MicroRNA signature in various cell types of mouse spermatogenesis: Evidence for stage-specifically expressed miRNA-221, -203 and -34b-5p mediated spermatogenesis regulation

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Background information. Recently, it became apparent that microRNAs (miRNAs) can regulate gene expression post-transcriptionally. Despite the advances in identifying the testis-expressed miRNAs and their role in spermatogenesis, only few data are available showing the spatiotemporal expression of miRNAs during this process.

Results. To understand how different miRNAs can regulate germ cells differentiation, we generated a transgenic mouse model and purified pure populations of premeiotic (PrM) cells and primary spermatocytes (meiotic cells). We also established spermatogonial stem cell (SSC) culture using relatively simple and robust culture conditions. Comparison of global miRNA expression in these germ cell populations revealed 17 SSC-, 11 PrM- and 13 meiotic-specific miRNAs. We identified nine miRNAs as specific for both SSC and PrM cells and another nine miRNAs as specific for PrM and meiotic cells. Additionally, 45 miRNAs were identified as commonly expressed in all three cell types. Several of PrM- and meiotic-specific miRNAs were identified as exclusively/preferentially expressed in testis. We were able to identify the relevant target genes for many of these miRNAs. The luciferase reporter assays with SSC (miR-221)-, PrM (miR-203)- and meiotic (miR-34b-5p)-specific miRNAs and 3'-untranslated region constructs of their targets, *c-Kit*, *Rbm44* and *Cdk6*, respectively, showed an approximately 30%–40% decrease in reporter activity. Moreover, we observed a reduced expression of endogenous proteins, c-Kit and Cdk6, when the testis-derived cell lines, GC-1 and GC-4, were transfected with miRNA mimics for miR-221 and miR-34b-5p, respectively.

Conclusions. Taken together, we established the miRNA signature of SSC, PrM and meiotic cells and show evidence for their functional relevance during the process of spermatogenesis by target prediction and validation. Through our observations, we propose a working model in which the stage-specific miRNAs such as miR-221, -203 and -34b-5p coordinate the regulation of spermatogenesis.

Supporting Information available online

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Abbreviations used: dpp, days post partem; dTg, *Stra8*/EGFP and *Sycp3*/DsRed double transgenic mice; EGFP, enhanced green fluorescent protein; miRNA, microRNA; OG2, *Oct4*/EGFP transgenic mice; PrM, premeiotic/premeiosis; SSC, spermatogonial stem cells.

Introduction

Spermatogenesis is a process common for all sexually reproducing species, which ultimately gives rise to mature haploid male gametes. It consists of three major phases: (i) self-renewal and proliferation, (ii) meiotic division and (iii) spermiogenesis. In mice, the process of spermatogenesis takes approximately 34 days and is strictly regulated by both transcriptional as well as post-transcriptional mechanisms (Bettgowda and Wilkinson, 2010). Although the transcriptional regulation mechanisms are well described (Kimmins et al., 2004; MacLean and Wilkinson, 2005; Bettgowda and Wilkinson, 2010), the post-transcriptional mechanisms are largely unknown. It is worth to mention that transcription is not a continuous process during spermatogenesis and is restrained by the differentiation of round spermatids into elongated spermatids (Monesi, 1964; Kleene, 2001). During this time, translation can occur only on mRNAs stored in cells. Hence, the stability of these mRNAs must be rigorously regulated for further translation.

Classical post-transcriptional control of gene expression includes binding of repressor protein to the poly(A)-tail or to other specific sequences in 3'-untranslated region (UTR) or 5'-UTR of mRNA (Braun, 1998; Yang et al., 2003). Several genes are known to be regulated through post-transcriptional mechanisms, but for some of them, no specific repressor protein was found (Kwon and Hecht, 1991; Steger, 2001). This suggests that there might be some other proteins or molecules which can execute this function. In support of this notion, it became apparent that one of these molecules could be microRNAs (miRNAs) (Lee et al., 1993; Wightman et al., 1993). miRNAs are small non-coding RNAs, about 22 nucleotides in length, which can regulate the gene expression either by target mRNA degradation or translation inhibition (Bartel, 2004). The biogenesis of miRNA is a complex process involving the processing of primary and precursor miRNA (pri- and pre-miRNAs, respectively) to yield mature miRNA (Hutvagner et al., 2001; Ketting et al., 2001). Mechanisms by which mature miRNA executes its function depend on the degree of complementarity between miRNA and its target mRNA; full complementarity causes mRNA degradation, whereas partial complementarity results in transla-

tion block (Thomas et al., 2010). First evidence for the importance of miRNA biogenesis during development came from a *Dicer* knockout (KO) mouse model, which showed embryonic lethality (Bernstein et al., 2003).

To unravel the role of miRNAs during spermatogenesis, three independent studies reported the generation of germ cell-specific *Dicer* KO mouse models and observed either infertility or sub-fertility, indicating the importance of miRNA biogenesis during gametogenesis (Hayashi et al., 2008; Maatouk et al., 2008; Korhonen et al., 2011). Further, in an attempt to identify known or novel miRNAs with a function in spermatogenesis, several research groups have undertaken a genome-wide or limited miRNA expression analysis using either partially purified germ cells or whole testis (Yu et al., 2005; Ro et al., 2007; Yan et al., 2007; Song et al., 2009). These results led to the identification of numerous miRNAs, many of which were shown to be expressed in various spermatogenic cell types or in specific testicular developmental stages (Yu et al., 2005; Ro et al., 2007; Yan et al., 2007; Song et al., 2009). Despite the advances in identifying the testis-specific miRNAs, there are only few data available showing the spatiotemporal expression of miRNAs and their potential function during this process.

