miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis

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SUMMARY
miR-18 belongs to the Oncomir-1 or miR-17–92 cluster that is intimately associated with the occurrence and progression of different types of cancer. However, the physiological roles of the Oncomir-1 cluster and its individual miRNAs are largely unknown. Here, we describe a novel function for miR-18 in mouse. We show that miR-18 directly targets heat shock factor 2 (HSF2), a transcription factor that influences a wide range of developmental processes including embryogenesis and gametogenesis. Furthermore, we show that miR-18 is highly abundant in testis, displaying distinct cell-type-specific expression during the epithelial cycle that constitutes spermatogenesis. Expression of HSF2 and of miR-18 exhibit an inverse correlation during spermatogenesis, indicating that, in germ cells, HSF2 is downregulated by miR-18. To investigate the in vivo function of miR-18 we developed a novel method, T-GIST, and demonstrate that inhibition of miR-18 in intact seminiferous tubules leads to increased HSF2 protein levels and altered expression of HSF2 target genes. Our results reveal that miR-18 regulates HSF2 activity in spermatogenesis and link miR-18 to HSF2-mediated physiological processes such as male germ cell maturation.

KEY WORDS: HSF, microRNA, miR-17–92, Speer4a, Ssty2, T-GIST, Mouse

INTRODUCTION
MicroRNAs (miRNAs) are small (~22 nucleotides in length) RNA molecules that regulate turnover or translational efficiency of target mRNAs through base-pairing with their 3′ UTR. The miRNAs are crucial for a multitude of cellular functions (Bartel, 2004) and for vertebrate development, as improper miRNA processing leads to embryonic lethality in mice (Bernstein et al., 2003; Liu et al., 2004). In addition, miRNAs are vital for normal spermatogenesis, and male mice lacking Dicer, which is required for miRNA biogenesis, have abnormal morphology and motility of sperm and hence acquire male infertility (He et al., 2009; Maatouk et al., 2008).

Oncomir-1, also called the miR-17–92 cluster, comprises six miRNAs (miR-17, -18a, -19a, -20a, -19b, -92a) and is transcribed as a polycistron that is subsequently processed to form individual miRNAs (Mendell, 2008; Tanzer and Stadler, 2004). Deletion of the cluster in mouse leads to neonatal lethality as well as defects in lung, heart and B-cell development (Ventura et al., 2008). The miR-17–92 cluster is an oncogene and frequently overexpressed in different types of tumours, including B-cell lymphomas, lung and colorectal carcinomas (Dews et al., 2006; Hayashita et al., 2005; Mendell, 2008; Ota et al., 2004; Volinia et al., 2006). The oncogenic potential of the cluster is associated with its ability to enhance cell cycle progression and to inhibit apoptosis, e.g. by regulating the expression of E2F1 (Novotny et al., 2007; O’Donnell et al., 2005). miR-18 is a member of the miR-17–92 cluster and has been shown to participate in tumour progression in a mouse model of colon cancer through targeting anti-angiogenic connective tissue growth factor, CTGF (Dews et al., 2006). In breast cancer cell lines, miR-18 has been suggested to repress estrogen receptor α (Leivonen et al., 2009). Although knowledge on the role of miR-18 and the whole miR-17–92 cluster in cancer is rapidly increasing, the physiological functions are still enigmatic.

