

Development 137, 3177–3184 (2010) doi:10.1242/dev.050955  
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# miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis

Johanna K. Björk<sup>1,2,\*</sup>, Anton Sandqvist<sup>1,2,\*</sup>, Alexandra N. Elsing<sup>1,2</sup>, Noora Kotaja<sup>3</sup> and Lea Sistonen<sup>1,2,†</sup>

## 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  $\alpha$  (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 spermatozoa during spermiogenesis (Papaioannou 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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,

<sup>1</sup>Department of Biosciences, Åbo Akademi University, 20520 Turku, Finland. <sup>2</sup>Turku Centre for Biotechnology, Åbo Akademi University and University of Turku, 20520 Turku, Finland. <sup>3</sup>Department of Physiology, University of Turku, 20520 Turku, Finland.

\*These authors contributed equally to this work

†Author for correspondence (lea.sistonen@btk.fi)

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 *Ssty2*. 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 CO<sub>2</sub> 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% CO<sub>2</sub> at 37°C. MCF-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In short,  $2.5 \times 10^5$  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-c-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 miRIDIAN 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-CACTAGTTCCTCCAGGAAGTGGACTTTAC-3' and 5'-CATCCAAG-CTTGAGAAAAATGGCCATTGAATCC-3'. The PCR fragment was digested with *SpeI* and *HindIII* and cloned into the pMIR-REPORT vector (Ambion). The construct containing the miR-18 binding site mutations was made by Quick Change site-directed mutagenesis (Stratagene) in two sequential reactions. The mutated sequence is shown in Fig. 3D, with the substituted bases underlined. The constructs were verified by sequencing. ST15A cells ( $10^5$  cells) were plated in a 24-well plate the day before transfection. The internal control pRL-SV40 (Clontech) and 180 ng of the luciferase reporter constructs, together with miRIDIAN miRNA mimics (Dharmacon) or a negative control, were incubated with 1 µl of Lipofectamine 2000 and transfected into cells. After 5 hours the transfection solution was replaced by fresh DMEM. Cell lysates were obtained 24 hours after transfection and assayed using the Dual-Luciferase Reporter Assay System (Promega) and Labsystem's Luminoscan. The firefly luciferase activity was normalized to the Renilla luciferase. MCF-7 cells were transfected with the luciferase reporter constructs and the internal control pMIR-REPORT β-gal (Ambion), together with pD40-His/V5-c-Myc (Yeh et al., 2004) or pcDNA3.1 (Invitrogen). Forty-eight hours after the transfection, cell lysates were analyzed for firefly luciferase activity as described above. The luciferase activity was normalized to β-galactosidase expression measured with Multiskan Ascent (Thermo Scientific).

### Quantitative real-time RT-PCR

RNA was isolated using the RNeasy Kit (Qiagen). For mouse GC-1 spg cells, reverse transcription was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For human cells, RNA was treated with RQ1 DNase (Promega) and reverse-transcribed using Moloney Murine Leukemia Virus RNase H(-) (Promega). ABsolute QPCR ROX Mix (Thermo Scientific) was used, and the PCR performed with ABI

Prism 7900HT (Applied Biosystems). Relative quantities of HSF2 were normalized against *GAPDH* (human) or β-actin (mouse), and fold-inductions were determined. The results were analyzed with SDS 2.3 and RQ manager software (Applied Biosystems). For analysis of transfected pachytene spermatocytes, RNA was isolated using the RNeasy Micro Kit (Qiagen). Reverse transcription was performed 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'-GGAGGAAACCCACACTAACG-3', *hHSF2* reverse 5'-ATC-GTTGCTCATCCAAGACC-3', *mHSF2* forward 5'-GGTGTGAGAA-CTAAGAGCAAAGC-3', *mHSF2* reverse 5'-CCAATGTAACAATA-AACTGGACAATC-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'-TGGCTCCTAGCACCATGAAGA-3', β-actin reverse 5'-GTGGACAGT-GAGGCCAGGAT-3', β-actin probe 5'-FAM CAAGATCATTGCTCC-TCCTGAGCGCA TAMRA-3'. *Ssty2* forward 5'-CAAGAAGAAGAGT-AGGAGGAAGCA-3', *Ssty2* reverse 5'-GAGAAATCTGCAGCCA-ACA-3', *Speer4a* forward 5'-CAAGCAGGAGTTCAAGAAGGAGCT-3', *Speer4a* reverse 5'-GCTGCAATATCGCCAACTTT-3'. The Taqman probes for *Speer4a* (no. 25) and *Ssty2* (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 mirPremier microRNA Isolation Kit (Sigma-Aldrich) and 15 ng of RNA was used in the RT reaction. The small nucleolar 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 (Obermosterer 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'-GTGTAACACGTCTATACGCCCA-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., 2004). The isolated stages were put in a 96-well dish with 50 µl 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  $\mu$ l Opti-MEM I (Gibco) together with 1.3  $\mu$ l Lipofectamine RNAiMAX. The mixture was incubated for 20 minutes and added to the wells. Following incubation at 34°C in 5% CO<sub>2</sub> 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  $\mu$ l 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  $\mu$ g/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 FACSVantage 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 $\times$ /1.4 Oil objective or a Plan-Neofluar 20 $\times$ /0.5 objective.