In the present study, we analysed miRNAs expression dynamics and their function in pure population of spermatogonial stem cells (SSCs), premeiotic (PrM) cells and primary spermatocytes (meiotic cells). Comparative miRNA expression profiling of SSCs, PrM cells and meiotic cells revealed stage-specific expression of several miRNAs indicating a spatiotemporal expression pattern. A number of PrM- and meiotic-specific miRNAs are found to be expressed exclusively/preferentially in testis compared with the other adult mouse tissues. We observed an inversely correlated expression pattern of SSC (miR-221)-, PrM (miR-203)- and meiotic (miR-34b-5p)-specific miRNAs and their spermatogenesis-related predicted target genes: *c-Kit*, *Rbm44* and *Cdk6*, respectively, suggesting functional significance of these miRNAs. Collectively, these observations led us to suggest a working model where the stage-specific expression of miRNAs may regulate their target mRNAs in a coordinated fashion for inhibition or activation of germ cell differentiation.

Results

Generation and characterisation of *Stra8*/EGFP and *Sycp3*/DsRed double transgenic animals

To identify miRNAs which are specifically expressed in SSCs as well as PrM and meiotic stages of spermatogenesis, we set out to specifically enrich these cell populations using cell culture and transgenic mouse model approaches. For simultaneous isolation of PrM and meiotic germ cells using flow cytometry, we have generated *Stra8*/EGFP and *Sycp3*/DsRed double transgenic (dTg) animals as outlined (Supplementary Figure S1A). The (enhanced) green fluorescent protein (EGFP) expression, driven by the *Stra8* promoter, marks all PrM (spermatogonia) cells in the testis of this transgenic mouse model (Nayernia et al., 2004). To specifically enrich meiotic cells, we generated a transgenic mouse line in which the red fluorescent protein (DsRed) is expressed under the control of the *Sycp3* minimal promoter, which is active only in primary spermatocytes (Botelho et al., 2001) (Figure 1A). The integration and the copy number of the *Sycp3*/DsRed transgenic construct were analysed using the GenomeWalker Kit (Clontech) and Southern blot analysis. The construct was found to be integrated as two copies in the intergenic region (Ensembl coordinates: 137456819–137456822) of mouse chromosome 2 (data not shown). A similar analysis for the *Stra8*/EGFP transgene revealed a single copy integration in the intergenic region (Ensembl coordinates: 123589305–123589308) of mouse chromosome 5 (data not shown).

Expression analysis of the DsRed transcript in adult homozygous *Sycp3*/DsRed transgenic animals revealed an expression only in testis, whereas the endogenous *Sycp3* showed high expression in testis and a very weak expression in brain and ovary (Figure 1B). To correlate the expression between endogenous *Sycp3* protein and the *Sycp3* promoter-driven DsRed expression, we performed immunoblotting for both proteins with testicular extracts of transgenic mice (Figure 1C). Expression of endogenous *Sycp3* protein is detected at 14.5 days post partum (dpp), whereas DsRed is detectable at 16.5 dpp (Figure 1C). To identify the DsRed expressing cell type(s) in the testis of transgenic animals, we performed immunostaining with a *Sycp3* antibody and found a specific expression of DsRed in primary spermatocytes as assessed by the typical paired chromosomal structures (Figure 1D).

Then, we generated *Stra8*/EGFP and *Sycp3*/DsRed double homozygous transgenic (dTg) animals and found a specific expression of EGFP and DsRed in PrM and meiotic germ cells, respectively, with no overlap between these two cell types (Figure 2A). We identified EGFP⁺ cells in basal compartment of all tubules, whereas DsRed⁺ cells were found only in adluminal compartment of few tubules (Figure 2A). We used the testicular cell suspension of 17.5 dpp dTg animals for simultaneous enrichment of both PrM (EGFP⁺) as well as meiotic (DsRed⁺) germ cells using fluorescence-activated cell sorting (FACS) as illustrated in Supplementary Figure S1A. The FACS analysis indicated approximately 53% of EGFP (green) positive PrM and approximately 1.1% DsRed (red) positive meiotic cells, further supporting the histological observations (Figures 2A and 2B). The reverse transcriptase PCR (RT-PCR) analysis of sorted EGFP⁺ cells showed the expression of SSC/PrM marker genes such as *Oct4*, *Sall4*, *Plzf* and *Stra8*, whereas DsRed⁺ cells showed no expression of SSC/PrM marker genes indicating the near homogeneity of the sorted cell types (Figure 2C). The expression of meiotic but not post-meiotic (PoM) marker genes was detectable in both cell types (Figure 2C).

