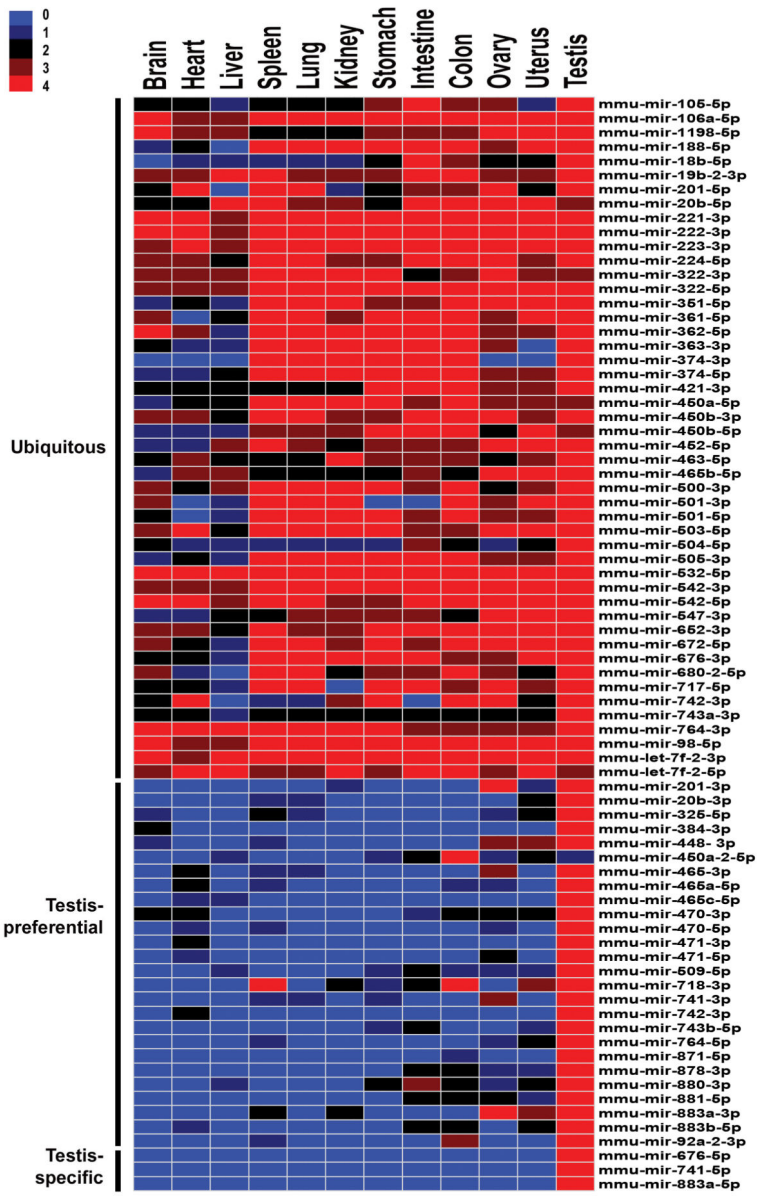

Figure 1.

Heat map representing expression levels of 77 currently known X-linked miRNAs in 12 organs of adult mice. Expression levels were determined by semi-quantitative PCR using small RNA cDNAs.