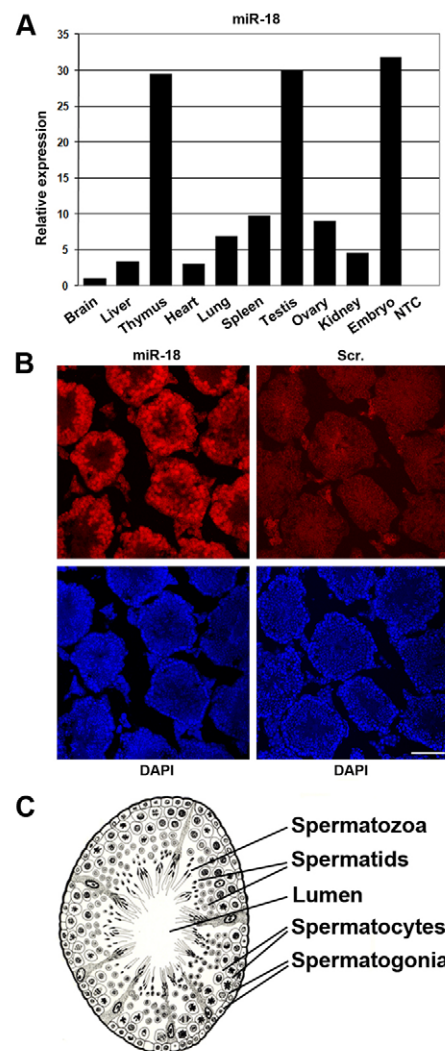
## RESULTS

### 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.

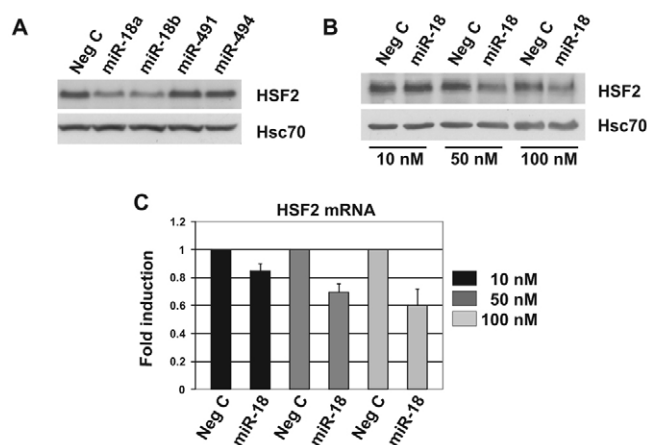
### miR-18 regulates HSF2 expression

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*<sup>-/-</sup> mice (Kallio et al., 2002; Wang et al., 2003; Åkerfelt et al., 2008). Furthermore, it has been



**Fig. 1. Expression of miR-18.** (A) miR-18 expression was analyzed in mouse tissues using real-time RT-PCR. Fold-induction was calculated by comparing expression levels to that of the brain, arbitrarily set to 1. Samples were derived from pooled tissues (7800, Ambion). Relative quantities of miR-18 were normalized to the internal control Sno202. NTC, no template control. (B) In situ hybridization of miR-18 (red) in cryosections of mouse testis. Nuclei were visualized with DAPI (blue). A non-targeting scrambled probe (Scr.) was used as control. Scale bar: 100  $\mu$ m. (C) Schematic cross-section of a seminiferous tubule showing the different spermatogenic cells. Adapted from Freeman and Bracegirdle (Freeman and Bracegirdle, 1969).

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



**Fig. 2. miR-18 downregulates HSF2.** (A) HEK 293T cells were transfected with the indicated miRIDIAN miRNA mimics or a negative control (Neg C). HSF2 expression was analyzed 48 hours later by western blot. Hsc70 serves as a loading control. (B) Concentration dependency of miR-18-mediated HSF2 regulation was monitored by transfecting mouse GC-1 spg cells with increasing amounts of miR-18 (10–100 nM) 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  $\beta$ -actin. Error bars indicate s.d.