Generation and characterisation of SSCs

To identify the SSC-specific miRNAs, we generated two independent SSC lines from dTg and *Oct4*/EGFP transgenic (OG2) (Yoshimizu et al., 1999) mice as outlined (Supplementary Figures S1A and S1B). To confirm the authenticity of the generated SSC lines, we characterised the OG2_SSC line extensively both *in vivo* and *in vitro*. These SSCs displayed typical grape-like morphology, were positive for alkaline phosphatase (AP) staining and express integrin- α -6 (Figures 2Da'–2Dd'). In addition, the expression of pluripotency marker genes (*Oct3/4*, *Nanog* and *Rex1*), as well as several PrM- (*Stra8*, *Piwi2* and *Rnf17*) and meiotic- (*Ovol1*, *Boll* and *Sycp3*) but not PoM marker genes, was detectable in the SSCs (Supplementary Figure S2A). The methylation analysis of imprinted genes revealed a hyper-methylation of the paternally methylated *Meg3* imprinting control region (ICR) and a hypo-methylation of the maternally methylated *Igf2r*, *Snrpn* and *Lit1* ICRs indicating an androgenic imprinting pattern of the SSCs (Supplementary Figure S2B). Further, to test the homing

Figure 1 | Generation and characterisation of *Sycp3*/DsRed transgenic mice

(A) The representative diagram showing the *Sycp3*/DsRed construct used to create the transgenic mouse line. (B) The RT-PCR analysis for the endogenous *Sycp3* and the transgene DsRed expression in various tissues of adult *Sycp3*/DsRed transgenic animals. The expression of the housekeeping gene *Hprt* was used as a positive control. (C) Immunoblot showing the expression of endogenous *Sycp3* and transgenic DsRed protein in indicated postnatal testicular developmental stages as well as in adult homozygous *Sycp3*/DsRed transgenic animals. The protein extract from wild-type adult mouse testis was used as a negative control for DsRed expression. All the blots were probed with an antibody against α -tubulin to show the equal loading of the protein samples and one representative blot is shown. (D) Immunohistochemical analysis of 17.5 dpp testis from *Sycp3*/DsRed transgenic animals showing the expression of endogenous *Sycp3* (green) in premeiotic (PrM) as well as meiotic cells (a'). The transgenic DsRed is expressed specifically only in meiotic cells (red), which are identified by typical synaptonemal complex structures (b'). The region highlighted in overlay (c') is enlarged (d'–f').

Figure 2 | Generation and characterisation of *Stra8*/EGFP and *Sycp3*/DsRed double transgenic (dTg) mouse

(A) Testis cross sections of a 17.5 dpp *Stra8*/EGFP and *Sycp3*/DsRed dTg mouse showing the expression of EGFP in peripheral cells (arrow) of the seminiferous tubule, whereas DsRed-expressing cells are located inside of the tubule (arrow head). (B) Flow cytometry analysis of dTg testis cell suspension showing the distinct green and red cell population. (C) RT-PCR analysis for SSC, PrM, meiotic and post-meiotic (PoM) marker genes in sorted EGFP⁺ and DsRed⁺ cells. WT-testis and water (negative control) were used as positive and negative controls, respectively. (D) SSC colonies showed the typical colony morphology (a'), as well as positivity for AP staining (b') and for the SSC cell surface marker integrin- α -6 (c').

and spermatogenesis reconstitution capacity of SSCs, we transplanted OG2_SSCs into one of the testis of busulfan-treated male mice. PCR-based genotyping showed the presence of the EGFP transgene in the OG2_SSC transplanted testicle, whereas the uninjected control testicle was negative for EGFP indicat-

ing the survival of transplanted cells (Supplementary Figure S2C). The histological analysis revealed the reconstitution of spermatogenesis (~31%–50% of the tubules) in OG2_SSC transplanted testis, whereas the control testis did not contain any tubule with spermatogenesis (Supplementary Figure S2D). The

PCR-based genotyping of sperm obtained from the epididymis of OG2_SSC transplanted testis also revealed the presence of the EGFP cassette (data not shown).

miRNA expression profiling in different stages of spermatogenesis

After isolation and characterisation of SSCs, PrM cells and meiotic cells, we performed miRNA expression profiling using the Agilent-Mouse_miRNA_Microarray. The principal component analysis showed that both SSC lines cluster together, whereas the green and red cells formed two distinct clusters (Figure 3A). The hierarchical clustering analysis revealed the collective clustering of the biological replicates of each cell type and the miRNA expression profile of each cell type was only distantly related to the expression profile of each other cell type (Supplementary Figure S3). For the identification of differentially expressed miRNAs with a strict classification, we selected miRNAs which are expressed above fourfold in one cell type compared with the other cell types. We identified 17 different miRNAs to be specifically expressed in SSCs, whereas 11 miRNAs were PrM specific and 13 were meiotically expressed (Figure 3B; Supplementary Table S1). We also identified miRNAs which are expressed commonly in two or all three cell types, *i.e.*, SSC + PrM (nine miRNAs), PrM + meiotic (nine) and SSC + PrM + meiotic (45) (Figure 3B; Supplementary Tables S1–S3). We could not identify any miRNA which is expressed equally in SSCs and meiotic cells. The generation of a heatmap from miRNA microarray data of all these identified miRNAs clearly reveals the spatiotemporal expression pattern of these miRNAs (Figure 3C). Further, we performed quantitative RT-PCR (qRT-PCR) for some of the selected miRNAs and detected an expression pattern similar to that seen in the microarray, confirming the quality and authenticity of the microarray data (Figure 3D).

