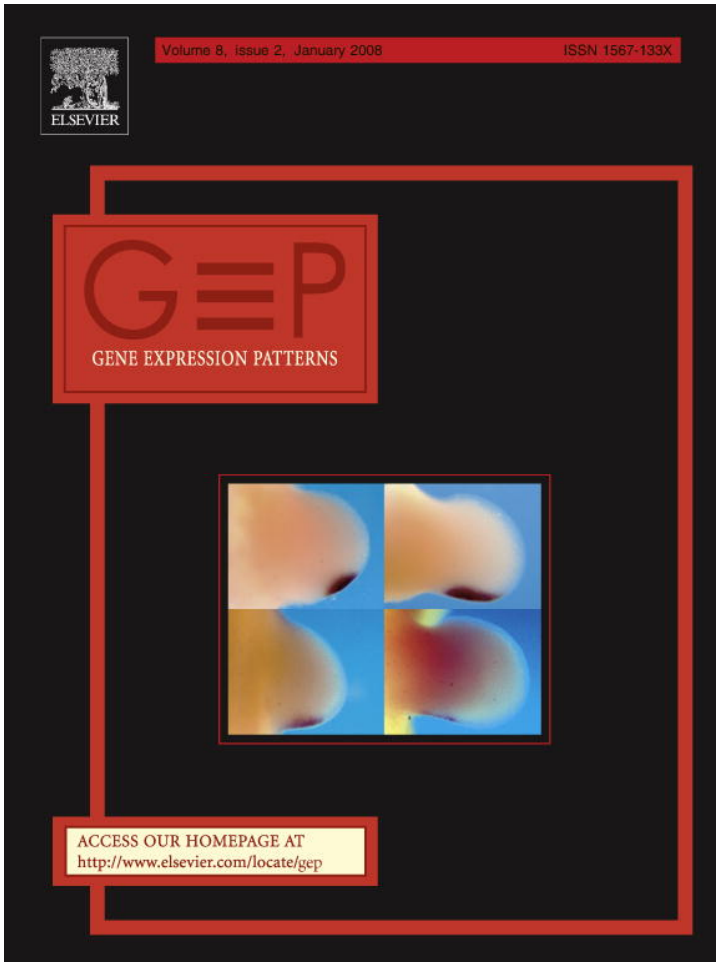

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Transcriptome analysis of differentiating spermatogonia stimulated with kit ligand

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Abstract

Kit ligand (KL) is a survival factor and a mitogenic stimulus for differentiating spermatogonia. However, it is not known whether KL also plays a role in the differentiative events that lead to meiotic entry of these cells. We performed a wide genome analysis of difference in gene expression induced by treatment with KL of spermatogonia from 7-day-old mice, using gene chips spanning the whole mouse genome. The analysis revealed that the pattern of RNA expression induced by KL is compatible with the qualitative changes of the cell cycle that occur during the subsequent cell divisions in type A and B spermatogonia, i.e. the progressive lengthening of the S phase and the shortening of the G2/M transition. Moreover, KL up-regulates in differentiating spermatogonia the expression of early meiotic genes (for instance: Lhx8, Nek1, Rnf141, Xrcc3, Tpo1, Tbca, Xrcc2, Mesp1, Phf7, Rtel1), whereas it down-regulates typical spermatogonial markers (for instance: Pole, Ptgs2, Zfpm2, Egr2, Egr3, Gsk3b, Hnrpa1, Fst, Ptch2). Since KL modifies the expression of several genes known to be up-regulated or down-regulated in spermatogonia during the transition from the mitotic to the meiotic cell cycle, these results are consistent with a role of the KL/kit interaction in the induction of their meiotic differentiation.