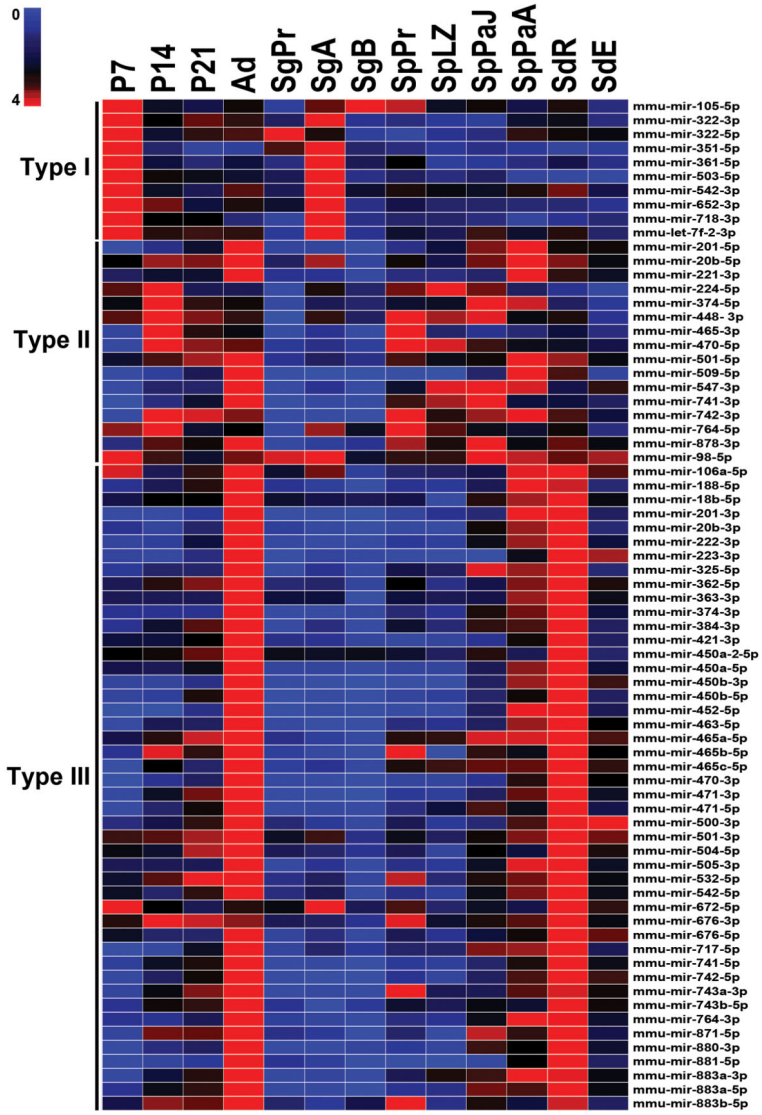

Figure 2.

Heat map representing expression levels of 77 X-linked miRNAs in developing testes and 9 purified spermatogenic cell populations. P7-21, postnatal days 7–21; Ad, adult; SgPr, primitive type A spermatogonia; SgA, type A spermatogonia; SgB, type B spermatogonia; SpPr, preleptotene spermatocytes; SpLZ, leptotene and zygotene spermatocytes; SpPaJ, pachytene spermatocytes from juvenile mice at postnatal day 18; SpPaA, pachytene spermatocytes from adult mice; SdR, round spermatids; SdE, elongated spermatids and residual bodies. Underlined X-linked miRNA genes also have one or more autosomal copies and thus the expression detected may be derived from the × chromosome and /or their autosomal copies.

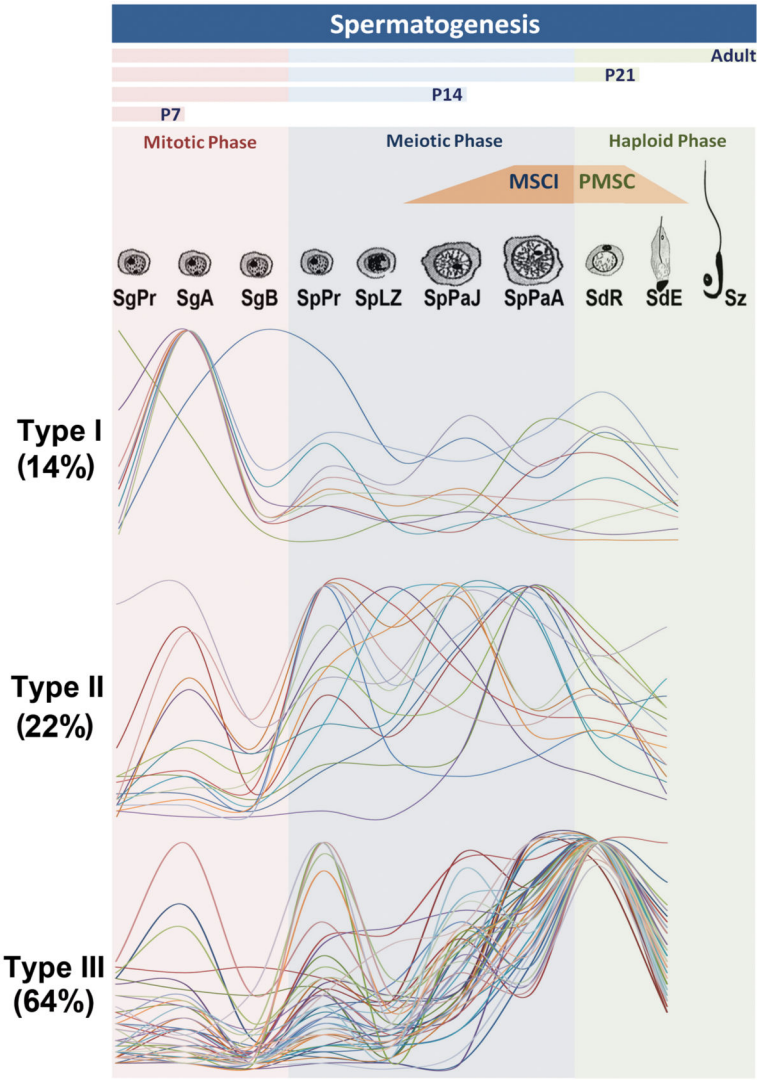

Figure 3.