To identify miRNAs which are specifically or preferentially expressed in testis, we comparatively analysed the expression of several miRNAs in different mouse tissues (Figure 4A; Supplementary Table S1). Although many analysed miRNAs showed a ubiquitous expression, some displayed a specific/preferential expression in testis (Figure 4A; Supplementary Table S1). The SSC-specific miRNAs (miR-294 and

-295) demonstrated testis-preferential expression with weak expression in few other tissues (Figure 4A). The PrM-specific miR-201 and miR-547 showed an expression only in testis and ovary (Figure 4A). The meiotically expressed miRNA-34 family members (miR-34b-3p, 34b-5p, -34c-3p and -34c-5p) and miR-449a displayed a nearly testis-preferential expression (Figure 4A; Supplementary Table S1). In our array, we found elevated expression of three newly identified X chromosome-linked miRNAs (miR-871-3p, miR-743-3p and 883b-3p) in all cell types. Previously, it was reported that most of the miRNAs expressed from the X chromosome are known to escape meiotic sex chromosome inactivation (MSCI) during spermatogenesis and are specifically expressed in testis with either no or weak expression in other adult tissues (Song et al., 2009). qRT-PCR analysis on various adult mouse tissues confirmed the testis-specific expression of the new X-linked miRNAs (miR-871-3p, miR-743-3p and 883b-3p), suggesting their escape from MSCI (Figure 4B; Supplementary Table S2).

Identification miRNA targets and their validation

To analyse the functional relevance of the identified miRNAs during spermatogenesis, we identified their putative targets, which are implicated in the process of spermatogenesis/gametogenesis (Supplementary Table S1). Interestingly, the disruption of many of these predicted target mRNAs was reported to result in either infertility or reduced fertility of the mouse (see two recent excellent reviews by Matzuk & Lamb, 2008, and Naz et al., 2009, for a comprehensive list of known gene KO mouse models of infertility). The quantitative RT-PCR (qRT-PCR) analysis of the selected target mRNAs showed an expression pattern which inversely correlates with the expression of the respective miRNA (Figures 5A–5D). The miR-221 is expressed specifically in SSCs (Figures 3C and 3D), whereas their targets *c-Kit* and *Tox* are expressed low in SSCs but highly in PrM cells in which the expression of miR-221 is either weak or absent (Figure 5A). Similarly, the expression of *Rsb1*, *Rbm44* and *Fndc3a*, which are targeted by SSC + PrM-specific miRNAs, is low in SSCs and PrM cells, whereas they are highly expressed in meiotic cells (Figure 5B). The expression of *Stag3*, a target of PrM-specific miR-34a, is low in SSCs and PrM cells, whereas its expression is upregulated in meiotic cells

Figure 3 | miRNA expression profiling of SSCs, PrM cells and meiotic cells

(A) The miRNA expression profiling and subsequent principal component analysis plot showing that the two SSC lines (OG2 and dTg) cluster together, whereas the PrM (EGFP^{+/ve}) and the meiotic (DsRed^{+/ve}) cells form distinct clusters. (B) Venn diagram showing the number of miRNAs in common and differentially expressed, respectively, in SSCs, PrM cells and meiotic cells. (C) Heat map showing the expression of cell-type specific miRNAs identified by miRNA microarray analysis. (D) Heat map representation of qRT-PCR data for miRNA microarray validation. Scale in (C) and (D) denotes an approximation of extent of miRNA relative upregulation in blue and downregulation in yellow.

Figure 4 | Specific/preferential expression of miRNAs in testis

(A) Heat map representation of qRT-PCR data showing the expression of SSC, PrM and meiotic-specific miRNAs in various adult mouse tissues. Scale denotes an approximation of extent of miRNA relative upregulation in blue and downregulation in yellow. (B) Bar graph showing the expression of X-linked miRNAs in various adult mouse tissues. miR-465a-3p was used as a positive control.

(Figure 5C). Further, the meiotic-specific miRNA target *Cdk6* is highly expressed in SSCs and PrM cells, whereas its expression is decreased in meiotic cells (Figure 5D). The gene ontology (GO) classification of all predicted mRNA targets showed that the global function of the identified miRNAs is to regulate various cellular functions such as developmental processes, mRNA transcription and regulation, cell cycle regulation, signal transduction and protein modification (Supplementary Table S1).

To validate the predicted targets, we have chosen *c-Kit*, *Rbm44* and *Cdk6* which are predicted to be targeted by SSC-specific (miR-221), SSC + PrM-specific (miR-203) and meiotic-specific (miR-34b-5p) miRNAs, respectively, and performed

luciferase reporter assays in testis-derived cell lines, GC-1 and GC-4 (Figures 6A–6C). The SSC-specific miR-221 inhibited the reporter activity of its target *c-Kit* to approximately 60%–70% in both cell lines (Figure 6A). The SSC + PrM-specific miRNA (miR-203) also reduced the reporter activity of its target *Rbm44* to approximately 60%–72% (Figure 6B). Likewise, miR-34b-5p, a meiotic-specific miRNA, inhibited the luciferase activity of its target *Cdk6* to approximately 70% (Figure 6C). To further confirm these results, we transfected GC-1 and GC-4 cells with either miRNA mimic (miR-221 or miR-34b-5p) or scramble miRNA and analysed the protein expression of their respective targets (Figures 6D and 6E). The miR-221 overexpression