Keywords: Kit ligand; Kit receptor tyrosine kinase; Retinoic acid; Bone morphogenetic protein 4; Spermatogonia; Spermatocytes; Cell cycle; Meiosis; Differentiation; DNA microarray; Spermatogenesis

1. Results and discussion

The kit tyrosine-kinase receptor and its ligand, (KL) are essential for the maintenance of primordial germ cells (PGCs) in both sexes. However, kit is known to play important roles also during post-natal stages of spermatogenesis (Sette et al., 2000). In the adult testis, the kit receptor is expressed in differentiating spermatogonia, but not in spermatogonial stem cells, whereas KL is expressed by Sertoli cells under FSH stimulation in both a soluble and a transmembrane form, which are generated by alternative splicing (Rossi et al., 1993). The soluble form of KL stimulates DNA synthesis in type A spermatogonia cultured in vitro, and injection of anti-kit antibodies blocks their proliferation in vivo (Rossi et al., 1993; Packer et al., 1995). A point mutation in the kit gene, which impairs KL-mediated activation of phosphatydilinositol 3-kinase, does not cause any significant reduction in PGCs number during embryonic development, nor in spermatogonial stem cell populations. However, males are completely sterile due to a block in the initial stages of spermatogenesis, associated to abolishment of DNA-synthesis in differentiating A_1–A_4 spermatogonia (Blume-Jensen et al., 2000; Kissel et al., 2000). With the onset of meiosis kit expression ceases, whereas a truncated kit product, tr-kit, a candidate sperm factor acting in egg activation at fertilization, is specifically expressed in post-meiotic stages of spermatogenesis (Sorrentino et al., 1991; Sette et al., 1997). Other Sertoli cell-secreted growth factors known to have direct effects on spermatogonia are glial cell line-derived

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neurotrophic factor (Gdnf), which acts on self-renewal of spermatogonial stem cells and inhibits their differentiation (Meng et al., 2000), and bone morphogenetic protein 4 (Bmp4), which has both a proliferative and differentiative effect on these cells, in which it stimulates kit expression (Pellegrini et al., 2003).

We have previously shown that in vitro addition of soluble KL to kit expressing A_1–A_4 spermatogonia from prepuberal mice stimulates their progression into the mitotic cell cycle and significantly reduces apoptosis in these cells (Dolci et al., 2001). However, it is not known whether, besides stimulating cell divisions and survival, KL also plays a role in the differentiative events which lead to meiotic entry, i.e. the transition from type B spermatogonia to primary spermatocytes, even though it has been reported that mouse germ cells cocultured with the 15-P1 cell line, expressing the transmembrane form of KL, can undergo transmeiotic progression in culture, whereas the soluble form of KL was reported to antagonize this effect (Vincent et al., 1998). Up to now, the only agent which has been postulated to have a (direct or indirect) role in the induction of meiotic entry is all-trans retinoic acid (ATRA) (van Pelt and de Rooij, 1991; Bowles et al., 2006; Koubova et al., 2006). Interestingly, Wang and Culty (2007) have recently found that ATRA stimulates kit expression in prepuberal spermatogonia. In order, to clarify whether kit, besides stimulating spermatogonial proliferation, also plays a direct role in the differentiative program at the onset of spermatogenesis, we performed microarray analysis of the variation in gene expression induced by a 24 h treatment with soluble KL of cultured spermatogonia from 7-day-old mice, a cell populations which is highly enriched in kit expressing cells.

The composition of the cell populations used for each RNA extraction was routinely evaluated by morphology and immunocytochemistry, as indicated in Section 2. The spermatogonial population purified from testes of 7-day-old mice consisted mainly of type A_1–A_4, intermediate and type B spermatogonia together with spermatogonial stem cells, and was contaminated by less than 5% of somatic cells, whereas germ cells in the meiotic prophase were virtually absent, in agreement with previous reports (Pellegrini et al., 2003; Dolci et al., 2001).

Spermatogonia were incubated for 24 h in the presence or absence of 100 ng/ml soluble KL. At the end of the incubation complementary RNAs (cRNAs) were prepared from the two different cell populations and hybridized with commercially available mouse Genome 430 2.0 GeneChip probe arrays (Affymetrix Inc.), containing 45,500 known mouse genes or EST sequences, and thus spanning approximately the whole mouse genome. The analysis was performed on duplicate chip arrays, using cRNAs from two different RNA pools, each obtained from two different culture experiments. Analogous experiments were also performed with other two agents known to be active on spermatogonia, i.e. Bmp4 (100 ng/ml) and ATRA (0.3 μM). The entire set of raw and normalized are available in the Array Express public repository at http://www.ebi.ac.uk/arrayexpress. (Accession No.: E-MEXP-1126).