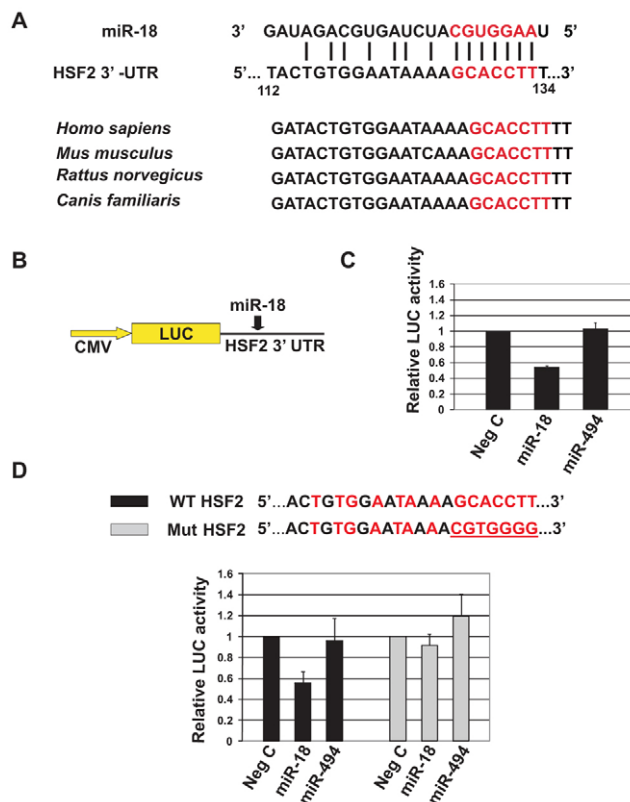
effect (Fig. 2A). Because miR-18b has not been found in tissues, and mice lacking the miR-18b locus show no obvious abnormalities (Ventura et al., 2008), we concentrated our study on miR-18a, hereafter called miR-18. Next, we transfected increasing amounts of miR-18 into mouse GC-1 spg cells and observed 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 and, as opposed to the negative control and the unrelated miR-494, only miR-18 prominently inhibited the luciferase activity (Fig. 3C). In order to define the site of interaction, we mutated the reporter construct in the putative binding region, which abolished the effect of miR-18 on the luciferase activity (Fig. 3D). These results confirmed *Hsf2* as a direct target of miR-18 and identified the site of interaction in the 3'UTR of *Hsf2* mRNA.

### 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

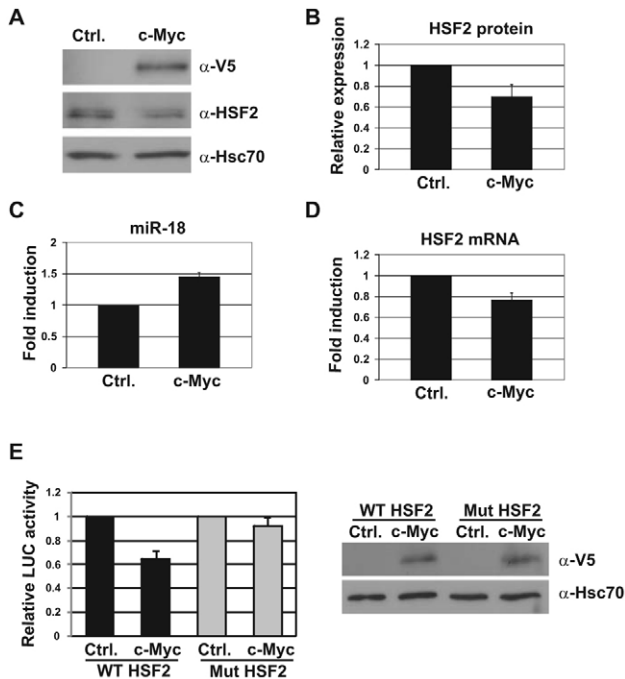


**Fig. 3. Identification of the miR-18 binding site.** (A) Putative duplex formation between miR-18 and the *Hsf2* 3'UTR. The binding site is conserved between several mammalian species. The seed recognising site is marked in red. (B) Schematic presentation of the luciferase (LUC) reporter used to identify 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.

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.