Figure 5 | Expression pattern of predicted miRNA targets in various germ cells

(A) The qRT-PCR data showing the expression of *c-Kit* and *Tox*, predicted as targets of SSC-specific miR-221 and -222 in various germ cells. (B) The qRT-PCR data showing the expression of *Rsb1*, *Rbm44* and *Fndc3a*, predicted as targets of SSC+PrM-specific miR-31, -203 and let-7f, respectively, in various germ cells. The qRT-PCR data showing (C) the expression of *Stag3*, a predicted as target of PrM-specific miR-34a and (D), the expression of *Cdk6*, the predicted target of miR-34 family, a meiosis-specific miRNA, in various germ cells.

in GC-1 cells reduced the expression level of *c-Kit* by approximately 75%, whereas GC-4 cells displayed no expression of *c-Kit* (Figure 6D). Overexpression of miR-34b-5p in GC-1 cells inhibited the expression of *Cdk6* by 25% and this effect was even more pronounced in GC-4 cells with approximately 55% inhibition in miR-34b-5p-transfected cells compared with the scramble miRNA-transfected cells (Figure 6E).

Discussion

Recent studies have highlighted the role of miRNAs in post-transcriptional control of various developmental and cellular processes including spermatogenesis (Papaioannou and Nef, 2010; McIver et al., 2012). In line with these observations, several studies have reported the global or limited miRNA expression signature in whole testicular cells or in partially purified germ cells using either microarrays or

Figure 6 | The stage-specific miRNAs regulate the expression of their predicted targets

(A–C) Bar graphs showing the luciferase activity of *c-Kit*_3'-UTR (A), *Rbm44*_3'-UTR (B) and *Cdk6*_3'-UTR (C) in either miRNA mimic- or scramble (control)-transfected GC-1 and GC-4 cell lines. The values which are statistically significant are indicated with asterisks ($p < 0.05$). (D) Western blot (upper panel) showing the c-Kit expression levels in GC-1 and GC-4 cells transfected with either miR-221 mimic or scramble miRNA (control). The c-Kit band intensities were quantified using ImageJ software and normalised to their respective tubulin band intensities and are represented as percentage of expression in a bar graph (lower panel). (E) Western blot (upper panel) showing the Cdk6 expression levels in GC-1 and GC-4 cells transfected with either miR-34b-5p mimic or scramble miRNA (control). The Cdk6 band intensities were quantified as described above and are represented as percentage of expression in a bar graph (lower panel).

cloning strategies (Yu et al., 2005; Ro et al., 2007; Yan et al., 2007; Marcon et al., 2008; Buchold et al., 2010). The study by Ro et al. (2007) described the expression of 28 new testis-expressed miRNAs in purified germ cell populations using a cloning method. Only 12 of these 28 miRNAs were annotated in the miRBase. In the following year, Marcon et al. (2008) reported the global miRNA expression profile in various germ cells. However, the analysed cells were suggested to be contaminated with different stages

of spermatogenic cells to various degrees, making it difficult to establish the clear blueprint of miRNAs in specific germ cells. On the contrary, three independent studies have reported the expression profile of miRNAs either by cloning or microarray strategies using immature and mature testis or whole testis at different stages of development (Yu et al., 2005; Yan et al., 2007; Buchold et al., 2010). Although the results of these studies suggest a possible correlation between the particular miRNA enrichment and

a specific stage of testicular development, no direct evidence could be established.

Isolation of various pure germ cell populations is critical for any downstream experiments and especially for whole-genome transcriptome and miRNA expression analysis. To derive pure germ cells population, *i.e.*, SSCs, PrM cells and meiotic cells with high certainty, we have generated a *Stra8*/EGFP and *Sycp3*/DsRed dTg mouse line. These mice expressed EGFP and DsRed protein specifically in PrM/spermatogonia and primary spermatocytes, respectively. We strongly suggest that the use of germ cells isolated from these particular transgenic mice for miRNA expression analysis minimises the cross-contamination of other germ cells.

The previously described culture medium for SSCs is composed of various defined growth factors, hormones and chemical molecules (Kanatsu-Shinohara et al., 2003; Ko et al., 2009). In the present study, we established culture conditions for the efficient generation of SSCs with minor modifications to the previously described protocols (Kanatsu-Shinohara et al., 2003; Ko et al., 2009). We observed that the addition of few growth factors such as glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) to the basic StemPro-34 culture medium is sufficient for the efficient derivation and maintenance of SSCs, in contrast to the previously reported cumbersome SSC culture protocols (Kanatsu-Shinohara et al., 2003; Ko et al., 2009). Addition of L-ascorbic acid (vitamin C) to the culture medium was found to enhance the proliferation of SSCs. Although vitamin C is known to inhibit the proliferation of a variety of cancer cells (Kang et al., 2005; Hahm et al., 2007; Lee et al., 2008), it was found to enhance the proliferation of human embryonic stem cells (ESCs) and also the generation of induced pluripotent stem cells (Esteban et al., 2010; Chen et al., 2011). These observations suggest that vitamin C might positively regulate the stem cell characteristics of both SSCs and ESCs. The SSCs generated using our modified protocol displayed all known characteristics, such as the expression of various pluripotency and germ cell marker genes, maintenance of androgenic imprinting and, more importantly, the reconstitution of spermatogenesis after transplantation into the testis. These SSC characteristics strengthen the authenticity of generated SSCs on

one hand and the establishment of a very simple and reliable culture medium for the generation of SSCs on the other hand.