In statistical analysis, only genes with detection parameter P in both duplicates for one of the two compared conditions (KL-treated and untreated cells) were taken into consideration, and in the subsequent comparative analysis only genes with change parameter I or D in both duplicate experiments were considered. We established a change in gene expression level between treated and untreated cells to be significant only if exceeding 1.5-fold up or down in both experimental duplicates. We chose this relatively low threshold considering that the cell population in the testis of 7-day-old mice is heterogeneous, i.e. approximately 50% of the spermatogonial population is expected to be KL-responsive, based on the percentage of kit-positive cells determined in spermatogonia isolated from 8-day-old mice (Prabhu et al., 2006).

We reasoned that the genes found to be up- or down-regulated by KL treatment assume significance especially when considered not as single genes, but as groups of functionally related genes, even though showing small quantitative changes in their expression level, since small but coordinated changes in gene expression have been shown to clearly distinguish biological phenotypes (Mootha et al., 2003).

Analysis of the data revealed that the transcripts up-regulated at least 1.5-fold by KL treatment in both experiments were 544, while those down-regulated at least 1.5-fold were 504. The complete list of up- and down-regulated genes is available in the first two files of the Supplementary data. The changes in spermatogonial gene expression pattern induced by KL appear to be relatively specific for this growth factor, since only 18% of these genes appeared to be regulated in a similar fashion also by treatment with Bmp4, and 12% also by treatment with ATRA (data available at http://www.ebi.ac.uk/arrayexpress with Accession No. E-MEXP-1126). One hundred and eighty-two of the KL-up-regulated and 128 of the KL-down-regulated transcripts corresponded to EST sequences for genes with unknown functions (unclassified). We grouped the remaining 362 KL-up-regulated and 376 KL-down-regulated transcripts in several functional classes (see the first two files in the Supplementary data for description of the single genes). Validity of the data of microarray analysis was confirmed by performing semi-quantitative (RT-PCR) analysis for randomly selected transcripts on RNA preparations obtained from different cell preparations (Fig. 1). After analysis of the encoded genes several interesting features emerged. We clustered the genes with similar functions and/or known developmental pattern of expression in the germ cell line in four main categories, which are summarized in Tables 1–4 (the indicated value of fold change is the average of the two separate experiments): (1) Cell cycle control; (2) Control of differentiation and meiotic entry; (3) Metabolism; (4) Growth factors, receptors, nucleic acid binding proteins and signal transducers.

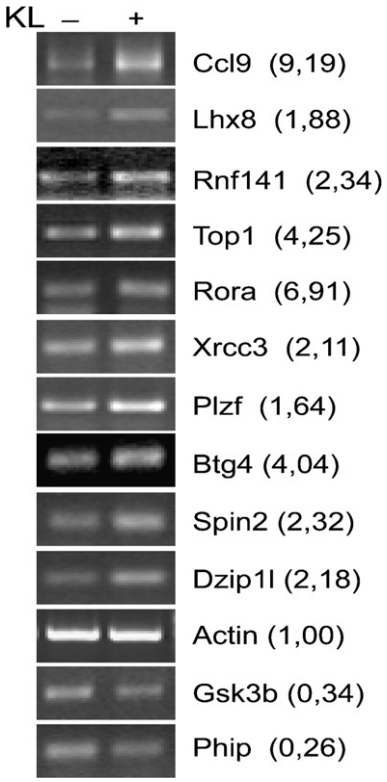

Fig. 1. Representative RT-PCR analysis of RNA from KL-treated and untreated spermatogonia from 7-day-old mice, using specific oligonucleotide primers for 12 of the 115 KL-regulated genes described in the text and in Tables 1–4. RT-PCR analysis was performed at least twice and provided similar results. The full list of the couples of oligonucleotide primers used for RT-PCR analysis of each gene transcript is shown in Section 2. Between parentheses, after the gene names, the average fold changes deduced from the two separate microarray analysis are reported.