Spermatogenesis is a complex process involving dramatic changes in cell proliferation, differentiation and morphology. It takes place in the seminiferous epithelium of the testis and is divided into three phases: mitosis, meiosis and spermiogenesis. In mitosis, spermatogonia divide, giving rise to primary spermatocytes. Meiosis leads to production of haploid spermatids that further differentiate into mature spermatoozoa during spermiogenesis (Papaoianmou and Nef, 2010; Russell et al., 1990). The entire process requires spermatogenic cell- and stage-specific gene expression, which are strictly controlled at the level of transcription, mRNA processing and translation. Spermatogenesis also features unique chromatin remodelling as somatic histones are replaced by DNA packing proteins, such as transition proteins and protamines which are exclusive to germ cells (Kleene, 2003; Pradeepa and Rao, 2007). Heat shock factor 2 (HSF2) is a transcription factor involved in corticogenesis and spermatogenesis that has either a stimulatory or repressive effect, depending on the target gene (Chang et al., 2006; Sandqvist et al., 2009; Östling et al., 2007; Åkerfelt et al., 2008). It has been shown that, in mouse testis, HSF2 binds the promoters of a large set of targets and regulates the transcription of multicopy genes residing on the sex chromosomes (Åkerfelt et al., 2008). Furthermore, Hsf2−/− mice display reduced size of testis and epididymis, altered morphology of the seminiferous tubules and a lowered number of spermatids (Kallio et al., 2002; Wang et al., 2003). Mature sperm in Hsf2−/− mice also exhibit deficient chromatin compaction, sperm head abnormalities and impaired quality (Åkerfelt et al., 2008).

In this study, we investigated the physiological role of miR-18 and found that it represses the expression of HSF2 by directly binding its 3′ UTR. During the epithelial cycle of spermatogenesis, miR-18 and HSF2 exhibit mutually excluding expression patterns,
suggesting that miR-18-mediated regulation occurs in developing germ cells. Using a novel method, called T-GIST (transfection of germ cells in intact seminiferous tubules), we inhibit miR-18 in spermatocytes residing inside cultured seminiferous tubules and demonstrate that miR-18 inhibition results in increased HSF2 protein levels, providing in vivo evidence that miR-18 targets Hsf2. Moreover, miR-18 inhibition leads to repression of the HSF2 target genes Speer4a and Stry2. Our results reveal that miR-18 controls the activity of HSF2 and establish miR-18 as a novel regulator of gene expression in spermatogenesis.

MATERIALS AND METHODS

Mice

Male C57BL/6N mice (60-80 days old) were used in the experiments. The pathogen-free mice were housed under controlled environmental conditions and fed with complete pellet chow and allowed tap water. The mice were sacrificed by CO2 asphyxiation and handled in accordance with the institutional animal care policies of the Åbo Akademi University (Turku, Finland).

Cell culture and transfection

GC-1 spg (spermatogonia), ST15A, MCF-7 and HEK 293T cell lines were cultured in DMEM supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin in 5% CO2 at 37°C. MCF-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In short, 2.5 × 105 cells were plated the day before transfection in a 12-well tissue culture dish. Transfection was performed with 1.6 μg DNA. The pD40-His/V5-e-Myc plasmid was described earlier (Yeh et al., 2004) and pEGFP-C1 (Clontech) was used to control for transfection efficacy and unspecific effects. HEK 293T and GC-1 spg cells were transfected with the indicated amounts of miR18 miRNA mimics (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

Luciferase assay

For production of a reporter construct, a 258-nucleotide-long fraction of the 3’UTR of human HSF2 was amplified using the primers: 5’-CATC-CACATGGTTCACCCGAAATGAGATTTTAC-3’ and 5’-CATCCAG-GTGGAGAAAAATGGCCATTTGAATCC-3’ (Dharmacon) or a negative control, were incubated with 1 μg_mRNA (Applied Biosystems). Reverse transcription was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and reverse primers were used as gene-specific RT primers.