Through our miRNA microarray analysis, we identified several miRNAs which are either specifically expressed in a particular stage of spermatogenesis or expressed equally in two or all three different spermatogenic stages. The SSC- and PrM/spermatogonia-specific miRNAs (miR-21, -199a-3p, -199b and let-7 family miRNAs let-7f, -7a and -7c) were recently described to be highly enriched in *Thy1*⁺ cells, which comprise of SSCs (Niu et al., 2011), further confirming the specific expression of these miRNAs. Our current study could identify 15 novel SSC-specific miRNAs including miR-290 family members (miR-291a, -294 and 295), whose function is crucial for maintenance of pluripotency (Houbaviy et al., 2003; Benetti et al., 2008; Sinkkonen et al., 2008). These observations suggest that the SSC-specific miRNAs together with miR-290 family members might maintain the stemness in SSCs. The expression of miR-322 and let-7f was previously described to be PrM/spermatogonia-specific (Song et al., 2009). In line with these observations, we could identify miR-322 and let-7f along with nine other PrM/spermatogonia-specific miRNAs. We report the identification of 12 novel meiosis-specific miRNAs, which could not be identified to be meiosis-specific in previous studies, possibly owing to the technical limitations of those studies (Ro et al., 2007; Yan et al., 2007; Marcon et al., 2008; Buchold et al., 2010; Niu et al., 2011). The miR-34c, which we identified to be meiosis specific, was recently characterised as pachytene spermatocytes- and round spermatid-specific miRNA (Bouhallier et al., 2010), further validating our microarray data. Interestingly, we found many miRNAs (45) which are highly expressed in all analysed germ cells. The majority of these miRNAs (16/45) are expressed from the X chromosome and 12 of them were previously described to be testis-specific/preferentially expressed, and to escape the MSCI during spermatogenesis (Ro et al., 2007; Song et al., 2009). In our array, we observed a testis-specific expression for three recently identified X-linked miRNAs, suggesting their possible escape from MSCI.

Using the miRNA body-map software, an interactive platform of all known miRNA target prediction softwares, we predicted the mRNA targets for all the

Figure 7 | Working model for the role of miRNAs in spermatogenesis

The expression of SSC-specific miRNAs might be necessary to maintain the self renewal and blockade of differentiation by inhibiting their target mRNAs. Next, the PrM-specific miRNAs might also regulate their targets, which are otherwise necessary for meiotic program activation or progression. Upon the activation of meiosis, the meiotically expressed miRNAs may block the cell proliferation and positively regulate the meiotic progression and subsequent development of haploid germ cells.

cell type-specific or preferentially expressed miRNAs and selected the targets based on their known or predicted function in spermatogenesis/gametogenesis. Interestingly, the expression of the predicted mRNA targets was inversely correlated with the expression of miRNAs, suggesting the functional significance of miRNAs. The expression of *c-Kit*, a target of miR-221, is detected in spermatogonia, but not in SSCs (Rossi et al., 2000), thus highlighting its crucial role in differentiation. Similarly, miR-221-mediated suppression of *c-Kit* in haematopoietic stem cells was shown to be essential for normal erythropoiesis (Felli et al., 2005). In our array, we found elevated miR-221 expression in SSCs and also could show inhibition of *c-Kit* reporter activity and protein levels upon miR-221 overexpression, suggesting that the miR-221 might be essential for the maintenance of SSCs by suppressing *c-Kit* (Figure 7). We identified miR-203 as one of the PrM-specific miRNAs, which is predicted to target *Rbm44*. The expression of *Rbm44* is highly detectable in primary and secondary spermatocytes, and is localised to intracellular bridges (Iwamori et al., 2011). We hypothesise that miR-203-mediated blockade of *Rbm44* expression might be important for the continuous morphological and structural maturation of PrM cells, before they enter into meiosis (Figure 7). The members of miR-34 family were shown to be highly upregulated from day 7 to day 14 of mouse testis development and in mature mouse testis, indicating that these miRNAs might play a crucial role in later stages of

spermatogenesis (Yan et al., 2007; Bouhallier et al., 2010; Buchold et al., 2010; McIver et al., 2012). In line with these observations, we found a very high expression of miR-34 family members in meiotic cells and showed the functional relationship between miR-34b-5p and its predicted target *Cdk6* by luciferase reporter assay and Western blot analysis of miR-34b-5p overexpressing cells. Through these observations, we suggest that the miR-34 family might be responsible for the cell cycle block and meiotic progression (Figure 7). On the basis of the above observations, we suggest a working model where the SSC-specific miRNAs regulate the expression of genes (e.g., *c-Kit*) to block SSC differentiation (Figure 7). Subsequently, the PrM/spermatogonia-expressed miRNAs (e.g., miR-203) regulate the entry of cells into meiotic phase of spermatogenesis (Figure 7). Finally, the meiotic-specific miRNAs inhibit the cell proliferation and promote the further stages of spermiogenesis (Figure 7).