1.1. Cell cycle control

Genes involved in cell cycle control up- or down-regulated by KL stimulation are reported in Table 1. Within these genes, three functional groups can be identified, i.e., G1/S transition inhibitors and G2/M promoters, which are up-regulated, and G1/S promoters, which are down-regulated. These data indicate that KL induces in kit expressing spermatogonia variations in their gene expression pattern, which might explain the occurrence of the qualitative changes in their mitotic cell cycle that have been previously described by pioneering autoradiographic studies by Monesi (Monesi, 1962). He showed that the whole duration of the cell cycle is relatively constant, but the average DNA synthetic time (S phase) becomes progressively longer during the subsequent cell divisions from type A to type B spermatogonia, up to the last premeiotic DNA synthesis in pre-leptotene spermatocytes (Monesi, 1962). On the contrary, the average post DNA-synthetic time (G2 phase) becomes progressively shorter during the subsequent cell divisions, and pre-leptotene spermatocytes enter the prophase of the first meiotic division shortly after the completion of the last round of DNA synthesis (Monesi, 1962). The present analysis reveals that, in agreement with the progressive lengthening of the S phase, KL up-regulates the expression of several genes which antagonize or delay G1/S progression, and down-regulates expression of genes which promote or accelerate G1/S progression. At the same time, in agreement with the progressive shortening of the G2/M phase, KL up-regulates the expression of several genes which stimulate mitotic entry and completion of mitosis.

1.2. Control of differentiation and meiotic entry

Genes known as spermatogonial or meiotic markers are reported in Table 2. Between these genes, three functional groups can be identified i.e., spermatogonial and late meiotic markers, which are down-regulated, and early meiotic markers, which are coordinately up-regulated.

These data indicate that stimulation by the soluble form of KL induces in kit expressing spermatogonia changes in their gene expression pattern compatible with the activation of their differentiative program culminating with entry into meiosis after the last round of DNA synthesis in pre-leptotene spermatocytes. Indeed, many genes known to be down-regulated in male germ cells at the onset of meiosis, actually decrease their expression following KL treatment, and, on the contrary, genes which are known markers of the early meiotic stages of spermatogenesis, or that start to be expressed from the lepto-zygotene stage onward, increase their expression after KL treatment.

The down-regulation of Gsk3b is interesting, since its homolog gene in *Saccharomyces cerevisiae*, Rim11p (regulator of inducer of meiosis), is pivotal for the control of meiosis entry in response to nutritional signals (Rubin-Bejerano et al., 2004; Kassir et al., 2003). Moreover, in vitro experiments have shown that Gsk3b inhibitors suppress DNA synthesis selectively in rat preleptotene spermatocytes, suggesting that Gsk3b might be required for the last round of DNA synthesis preceding meiosis (Guo et al., 2003).

The up-regulation of Tbca is also interesting. The precise role of Tbca is still unknown, though findings on Rbl2p (its yeast homolog) suggest that it might play a role in meiosis: Rbl2p has been hypothesized to serve as a b-tubulin storage protein, and to intervene specifically along meiosis but not mitosis (Archer et al., 1995).

Particularly intriguing is also the KL-mediated induction of the homeobox gene Lhx8, a gene highly expressed in the adult testis, in view of its possible role in regulation of spermatogonial differentiation into spermatocytes, downstream from spermatogenesis and oogenesis basic helix–loop–helix transcription factor 1 (Sohlh1) (Pangas et al., 2006; Ballow et al., 2006).