Primers for Hsf2 were designed by Universal Probe Library Assay Design Center (Roche Applied Biosciences) and were as follows: hHSF2 forward 5’-GGGAGGAAAACCCACACTAAGG-3’, hHSF2 reverse 5’-ATCG-GTGTCTGCTCAAGAGACCC-3’, mHSF2 forward 5’-GGTGTCAAGAATAAGAAGGAA-3’, miR18 reverse 5’-CCAATGATCACATTAACCGCCAGAATTTCTCC-3’. The fluorescent Taqman probes were obtained from Roche (no. 36 for hHSF2 and no. 3 for mHSF2). Primers and probes for mouse β-actin were purchased from Oligomer: β-actin forward 5’-TGGCTCTTACACCACATGAAGA-3’, β-actin reverse 5’-GTGGACAGTGGGCGGATATG-3’, β-actin probe 5’-FAM CAAGATCATGTTTTCATCCGCAC-3’, mHSF2 forward 5’-FAM CAAGATCATGTTTTCATCCGCAC-3’, reverse 5’-GAGAATCTTGTGACAGGACA-3’, Stry2 forward 5’-CAAGAGAGAGGGACTTTGAC-3’, Stry2 reverse 5’-GAGAATCTTGTGACAGGCAC-3’. Stry2 reverse 5’-GAGAATCTTGTGACAGGACA-3’, Speer4a reverse 5’-GCTGCAAATATCGCCAACTTT-3’, Stry2 reverse 5’-GAGAATCTTGTGACAGGACA-3’, Speer4a reverse 5’-GCTGCAAATATCGCCAACTTT-3’, the Taqman probes for Stry2 (no. 25) and Stry2 (no. 81) were obtained from Roche. Primers and probes for hGAPDH have been described earlier (Östling et al., 2007).

miRNA quantitative real-time RT-PCR

TaqMan miRNA Assays (Applied Biosystems) were performed according to the manufacturer’s instructions. For analysis of human MCF-7 cells, RNA was isolated using miPremier microRNA Isolation Kit (Sigma-Aldrich) and 15 ng of RNA was used in the RT reaction. The small nuclear RNA RNU44 was used as an endogenous control. For analyses in mouse, total RNA derived from pooled tissues from Swiss Webster mice were used as starting material (7800, Ambion). The small RNA Sno202 was used for normalization. All PCR reactions were performed in triplicates and analyzed as above.

Western blot analysis

Cells were lysed in Laemmli buffer and subjected to SDS-PAGE followed by transfer to nitrocellulose membrane. Antibodies used were: HSF2 (Sarge et al., 1993); Hsc70 (SPA-815, Stressgen); V5 (MCA1360GA, AbD Serotec); and β-actin (A4700, Sigma). Secondary antibodies conjugated to horseradish-peroxidase were purchased from Promega or GE Healthcare. Blots were developed by ECL (GE Healthcare).

In situ hybridization

In situ hybridization was done as previously described (Obernosterer et al., 2007), with minor changes. Mouse testes were prefixed in 4% paraformaldehyde (PFA) for 2 hours, incubated overnight in 0.3 M sucrose, embedded in Tissue Tek OCT (Sakura) and stored at −80°C. Ten micrometer cryosections were postfixed in PFA, acetylated, treated with proteinase K and prehybridized. Digoxigenin-labeled LNA scrambled probe and miR-18 antisense probe (Exiqon, 15 nM) were hybridized to the slides at 51°C overnight. Following washes and blocking, anti-digoxigenin antibody (Roche, 1:2000) was applied. Alkaline phosphatase colour reaction was performed with Fast Red Substrate (Dako). Slides were mounted and DNA visualized using Vectashield mounting medium with DAPI (Vector Laboratories). The sequences of the miR-18 probe and the scrambled probe were 5’-CTATCTGCACTAGATGCACCTTA-3’ and 5’-GGTGAACACGTCTATACGCCCA-3’, respectively.