Material and methods

Generation of transgenic mouse lines

For generating *Sycp3*/DsRed transgenic mice, the genomic sequence of the *Sycp3* minimal promoter (−694 to −1 bp from transcriptional start site), which was previously described by Botelho et al. (2001), was PCR amplified and cloned into *Xba* I and *Sac* II restriction sites of pDsRed-Express-1. The resulting p*Sycp3*/DsRed construct was linearised with *Bgl* II, injected into the male pronuclei of 1 cell-stage mouse embryos and transferred into FVB pseudopregnant mothers. Transgenic animals were identified by PCR genotyping and the positive founder

animals were bred with wild-type FVB animals to establish the *Sycp3*/DsRed transgenic mouse line. The copy number and the genomic location of the *Sycp3*/DsRed transgene were evaluated using the GenomeWalker Kit (Clontech) and Southern blot analysis to establish the homozygous *Sycp3*/DsRed transgenic mouse line. The generation of the *Stra8*/EGFP transgenic mouse line was previously described (Nayernia et al., 2004). The genomic location of the *Stra8*/EGFP transgene was evaluated essentially as described for *Sycp3*/DsRed mouse line to establish the homozygous *Stra8*/EGFP transgenic mouse line. The dTg *Sycp3*/DsRed and *Stra8*/EGFP mouse line was generated by crossing both individual transgenic animals. The genotyping primers are provided in Supplementary Table S4.

Isolation of EGFP⁺ and DsRed⁺ germ cells by FACS

EGFP- and DsRed-positive cells were isolated from the testis of 17.5 dpp dTg animals using a previously described protocol (Guan et al., 2006). Briefly, the animals were sacrificed and the testicles were isolated, washed, stripped from *Tunica albuginea* followed by mechanical separation of seminiferous tubules. Further, the tubules were digested by two-step enzymatic digestion as previously described (Kanatsu-Shinohara et al., 2003). EGFP⁺ and DsRed⁺ cells were separated by FACS using BD FACSAria II cell sorter.

Derivation and culture of SSCs from mouse testis

SSCs lines were generated from *Oct4*/EGFP (OG2) (Yoshimizu et al., 1999) and dTg animals. The single cell suspension (obtained after two step enzymatic digestion of testicular tissue) was subjected to magnetic-activated cell sorting (MACS) using MACS cell separation kit (Miltenyi Biotec) and an integrin- α -6 antibody to enrich the SSC population. The MACS elution fraction was cultured in StemPro-34 medium (Invitrogen) supplemented with Stem pro supplement (Invitrogen), 1% fetal calf serum (FCS), 2 mM L-glutamine (PAN), 1 mM sodium pyruvate (Invitrogen), 1 $\times$ MEM non-essential amino acid (Invitrogen), 100 μ M β -mercaptoethanol (Invitrogen), 50 μ g/ml vitamin C (Sigma) and a cocktail of growth factors; 1000 U/ml LIF (Chemicon), 10 ng/ml human GDNF (Invitrogen), 20 ng/ml mouse EGF (Sigma) and 10 ng/ml human bFGF (Invitrogen). The SSC colonies were picked after 2–3 wk of initial plating and were maintained on mitomycin C-inactivated mouse embryonic fibroblasts. To test the ability of SSCs to reconstitute spermatogenesis, OG-2 SSCs were transplanted into busulfan-treated male mice as described previously (Guan et al., 2006).

DNA extraction and Southern blot analysis

Genomic DNA (gDNA) isolation from mouse tail or liver was performed using standard procedures. Briefly, tissues were homogenised, resuspended in lysis buffer (100 mM Tris/HCl pH 8.5, 5 mM EDTA pH8, 0.2% SDS, 200 mM NaCl and 100 μ g/ml proteinase K in dH₂O) and incubated overnight at 55°C. The next day, gDNA was purified from the supernatant by using standard phenol-chloroform purification method. The gDNA was used either for PCR genotyping or Southern blot analysis. For Southern blot analysis, DNA was digested overnight at 37°C with either *Bam*HI or *Hind*III restriction enzymes (Invitrogen), separated on agarose gel and

transferred to Hybond-XL nitrocellulose membrane (Amersham Bioscience). The DsRed-specific probe was generated by restriction digestion of pDsRed-Express-1 vector with *Bam*HI and *Not*I restriction enzymes (Invitrogen) and subsequent P-32 isotope labelling to confirm the copy number and genomic integration of the *Sycp3*/DsRed transgene.

Protein isolation and Western blot analysis

Tissues were homogenised in liquid nitrogen and immediately transferred to lysis buffer-I (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 2.5% SDS, 1 mM PMSF and protease inhibitor cocktail). The total protein extracts from GC-1 and GC-4 cell lines transfected with either miRNA mimic or scramble miRNA were prepared by using lysis buffer-II (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1.0% Nonidet P40, 0.5% sodium deoxycholate and protease inhibitor cocktail). The protein extracts (~50 μ g) were separated on NuPAGE 4%–12% Bis-Tris Gel (Invitrogen) and transferred onto Hybond-C Extra membrane (Amersham Bioscience). Membranes were blocked in 5% milk powder in Tris-buffered saline and Tween 20 (TBST) (25 mM Tris pH 8.3, 150 mM NaCl and 0.1% Tween 20) followed by overnight incubation at 4°C with primary antibodies diluted in 2% milk powder in TBST. Further, membranes were washed and incubated with 2% milk containing horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. The specific bands were detected using Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology, Inc.). The sources of primary antibodies are listed in Supplementary Table S5.