KL influence also the expression of other genes whose homologs play a role in meiosis in other species, even though details about their expression during mammalian spermatogenesis are not yet available. For instance, two DSB repair enzymes Xrcc3 and Xrcc2 are up-regulated by KL treatment. The Arabidopsis and Drosophila homo-

Table 1
Cell cycle control

Functional groups of genes	Target ID	Fold change vs. control	Gene symbol	Description and functional notes
Inhibitors of G1/S transition	1426520_at	4.04	Btg4	Negative regulator of progression through mitotic cell cycle, expressed at high levels in the testis and in oocytes. (Buanne et al., 2000). Also called PC3B
	1457356_at	2.85	Igf2r	Antagonist of Igf2-stimulated growth, known to be expressed in spermatogenic cells (Tsuruta and O'Brien, 1995)
	1426210_x_at	2.67	Parp3	Poly(ADP-ribosyl)transferase. Its overexpression interferes with G1/S transition (Augustin et al., 2003)
	1430164_a_at	2.62	Grb10	Potent growth inhibitor which is a specific endogenous suppressor of Igf-I-stimulated cell signaling and DNA synthesis (Dufresne and Smith, 2005)
	1422574_at	2.45	Mxd4	Myc antagonist and inhibitor of cell cycle entry (Hurlin et al., 1995)
	1431134_at	2.00	Ing5	Its overexpression in cancer cell lines decrease cell population in S phase (Shiseki et al., 2003)
	1427708_a_at	1.96	Nf2 (merlin)	A tumor suppressor which inhibits cell proliferation and cell cycle progression by repressing cyclin D1 expression (Xiao et al., 2005)
	1422477_at	1.82	Cables1	Inhibits cdk2 activity by enhancing phosphorylation of its tyrosine 15 by Wee1 (Wu et al., 2001)
	1441183_at	1.79	Jmjd2b	Member of a family of retinoblastoma-binding proteins which potentiate pRb-mediated repression of E2F-regulated promoters (Gray et al., 2005)
	1438938_x_at	1.73	Phb2	Prohibitin 2. An inhibitor of cell proliferation (Tatsuta et al., 2005)
	1448496_a_at	1.58	Ing1	Negative regulator of cell cycle progression analogous to Ing5 (Garkavtsev et al., 1996)
Promoters of G1/S transition	1422535_at	0.53	Ccne2	Cyclin E2. A well known stimulator of entry into the S phase (Payton and Coats, 2002)
	1435176_a_at	0.51	Id2	Transcription factor known as a positive regulator of cell proliferation and negative regulator of cell differentiation, which causes inappropriate DNA synthesis in meiotic cells when up-regulated in Ovol1-deficient mice (Li et al., 2005)
	1433640_at	0.45	Fubp1	Transcription factor required for c-myc expression and cell proliferation (He et al., 2000)
	1423635_at	0.37	Bmp2	Reported to stimulate spermatogonial proliferation (Puglisi et al., 2004)
	1448254_at	0.32	Ptn	Pleiotropin. A known stimulator of spermatogonial proliferation essential for spermatogenesis (Zhang et al., 1999)
	1451805_at	0.26	Phip	Stimulator of insulin and Igf signaling (Farhang-Fallah et al., 2002)
	1458994_at	0.16	Csnk1g3	Casein kinase isoform whose yeast homologs are essential for cell growth (Zhai et al., 1995)
Promoters of G2/Mtransition	1450073_at	2.45	Kif3b	Kinesin family member important for the proper progression of mitosis (Haraguchi et al., 2006)
	1419092_a_at	2.31	Slk	Required for progression through G2 upstream of H1 kinase activation (O'Reilly et al., 2005)
	1432043_at	2.16	Smu1	Involved directly or indirectly in activation of cdc2 kinase (cdk1), and spindle assembly (Sugaya et al., 2005)
	1427679_at	2.15	Lats1	Promoter of mitotic exit (Bothos et al., 2005)
	1419943_s_at	1.77	Ccnb1	Cyclin B1. A well known inducer of the M phase (Takizawa and Morgan, 2000)
	1448000_at	1.72	Cdca3 (Tome-1)	A cytosolic protein required for proper activation of cdk1/cyclin B and mitotic entry (Ayad et al., 2003)

logs of Xrcc3 play an essential role in meiosis (Bleuyard and White, 2004), and Xrcc2 is known to be expressed at high levels in the mammalian testis, so a possible role in meiosis for this gene has been postulated (Cartwright et al., 1998). A recent report indicates that Xrcc3 plays a role during meiotic events in the mammalian testis (Liu et al., 2007).