Transfection of germ cells in intact seminiferous tubules (T-GIST)

For a detailed description, see Fig. S3 in the supplementary material. Mouse testes were isolated and decapsulated in DMEM:Ham’s F12 (D8437, Sigma). Stage IX of the seminiferous epithelial cycle was identified and segments were cut as previously described (Kotaja et al., 2007). The isolated stages were put in a 96-well dish with 50 μl m medium and transfected with FITC-labelled miRCURY LNA knockdown oligonucleotides (Exiqon, 200 nM). A scrambled control or a knockdown
oligonucleotide specific for miR-18 was diluted in 30 µl Opti-MEM I (Gibco) together with 1.3 µl Lipofectamine RNAiMAX. The mixture was incubated for 20 minutes and added to the wells. Following incubation at 34°C in 5% CO₂ for 5 hours, DMEM:Ham’s F12 supplemented with antibiotics was added and the tubules were further cultivated for 35 hours. Transfected germ cells were identified and analyzed with fluorescence-based methods such as squash preparation and FACS; see below.

Fluorescence-activated cell sorting (FACS) of transfected spermatocytes

Seminiferous tubules were transfected as described above. After cultivation, cells were released from the tubule segments using scissors, centrifuged at 600 g for 5 minutes and resuspended in PBS. For fixation, ethanol was added to a final concentration of 70% in 150 µl and samples were stored at −20°C for at least 24 hours. Prior to Propidium Iodide (PI) staining, the cells were centrifuged. PI was used at 40 µg/ml diluted in PBS, 0.05% Tween 20. Transfected pachytene spermatocytes were collected based on the DNA content (4N) and the signal from FITC-tagged inhibitors with a FACS Vantage SE cell sorter (BD).

Squash preparation and immunofluorescence

Squash preparations were performed as in (Kotaja et al., 2004). For immunofluorescence, the preparations were postfixed for 10 minutes in 4% PFA, washed and permeabilized in 0.2% Triton X-100 for 5 minutes. After washes, slides were blocked in FCS in B1 solution (0.1 M Tris pH 7.5, 0.15 M NaCl), incubated with a polyclonal rabbit anti-HSF2 antibody (4506, produced in the Sistonen laboratory; see Fig. S2 in the supplementary material) and a secondary antibody (anti-rabbit Alexa 546, Molecular Probes). Nuclei were visualized as described above. Cryosections for staining were prepared as for in situ hybridization. Following sectioning, slides were air-dried, blocked and stained as above.

Image acquisition

Cryosections and squash preparations were analyzed with a LSM510-Meta scanning confocal microscope (Carl Zeiss) equipped with the SP2 (version 3.2) software. The images were acquired using a Plan-Apochromat 40×/1.4 Oil objective or a Plan-Neofluar 20×/0.5 objective.

RESULTS

Cell-type-specific expression of miR-18 in spermatogenesis

To assess the function of miR-18 in normal physiological processes, we analyzed its expression levels in various mouse tissues using real-time RT-PCR. miR-18 was present in all tissues tested, but was most abundant in the testis, thymus and developing embryo [embryonic day (E) 10-12; Fig. 1A]. We chose to investigate the role of miR-18 in mouse testis and performed in situ hybridization on testis cryosections to get a detailed view of the expression pattern. Fig. 1B shows cross-sections of seminiferous tubules in different developmental stages of the epithelial cycle that constitutes spermatogenesis (for details, see Fig. 5B). Each stage contains a specific association of spermatogenic cells in different phases of maturation, arranged in a typical pattern with the most mature germ cells closest to the lumen (Fig. 1C). Interestingly, miR-18 displayed a cell-type-specific expression, with highest intensity in the spermatocytes (Fig. 1B,C), indicating a role for miR-18 in the development of male germ cells.