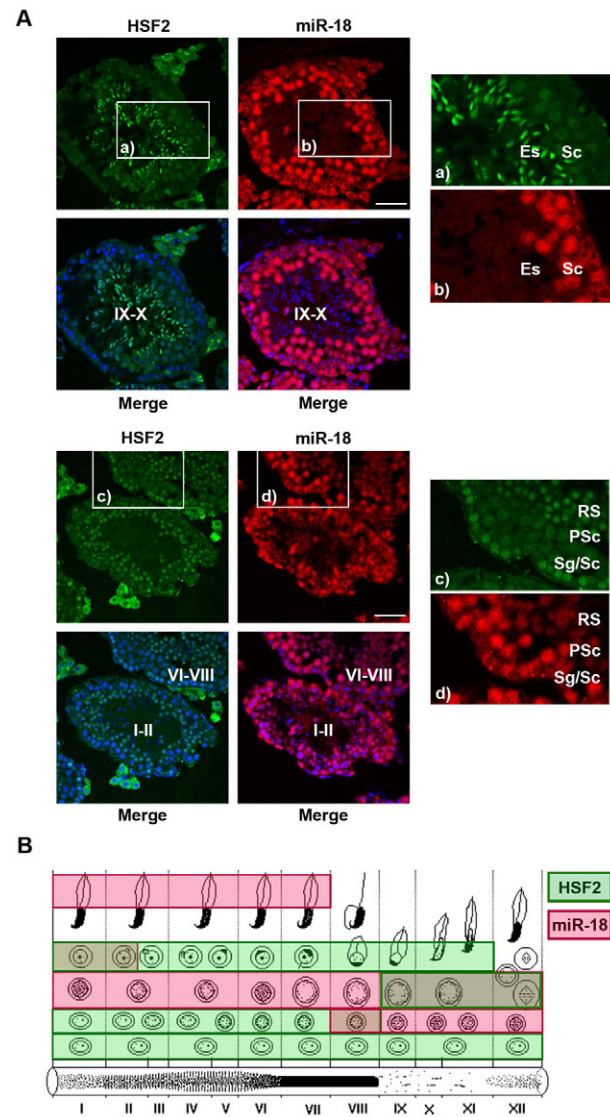
### HSF2 and miR-18 display inverse correlation in spermatogenesis

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



**Fig. 4. c-Myc upregulates endogenous miR-18, which leads to decreased expression of HSF2.** (A) Western blot of whole-cell lysates of MCF-7 cells after transfection with V5-tagged c-Myc or GFP as a control (Ctrl.). Antibodies against HSF2, V5 for exogenous c-Myc detection and Hsc70 were used. (B) Quantification of HSF2 protein levels, as detected by western blot, was performed from four individual experiments using Image J software and normalized to the loading control. Control transfection was arbitrarily set to 1. (C,D) Real-time RT-PCR analysis of miR-18 (C) and *Hsf2* (D) after overexpressing c-Myc. Fold-induction was calculated by comparing transcription in the c-Myc overexpressing cells with the control cells, which was arbitrarily set to 1. The data represent four biological samples, and the relative quantities of RNA were normalized to RNU44 and *Gapdh*, respectively. (E) MCF-7 cells were transfected with the luciferase (LUC) reporter carrying either a wild-type (WT) sequence of *Hsf2* 3'UTR or a mutated sequence (Mut; see Fig. 3D), together with c-Myc or empty vector as a control (Ctrl.). Left panel: the firefly luciferase activity was normalized to  $\beta$ -galactosidase. The data represent three biological samples. Right panel: a representative western blot showing overexpressed c-Myc detected with anti-V5 antibody. Hsc70 is used as a loading control. All 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



**Fig. 5. Inverse correlation between HSF2 and miR-18 expression.** (A) In situ hybridization of miR-18 (red) and immunostaining of HSF2 (green) performed on consecutive sections of mouse testis. The stages of the seminiferous epithelial cycle (I-XII, see panel B) are denoted by Roman numerals and were identified based on the appearance of the DAPI-stained germ cell nuclei (blue). Representative stages and higher magnifications of them are shown in a-d. For more stages and antibody controls, see Figs S1 and S2 in the supplementary material. Es, elongated spermatids; PSc, pachytene spermatocytes; RS, round spermatids; Sc, spermatocytes; Sg, spermatogonia. Scale bars: 50  $\mu$ m. (B) Schematic presentation of the expression of miR-18 (red) and HSF2 (green) in the seminiferous epithelial cycle, based on analyses of all twelve stages. The development of germ cells starts from the left lower corner and proceeds to the right, moves up one row and again starts from the left, until the cycle is completed with sperm release. The transillumination pattern of a tubule is shown at the bottom.