The down-regulation of several genes considered to be markers of later stages of the meiotic prophase or of post-meiotic germ cells, but which are not expressed in early meiosis (i.e. in lepto-zygotene spermatocytes), is not conflict with the "pro-meiotic" action of KL in spermatogonia. Indeed, this might be explained if the main action of soluble form of KL is to induce an "early meiotic" phenotype, while inhibiting or delaying further progression of the meiotic prophase, which is actually known to be a lengthy process. Moreover, this would give a rational explanation to the previously reported inhibitory role exerted by the soluble form of KL in the relatively rapid transmeiotic progression of mouse germ cells in vitro (detected as expression of postmeiotic genes), which would be instead stimulated by transmembrane KL (Vincent et al., 1998).

1.3. Metabolism

KL treatment appears to down-regulate in spermatogonia several genes which are important in the control of several anabolic pathways and energy production (Table 3)

Table 2
Control of differentiation and meiotic entry

Functional groups of genes	Target ID	Fold change vs. control	Gene symbol	Description and functional notes
Early meiotic markers	1459672_at	4.25	Top1	DNA topoisomerase 1. Expressed spermatogonia and spermatocytes, with the highest levels in lepto-zygotene (Cobb et al., 1997). Localized in the nuclei of meiotic cells in C. elegans (Lee et al., 2001)
	1434588_x_at	3.17	Tbca	Tubulin cofactor a. Involved in beta-tubulin folding. Abundant in testis, it is progressively up-regulated from the onset of meiosis through spermiogenesis (Fanarraga et al., 1999)
	1432386_a_at	2.51	Phf7	PHD finger protein 7, also called Nyd-sp6. A transcription factor highly expressed in the human testis, and absent in spermatocytic arrest (Xiao et al., 2002). One of the most differentially expressed genes between spermatocytes and spermatogonia in mice (Rossi et al., 2004)
	1425641_at	2.48	Aff1	AF4/FMR2 family, member 1, also called AF5q31, Af4, Rob, Mllt2h, Alf4. A transcriptional regulator essential for male germ cell differentiation and survival (Urano et al., 2005)
	1418466_at	2.34	Rnf141	Ring finger protein 141, also called Zfp36 or Znf230. Abundant in the human testis and absent in azoospermic patients (Zhang et al., 2001). First detected at day 6 after birth in the mouse testis, it reachs adult levels between day 14 and 21 (Qiu et al., 2003)
	1432230_at	2.25	Hip1	Huntingtin interacting protein 1. A protein required for differentiation, proliferation, and/or survival of spermatogenic progenitors (Rao et al., 2001)
	1436354_at	2.18	Dzip1l	DAZ interacting protein 1-like An homolog of Dzip1, a RNA binding protein expressed in pre-meiotic spermatogonia (Curry et al., 2006)
	1452748_at	2.11	Xrcc3	X-ray repair complementing defective repair in Chinese hamster cells 3. A double-strand DNA break (DSB) repair enzyme member of the recA/Rad51 family, which binds to Rad51C, highly expressed in the mammalian testis (Dosanjh et al., 1998)
	1442660_at	2.05	Ypel1	Yippee-like 1. A protein localized to the centrosome, the nucleolus and the mitotic apparatus. Highly expressed within the testis in meiotic cells (Hosono et al., 2004; Maratou et al., 2004)
	1429923_x_at	2.02	Spata3	Spermatogenesis associated 3, also called Tsarg1 A testicular member of the DnaJ/HSP40 protein family, cloned by subtraction with cryptorchid testis (Yang et al., 2005)
	1449912_at	1.90	Ssxb1	Synovial sarcoma, X member B, breakpoint 1. A X-linked gene showing tissue-restricted mRNA expression to testis (Chen et al., 2003)
	1427300_at	1.88	Lhx8	LIM homeobox protein 8. Highly expressed only in the post-natal testis and in fetal oocytes, in which mRNA is detectable after meiotic entry (Ballow et al., 2006; Pangas et al., 2006)
	1454263_at	1.86	Gscl	Goosecoid-like. A transcription factor expressed in both PGCs and in the adult testis (Galili et al., 2000)
	1457708_at	1.85	Mbd4	Methyl-CpG binding domain protein 4. Possibly involved in DNA mismatch repair or in gene silencing imposed by methylated DNA. Expressed in fetal gonads and adult testis (Fukushige et al., 2006)
	1453612_at	1.81	Nek1	NIMA-never in mitosis gene a-related expressed kinase 1. A kinase possibly involved early in the DNA damage response. In the testis is expressed at the beginning of the meiotic phase (Arama et al., 1998; Letwin et al., 1992). Essential for male fertility (Upadhya et al., 2000)
	1426557_at	1.77	Mesp1	Mesoderm posterior 1. A spermatocyte-specific transcription factor absent in spermatogonia (Rossi et al., 2004)
	1455335_at	1.75	Xrcc2	X-ray repair complementing defective repair in Chinese hamster cells 2. DSB repair enzyme member of the recA/RAD51 family, highly expressed in the testis (Cartwright et al., 1998)
	1456729_x_at	1.73	Rtel1	Regulator of telomere elongation helicase 1. An essential gene that regulates telomere length and prevents genetic instability. Highly expressed in the testis, mainly in the spermatogonia and spermatocytes, where it could prevent illegitimate recombination in early meiosis (Ding et al., 2004)
	1427498_a_at	1.66	Spag5	Sperm associated antigen 5. A microtubule-binding protein found in the mitotic spindle (also called Deepest, MAP126, Mastrin, Astrin). It starts to be expressed in the testis when meiosis begins (Shao et al., 2001)
Spermatogonial markers	1428639_at	0.58	Lin9	Required for the expression of the spermatogonial marker B-Myb (Latham et al., 1996; Pilkinton et al., 2007)
	1422655_at	0.57	Ptch2	Patched homolog 2. A receptor for sonic hedgehog, expressed in spermatogonia, and at lower levels in spermatocytes (Szczepny et al., 2006)