miR-18 regulates HSF2 expression

We initiated our search for miR-18 targets by using the target prediction programs TargetScan, miRanda, PicTar and miRBase (Betel et al., 2008; Griffiths-Jones et al., 2008; Krek et al., 2005; Lewis et al., 2003). Among the putative targets, we focused on HSF2, which is needed for proper spermatogenesis, as evident from the phenotype of the Hsf2−/− mice (Kallio et al., 2002; Wang et al., 2003; Åkerfelt et al., 2008). Furthermore, it has been demonstrated that HSF2 can become transcriptionally active by simply raising its concentration in cells (Sandqvist et al., 2009), indicating that the level of HSF2 is under strict control. Therefore, to test whether miR-18 is involved in the regulation of HSF2, we first transfected miRNA mimics into human HEK 293T cells. As detected by western blot analysis, miR-18 downregulated the protein levels of HSF2 (Fig. 2A). HSF2 was similarly downregulated by overexpressing miR-18 in rat ST15A cells (data not shown). As miR-18 exists in two paralogous forms, a and b, located in the miR-17−92 and miR-106a−363 clusters, respectively (Mendell, 2008), we examined the effect of both forms. The nucleotide sequences of the paralogs are very similar and both were able to downregulate HSF2, whereas unrelated miRNAs had no
effect (Fig. 2A). Because miR-18b has not been found in tissues, and mice lacking the miR-18b locus show no obvious abnormalities (Ventura et al., 2008), we concentrated our study on miR-18a, hereafter called miR-18. Next, we transfected increasing amounts of miR-18 into mouse GC-1 spg cells and observed a concentration-dependent decrease in the HSF2 protein (Fig. 2B). Interestingly, the mRNA levels of Hsf2 also decreased in relation to the transfected amount of miR-18 (Fig. 2C), suggesting that miR-18 is able to operate through destabilizing Hsf2 mRNA.

**Hsf2 is a direct target for miR-18**

As illustrated in the upper panel of Fig. 3A, alignment of human HSF2 with miR-18 revealed a putative target site at position 112-134 of the HSF2 3’UTR, with an exact match in the seed region and position 8, i.e. a 7mer-m8 site (Grimson et al., 2007). This site is located in a conserved region of the Hsf2 3’UTR of several species (Fig. 3A, lower panel). To investigate whether Hsf2 is a direct target of miR-18, we generated a reporter construct bearing the 3’UTR of Hsf2 downstream of the luciferase gene (Fig. 3B). Rat ST15A cells were transfected with the indicated miRNA mimics or a negative control (Neg C). Samples were analyzed as in A. (C) Real-time RT-PCR of Hsf2 on cells transfected as in B. For fold-induction, mRNA amounts in the miR-18-expressing cells were compared with the control cells, arbitrarily set to 1. The data represents three biological samples and relative Hsf2 mRNA quantities were normalized to β-actin. Error bars indicate s.d.

**Endogenous miR-18 downregulates HSF2**

It is well established that miR-18, in conjunction with the whole miR-17–92 cluster, is regulated by c-Myc, and that c-Myc upregulation increases the expression of the different cluster members (Dews et al., 2006; O’Donnell et al., 2005). To examine whether endogenous miR-18 can regulate HSF2, we induced miR-18 by overexpressing c-Myc in MCF-7 cells (Fig. 4A). As measured by real-time RT-PCR, the level of miR-18 increased in comparison to the control transfection (Fig. 4C). When monitoring HSF2 protein (Fig. 4A,B) and mRNA levels (Fig. 4D), a decrease was evident in cells where c-Myc, and consequently miR-18, were upregulated. Furthermore, to convincingly demonstrate that the decrease in HSF2 occurs through the action of miR-18, we measured the effect on the luciferase reporter construct carrying the 3’UTR of Hsf2. After overexpressing c-Myc (Fig. 4E, right panel), a clear decrease in the luciferase activity was evident, whereas the luciferase activity was rescued using a construct mutated in the miR-18-binding region (Fig. 4E, left panel). We therefore conclude that endogenous miR-18 is able to regulate the expression of HSF2.