Three types of X-linked miRNAs grouped according to their expression patterns during spermatogenesis. Type I X-linked miRNAs are expressed in premeiotic spermatogonia followed by decreased expression in spermatocytes and spermatids, indicating that they are subject to MSCI and PMSC. Type II X-linked miRNAs show increased expression in spermatocytes indicating escape from MSCI, followed by decreased expression in spermatids indicating that they are subject to PMSC. Type III X-linked miRNAs display elevated expression in both spermatocytes and spermatids indicating escape from both MSCI and PMSC. SgPr, primitive type A spermatogonia; SgA, type A spermatogonia; SgB, type B spermatogonia; SpPr, preleptotene spermatocytes; SpLZ, leptotene and zygotene spermatocytes; SpPaJ, pachytene spermatocytes from juvenile mice at postnatal day 18; SpPaA, pachytene spermatocytes from adult mice; SdR, round spermatids; SdE, elongated spermatids and residual bodies; Sz, spermatozoa.

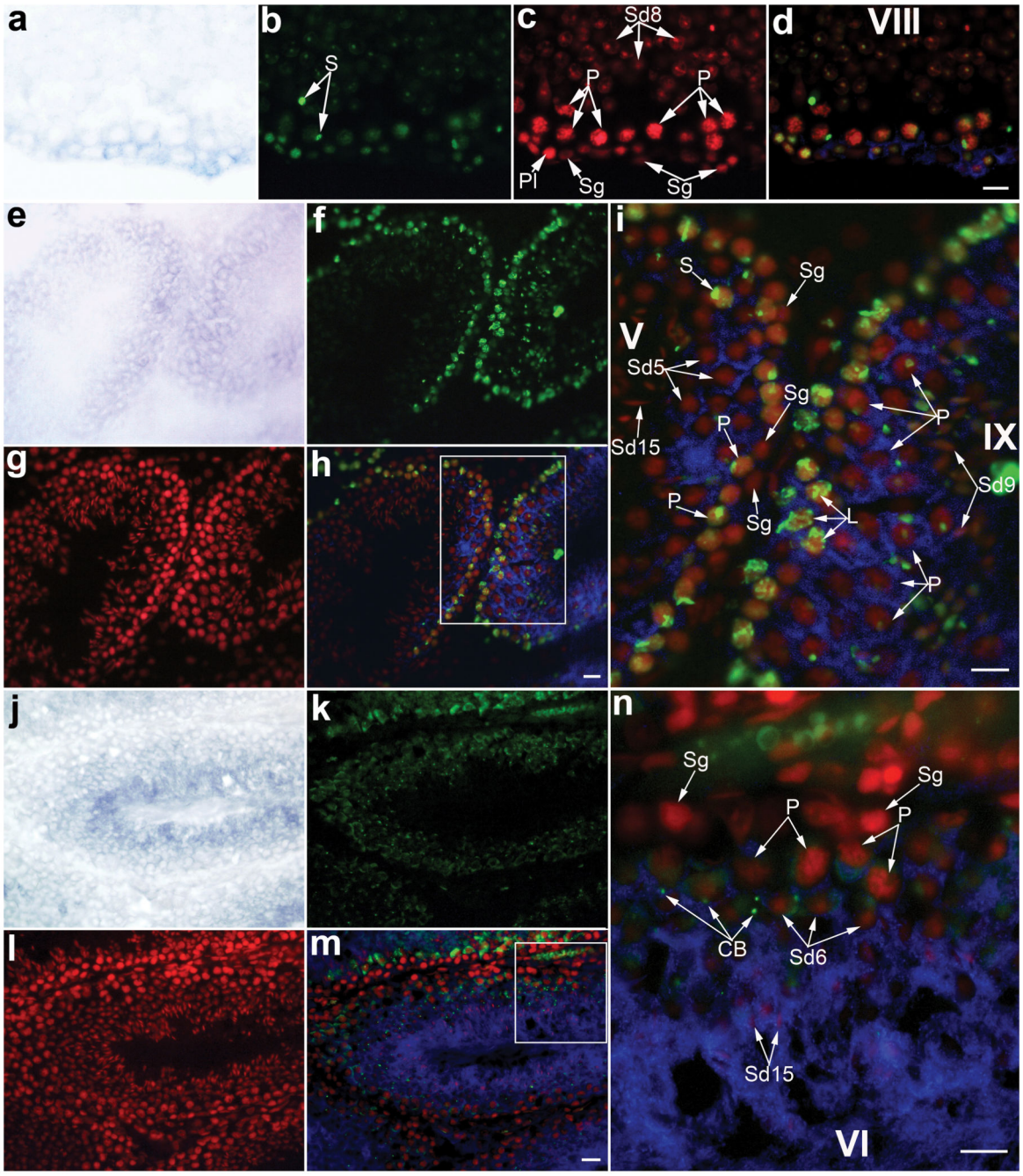

Figure 4.