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). In spermatocytes, the expression of HSF2 was low, but reappeared in round spermatids (Fig. 5Ac) and elongating spermatids (Fig. 5Aa), where miR-18 was present only at low levels (Fig. 5Ab,d).

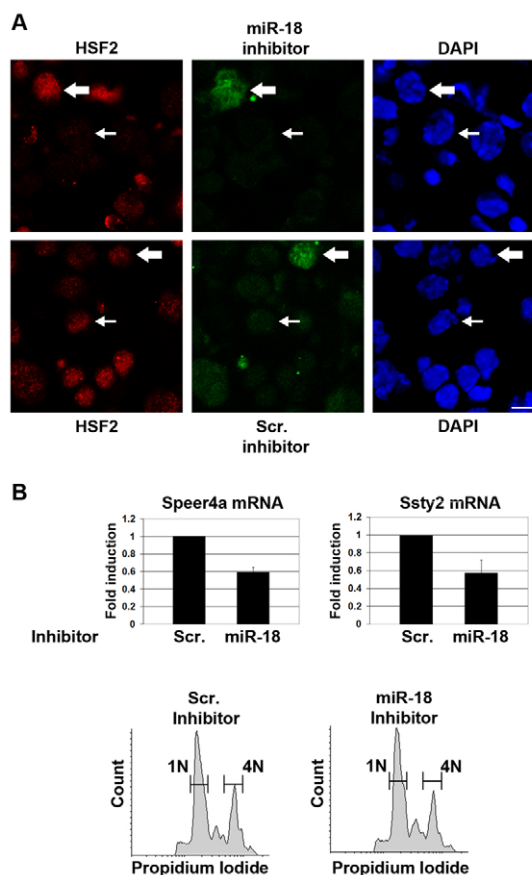
This pattern of expression, exhibiting an inverse correlation between miR-18 and HSF2 (Fig. 5B), strongly suggests that miR-18 targets HSF2 in spermatogenesis.

### 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.



**Fig. 6. HSF2 target genes are repressed upon miR-18 inhibition.**

(A) Squash preparations of seminiferous tubules of stage IX were made as in Fig. S3 in the supplementary material. The image displays different cell types present in stage IX, as identified based on the appearance of the DAPI-stained nuclei. Pachytene spermatocytes transfected with FITC-tagged miR-18 inhibitor or scrambled control (Scr.) are marked with thick arrows and untransfected cells of the same type with thin arrows. HSF2 expression was detected by immunostaining (red). Note that different cell types display different levels of HSF2 protein and that the amount of HSF2 increases upon inhibition of miR-18 specifically in spermatocytes. The images represent a typical experiment. Scale bar: 10  $\mu$ m. (B) Upper panels: real-time RT-PCR analysis of *Speer4a* (left) and *Ssty2* (right) mRNAs in spermatocytes, transfected with the specific miR-18 inhibitor or scrambled control (Scr.). For fold-induction, transcription in the miR-18-inhibited samples was compared with the control samples, arbitrarily set to 1. The data represent at least three biological samples and relative quantities of *Speer4a* and *Ssty2* mRNAs were normalized to  $\beta$ -actin. Error bars indicate s.d. Lower panels: FACS profiles of isolated cells from seminiferous tubules demonstrating that corresponding cell populations were purified for the analysis of cells transfected with the scrambled control or miR-18 inhibitor. The 4N peak corresponds to spermatocytes, among which the FITC-positive cells were collected for real-time RT-PCR analysis. The FACS profiles stem from one representative experiment.

### 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, 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.

#### Acknowledgements

We thank all members of our laboratory for contributions and review of the manuscript. Martti Parvinen, Perttu Terho, Heidi Bergman, Pernilla Stenroos and the laboratory of Javier Martinez are acknowledged for excellent technical assistance. Rosalie Sears is thanked for the pD40-His/V5-c-Myc plasmid. The work was supported by the Academy of Finland, the Sigrid Jusélius Foundation, the Finnish Cancer Organizations and the Åbo Akademi University (L.S.). J.K.B. and A.S. were supported by K. Albin Johansson's Foundation, Lounaisuomalaiset Syöpäjärjestöt, Stiftelsen för Åbo Akademi forskningsinstitut, and J.K.B., A.S. and A.N.E. by the Turku Graduate School of Biomedical Sciences.

## Competing interests statement

The authors declare no competing financial interests.

## Supplementary material

Supplementary material for this article is available at

<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050955/-DC1>

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