**HSF2 and miR-18 display inverse correlation in spermatogenesis**

miR-18 was expressed in a cell-type-specific manner in testis (Fig. 1B). Therefore, we investigated the physiological link between miR-18 and HSF2 by surveying their expression in the twelve species (Fig. 3A, lower panel). The seed recognising site is marked in red. (B) Schematic presentation of the luciferase (LUC) reporter used to identify the target site of miR-18. (C) ST15A cells were transfected with the reporter together with 10 nM of miRIDIAN miRNA mimics or a negative control. The firefly luciferase activity was normalized to Renilla luciferase. (D) Schematic description of the wild-type (WT) sequence of Hsf2 3’UTR containing the putative binding site of miR-18 in red. The mutated sequence (Mut) has the altered nucleotides underlined. Samples were assayed as in C. The data represent at least three biological samples. Error bars indicate s.d.
stages that make up the epithelial cycle of mouse spermatogenesis, where each stage comprises germ cells in different phases of development (Fig. 5B) (Kotaja et al., 2004). The precise stage of the seminiferous epithelial cycle was determined based on the appearance of the DAPI-stained germ cell nuclei. The criteria used for staging were the presence of specific germ cell types and combinations of different cell types, the organization and position of different cell types in the epithelium, as well as the size of late spermatocytes. Using in situ hybridization and immunostaining on consecutive sections, we were able to compare the expression of miR-18 and HSF2 in individual cells. Similarly to miR-18 (Fig. 1B), HSF2 showed a clear cell- and stage-specific localization (Fig. 5; see Fig. S1 in the supplementary material). Remarkably, its expression pattern was complementary to that of miR-18; cells expressing miR-18 were negative for HSF2 and vice versa. HSF2 was present in the outer cell layers that consist mainly of spermatogonia (Fig. 5Ac), whereas the expression of miR-18 was minute in these cells. Instead, miR-18 was highly expressed in the following differentiation phase, i.e. the spermatocytes (Fig. 5Ab,d).
This pattern of expression, exhibiting an inverse correlation between miR-18 and HSF2 (Fig. 5B), strongly suggests that miR-18 targets HSF2 in spermatogenesis.

**Inhibition of miR-18 in spermatocytes leads to elevated HSF2 levels**

To verify that HSF2 is regulated by miR-18 in vivo, we developed a new method, called T-GIST. This method allowed us to manipulate miR-18 in spermatogenic cells within intact seminiferous tubules, using inhibitors antisense to miR-18 (see Materials and methods; see Fig. S3 in the supplementary material). Transfection of cells residing in their natural environment was a crucial step to facilitate their survival. It also allowed subsequent culture, which provided time for an effect on the miRNA target to occur. We chose to transfect stage IX, where HSF2 and miR-18 colocalised in spermatocytes (Fig. 5B). Subsequently, squash preparations were made, resulting in a monolayer of all the different cell types present in stage IX, and the cells were stained for detection of HSF2. Indeed, inhibition of miR-18 in pachytene spermatocytes led to considerably increased levels of HSF2, as compared with untransfected cells of the same type (Fig. 6A, upper panel). By contrast, no increase in HSF2 was observed when spermatocytes were transfected with a scrambled inhibitor (Fig. 6A, lower panel). These results clearly demonstrate that miR-18 downregulates HSF2 in spermatogenesis.