RNA isolation, RT-PCR and qRT-PCR

Total RNA from tissues was isolated by either TriFast (Peqlab) or miRNeasy mini kit (Qiagen). For mRNA expression, cDNA synthesis was performed using oligo-dT primer (Invitrogen) and SuperScriptII system (Invitrogen) followed by qRT-PCR detection using SYBR Green PCR MasterMix (Qiagen). For miRNA expression analysis, cDNA synthesis and qRT-PCR detection of miRNAs was carried out according to manufacturer's protocols using the miScript Reverse Transcription Kit and the miScript SYBR Green PCR Kit (Qiagen), respectively. All qRT-PCR experiments were run on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The qRT-PCR data were first normalised to either U6 (a non-coding housekeeping RNA) or *Sdh*a (a housekeeping gene), for miRNA and mRNA expression analysis, respectively, and represented as relative expression to one of the cell-types/tissues. The primers used to detect both mRNA and miRNA are listed in Supplementary Tables S6 and S7.

Fluorescence microscopy and AP staining

To identify the EGFP⁺ and DsRed⁺ cells within the seminiferous tubules, the dTg mouse testes [postnatal day 17 (P17) or adult] were used to prepare cryosections (5 μ m) and images were acquired using Olympus BX60 fluorescence microscope equipped with UplanFI 20X/0.50 objective lens and CellF software. Immunostaining was performed as previously described (Khromov et al., 2011) and images were acquired as described above. AP staining was performed according to the manufacturer's protocol provided in the AP staining kit (Sigma).

Methylation analysis of imprinted gene

Genomic DNA was isolated from OG2_SSC lines. Bisulphite pyrosequencing of genomic DNA was performed as previously described (Zechner et al., 2009).

Whole-genome miRNA expression analysis

The whole-genome miRNA expression analysis was performed on SSCs, EGFP⁺ (PrM) cells and DsRed⁺ (meiotic) cells using Agilent-031184 Unrestricted Mouse miRNA V16.0 Microarray. RNA extraction, labelling and detection of miRNAs expression were performed according to the protocol supplied by the manufacturer.

Luciferase reporter assay

Luciferase reporter assays were performed as described previously (Zovoilis et al., 2009). PCR fragments including the 3'-UTRs of *c-Kit*, *Rbm44* and *Cdk6* were cloned into the multiple cloning site of pMIR-Report Luciferase vector (Life Technologies). Detailed information about the cloned fragments is available in Supplementary Table S8. We used testis-derived GC-1 and GC-4 cell lines, which are regarded as equivalent to spermatogonia and spermatocytes, respectively (Tascou et al., 2000). The GC-1 and GC-4 cells were transiently transfected with the indicated luciferase vector, pMIR-Report β -Gal (control plasmid for use in normalisation of the results) (Life technologies) and the pre-miR molecules (Qiagen) using Lipofectamine 2000 transfection reagent (Invitrogen). Briefly, 1 day before transfection, cells were trypsinised and transferred (1×10^5 cells/well) to a fresh well of a six-well plate. The optimised DNA transfection amounts for each vector were 3 μ g (for both luciferase and β -Gal vector a total of 6 μ g) per well of cells. The optimised miRNA precursor final concentrations per well of cells were 10 nM for each miRNA. Per well, 10 μ l transfection reagent was used and, with these reagent and miRNA concentrations, no cell toxicity effects were observed. Transfections including only the vectors or the pre-miR molecules and comparisons with mock transfection controls were initially performed for validation of the assay. After validation, cells were transfected in triplicate and each transfected well was evaluated in triplicate after 24 h using the Luciferase Assay System (Promega) according to manufacturer's recommendations in a SynergyMx Luminometer (BioTek). Luciferase activity was normalised to that of the β -Gal vector activity.

Bioinformatics and statistical analysis

For computational prediction of miRNA targets, we used the miRNA Body-map software, which combines all known miRNA target prediction softwares (<http://www.mirnabodymap.org/>). A gene was regarded as a potential target when the gene was hit at least by four independent softwares and known or predicted to have a function in spermatogenesis process. For functional annotation of miRNA targets, we used the data mining environment provided by the DAVID platform (Huang da et al., 2009). The functional annotation module was applied for GO terms in PANTHER database using an EASE score of 0.1 and a minimum number of two counts. For statistical analysis, we used Graphpad Prism software and performed paired *t*-tests. Data are expressed as the mean $\pm$ SD and *p* value <0.05 was considered statistically significant. The microarray and qPCR

data were used to generate Heatmaps using CIMminer software (<http://discover.nci.nih.gov/cimminer/oneMatrix.jsp>).

Author contribution

L.S., J.N., W.E. and D.V.K.P. participated in the design of the study. L.S. and Y.Z. carried out the experiments. L.S., U.Z., W.E. and D.V.K.P. helped in drafting the manuscript. J.N., U.Z. and D.V.K.P. helped in coordination of the study. W.E. and U.Z. gave financial support to the study.

All authors read and approved the final manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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