Localization of three X-linked miRNAs in adult mouse testes using locked nucleic acid-based miRNA *in situ* hybridization. (**a**) Hybridization signals (blue) for mir-718-3p in stage VIII seminiferous tubule section. (**b**) Immunofluorescence staining of γH2AX (green) as a marker for spermatocytes in the same section shown in **a**. (**c**) Propidium iodide (PI) staining (red) of cell nuclei of the same section shown in **a**. (**d**) Merge of **a, b** and **c**. (**e**) Hybridization signals (blue) for mir-883a-3p in stage V (left) and stage IX (right) seminiferous tubule sections. (**f**) Immunofluorescence staining of γH2AX (green) in the

same section shown in **e**. (**g**) PI staining of cell nuclei (red) of the same section shown in **e**. (**h**) Merge of **e, f** and **g**. (**i**) High-power image of the framed area in **h**. (**j**) Hybridization signals (blue) for mir-883a-5p in stage VI seminiferous tubule section. (**k**) Immunofluorescence staining of mouse MIWI (green) as a marker for round spermatids in the same section shown in **j**. (**l**) PI staining of cell nuclei in the same section shown in **j**. (**m**) Merge of **j, k** and **l**. (**n**) High-power image of the framed area in **m**. Scale bars= 10μm. Panels a–d, e–h and j–m are shown at the same magnifications, respectively. S, sex body (also called XY body); CB, chromatoid body; Sg, spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; P, pachytene spermatocytes; Sd5, 8, 15, step 5, 8 and 15spermatids, respectively.

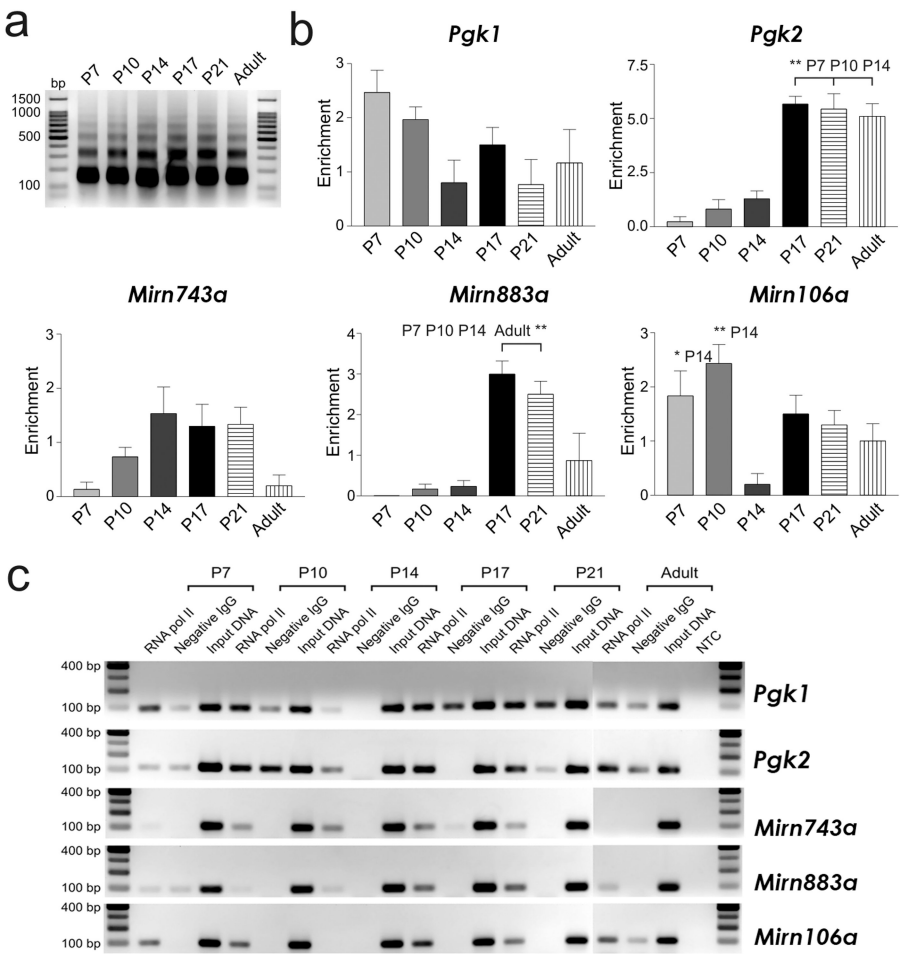

Figure 5.