**Speer4a and Ssty2 are repressed upon inhibition of miR-18**

Finally, we investigated the consequence of miR-18-mediated regulation of HSF2. The mRNAs of Speer4a, a member of the SPErm-associated glutamate (E)-Rich protein (SPEER) family, and the multicopy gene Ssty2 (spermiogenesis specific transcript on the Y 2) were analyzed, as HSF2 is known to bind their promoters (Åkerfelt et al., 2008). The possibility that miR-18 would target Speer4a or Ssty2 directly was excluded using the target prediction programs TargetScan, miRanda, PicTar and miRBase (Betel et al., 2008; Griffiths-Jones et al., 2008; Krek et al., 2005; Lewis et al., 2003). Mouse seminiferous tubules were transfected using the T-GIST protocol to inhibit the function of miR-18. Spermatogenic cells were then isolated from the tubules and the transfected cells were collected using fluorescence-activated cell sorting (FACS) based on the FITC-tagged inhibitors. To enrich pachytene spermatocytes, we took advantage of their doubled DNA content, 4N (see Fig. S3 in the supplementary material). In support for the increase in HSF2 protein detected in spermatocytes (Fig. 6A), inhibition of miR-18 also led to a modest but consistent increase (8%) in Hsf2 transcripts (data not shown). This relatively small effect on Hsf2 expression at the level of mRNA might reflect the function of miRNAs; they are able to both repress translation and/or promote mRNA degradation (Bartel, 2004), and therefore, the abundance of mRNAs does not strictly mirror the level of protein expression. Analysis of the Speer4a and Ssty2 mRNA levels revealed a prominent decrease in response to transfection with the specific miR-18 inhibitor (Fig. 6B). This repressive effect on Speer4a and Ssty2, resulting from increased HSF2 levels, is in accordance with our previous studies, where HSF2 has been demonstrated to act either as an activator or a repressor in a target-gene-specific manner (Sandqvist et al., 2009; Östling et al., 2007; Åkerfelt et al., 2008). Taken together, our results delineate a novel miR-18-dependent transcriptional regulatory pathway during the maturation of male germ cells.

**DISCUSSION**

There is a wealth of evidence for the role of the miR-17–92 cluster in cancer. By contrast, very little is known about the physiological functions of the cluster and especially of the functions of its individual members. Here, we demonstrate that miR-18 targets a transcription factor, HSF2, in testis. We show that miR-18 mediates cell- and stage-specific expression of HSF2 during spermatogenesis.
spermatogenesis, for which carefully controlled gene expression is a prerequisite. During spermatogenesis, thousands of genes need to be temporally expressed (Eddy, 1998; Grimes, 2004) and, for HSF2, miR-18-mediated regulation provides a mechanism bypassing transcriptional regulation. Indeed, regulation of expression through translational control is a striking feature of spermatogenesis, particularly in meiotic and haploid germ cells where most mRNA species are at times translationally repressed and sequestered in ribonucleoprotein particles, e.g. in the chromatoid body (Kleene, 2003). Similarly, many miRNAs and components of the miRNA pathway are stored in the chromatoid body (Kotaja et al., 2006), enabling rapid mobilization. Consequently, miRNAs are suitable candidates to participate in the correct timing of protein expression at specific stages during male germ cell maturation.

HSF2 belongs to a family of transcription factors, of which HSF1 is the most thoroughly investigated member. In contrast to HSF1, whose activity is regulated through extensive post-translational modifications such as phosphorylation, sumoylation and acetylation (Anckar and Sistonen, 2007), the mechanisms of HSF2 regulation have remained obscure. Previously, a positive correlation between HSF2 expression and activity has been described (Mezger et al., 1989; Murphy et al., 1994) and, in embryogenesis, HSF2 DNA binding activity was found to correlate temporally with HSF2 expression (Min et al., 2000; Rallu et al., 1997). More recently, it was demonstrated that increased expression of HSF2 induces nuclear localization and transcription of HSF2 targets genes, suggesting that HSF2 is activated by its elevated expression (Sandqvist et al., 2009). We now provide evidence that, upon inhibition of miR-18, the protein levels of HSF2 are increased in vivo, which leads to altered expression of the HSF2 target genes Speer4a and Ssty2. Hence, although additional mechanisms might exist, our results indicate that miR-18-mediated regulation provides a rheostat for the adjustment of HSF2 expression and activity during germ cell development.

The survival of spermatogenic cells depends largely on the specific milieu in testis. Inside the seminiferous tubules they are surrounded by Sertoli cells, which provide structural support and facilitate development, germ cell movement and release of mature germ cells to the lumen. Endocrine communication between testicular cell types is also vital for differentiation and the process of spermatogenesis as a whole (Wang et al., 2009). These requirements of germ cells make examination of gene expression during spermatogenesis challenging, especially as there are no cell lines representing the later stages of spermatogenesis. To provide in vivo evidence for miR-18-mediated regulation of HSF2, we developed a novel method, T-GIST, which enabled inhibition of miR-18 in germ cells in their natural environment, i.e. the seminiferous tubules. In T-GIST, stages of the seminiferous tubules are isolated based on their transillumination pattern (Kotaja et al., 2004). The stages are then transfected with microRNA inhibitors tagged with fluorescent markers and cultured to allow target inhibition. Analysis is performed with fluorescence microscopy or real-time RT-PCR, for which the cells are first sorted based on their DNA content. Importantly, as both approaches permit staining of DNA, they provide means to selectively study a specific cell type. Furthermore, the fluorescent markers make possible the identification of transfected cells. In summary, T-GIST allows transfection of germ cells in seminiferous tubules and subsequent investigation of gene expression on the level of protein and mRNA.

Recently, HSF2 was also discovered to bind a multitude of target genes in testis and to affect the quality of sperm (Åkerfelt et al., 2008). To investigate the downstream effects of HSF2 regulation by miR-18, we inhibited miR-18 by T-GIST and analyzed the protein levels of HSF2 as well as the expression of the testis-specific genes Speer4a and Ssty2, known target genes of HSF2 (Åkerfelt et al., 2008). The SPEER proteins are thought to be nuclear matrix proteins involved in reorganization of the postmeiotic nucleus (Spiess et al., 2003), and HSF2 also binds promoters of other members of the family, such as Speer4b/c/d (M. Åkerfelt, unpublished). When miR-18 was inhibited in pachytene spermatocytes, the concentration of HSF2 was increased and expression of Speer4a decreased, suggesting that activation of HSF2 occurs in these cells.

Similarly, the expression of Ssty2 was repressed upon inhibition of miR-18 in pachytene spermatocytes. In the postmeiotic spermatids, where Ssty2 is thought to regulate chromatin remodelling vital for correct germ cell development, HSF2 has been shown to upregulate the expression of Ssty2 (Toure et al., 2004; Åkerfelt et al., 2008). We hypothesize that HSF2 represses the expression of Ssty2 in the preceding cell type pachytene spermatocytes, as the function of Ssty2 is required only after meiosis. Although further studies are warranted, these results indicate that HSF2 is a multifaceted regulator of spermatogenesis, with distinct roles during different stages of germ cell maturation. Based on our results, it is probable that other targets of HSF2 are also regulated by miR-18 and that miR-18 participates in the processes attributed to HSF2, such as correct chromatin organization and sperm maturation (Kallio et al., 2002; Wang et al., 2003; Åkerfelt et al., 2008). Taken together, our results point to a cascade of events where a miRNA, single-handedly or in cooperation with other factors, downregulates the expression of a transcription factor, thereby facilitating transcriptional regulation of a plethora of downstream target genes.

In this study, we describe miR-18-mediated regulation of HSF2 in spermatogenesis. In addition to testis, we show that miR-18 was also strongly expressed in the midterm embryo. A study elucidating the expression profiles of miRNAs in mouse embryos reported that miR-18 exhibits temporal expression during prenatal development when comparing E9.5, E10.5 and E11.5 embryos (Mineno et al., 2006). Similarly, the expression of HSF2 varies during embryogenesis; HSF2 is abundant in E9.5 embryos, whereas during the second half of gestation, the expression becomes restricted to the central nervous system (Kallio et al., 2002; Rallu et al., 1997). It is tempting to speculate that regulation of HSF2 by miR-18 is not restricted to spermatogenesis but might also occur during certain stages of embryonic development. Moreover, miR-18 appears to be ubiquitous in the adult mouse as we found miR-18 expression in all tissues analyzed, prompting further studies on the role of miR-18 in physiology in general.

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