ChIP-qPCR analyses of interactions between RNA polymerase II (pol II) and three X-linked miRNA genes. (**a**) Formaldehyde cross-linked genomic DNAs from postnatal day 7 (P7), P10, P14, P17, P21 and adult testes were reduced to fragment sizes of 180 bp to 330 bp *via* enzymatic digestion. (**b**) qPCR analyses of levels of *Pgk1*, *Pgk2*, *Mirn743a*, *Mirn883a* and *Mirn106a* in RNA pol II ChIP products from developing mouse testes. Values are presented as means ±SEM, n=3. Significantly different values are marked with *($p \leq 0.05$) or ** ($p \leq 0.01$). (**c**) Analysis of qPCR products on 2% agarose gels.

Table 1

Semi-qPCR assays of levels of primary miRNAs for 4 type II and 29 type III X-linked miRNAs in 8 spermatogenic cell types[a]

Type	Pri-miRNA	SgA	SgB	SpPr	SpLZ	SpPaJ	SpPaA	SdR	SdE
II	Pri-mir-221	−	−	−	−	±	+++	++	++
II	Pri-mir-374	−	−	±	+	+++	+	+++	−
II	Pri-mir-470	−	±	+++	+++	++	+	±	±
II	Pri-mir-741	−	−	±	+	+++	±	+	±
III	Pri-mir-106a	+	−	±	±	+	+++	++	+
III	Pri-mir-188	−	−	−	−	±	++	+++	±
III	Pri-mir-201	−	−	−	−	−	++	+++	++
III	Pri-mir-20b	+	−	±	±	++	+++	++	+
III	Pri-mir-222	−	−	−	−	±	+	+++	±
III	Pri-mir-223	−	−	−	−	−	±	+++	±
III	Pri-mir-325	−	−	±	±	+	++	+++	−
III	Pri-mir-362	±	−	±	−	±	++	+++	+
III	Pri-mir-363	±	−	±	±	−	++	+++	+
III	Pri-mir-384	−	−	±	±	+	+++	++	±
III	Pri-mir-450a	−	−	−	−	±	+++	++	+
III	Pri-mir-450b	−	−	−	−	−	+	+++	±
III	Pri-mir-452	−	−	−	−	−	+	+++	+
III	Pri-mir-463	−	−	−	−	−	+	+++	+
III	Pri-mir-465a	±	±	+	+	+	++	+++	+
III	Pri-mir-465c	−	−	±	±	+	++	+++	±
III	Pri-mir-471	−	−	−	−	±	++	+++	+
III	Pri-mir-500	−	−	±	−	±	+++	+++	+
III	Pri-mir-501	±	+	±	±	+	++	+++	−
III	Pri-mir-505	−	−	−	−	±	+++	+++	±
III	Pri-mir-532	−	−	+	±	±	+	+++	−
III	Pri-mir-542	−	−	−	±	±	++	+++	++
III	Pri-mir-672	+	±	±	±	±	±	+++	+
III	Pri-mir-676	−	−	±	±	±	+	+++	±

Type	Pri-miRNA	SgA	SgB	SpPr	SpLZ	SpPaJ	SpPaA	SdR	SdE
III	Pri-mir-742	−	−	−	−	±	+	+++	+
III	Pri-mir-743a	−	−	++	+	++	++	+++	±
III	Pri-mir-743b	±	±	+	+	++	++	+++	±
III	Pri-mir-764	−	−	−	−	−	+++	+++	±
III	Pri-mir-883a	−	−	−	−	±	++	+++	+

[a]Relative levels of expression were compared among 8 purified spermatogenic cells including type A spermatogonia (SgA)), type B spermatogonia (SgB), preleptotene spermatocytes (SpPr), leptotene +zygotene spermatocytes (SpLZ), juvenile pachytene spermatocytes (SpPaJ), adult pachytene spermatocytes (SpPaA), round spermatids (SdR) and elongated spermatids (SdE). “− ”stands for non-detectable by the PCR method used; “±”, “+”, “++” and “+++” represent barely detectable, weak, moderate and strong bands, respectively.