Table 2 (*continued*)

Functional groups of genes	Target ID	Fold change vs. control	Gene symbol	Description and functional notes
	1436549_a_at	0.54	Hnrpa1	RNA binding protein expressed in the fetal testis but not in the fetal ovary. After birth it is highly expressed only in early spermatogonia (Kamma et al., 1995)
	1434458_at	0.52	Fst	Follistatin. An activin-binding protein located in type B spermatogonia, and primary spermatocytes, with the exception of the late leptotene and early zygotene stages (Meinhardt et al., 1998)
	1421700_at	0.50	Tex16	Testis expressed gene 16, an X-linked spermatogonial marker (Wang et al., 2001)
	1436329_at	0.49	Egr3	Transcription factor specific for early A spermatogonia (Hamra et al., 2004)
	1448460_at	0.48	Acvr1	Activin A receptor, type 1, also called Alk2 or ActRIA. Expressed in PGCs and in mouse spermatogonia (Puglisi et al., 2004), in which activin is bound with higher intensity with respect to leptotene or zygotene spermatocytes (Woodruff et al., 1992)
	1438921_at	0.45	Atr	Ataxia telangiectasia and rad3 related). A DNA damage checkpoint protein. Expressed in all germ cell types with exception of the early meiotic stages (Bellani et al., 2005)
	1427682_a_at	0.39	Egr2	Transcription factor highly expressed in type A and B spermatogonia and downregulated in preleptotene spermatocytes (Guo et al., 2004)
	1439949_at	0.34	Gsk3b	In mice, it is expressed in type B spermatogonia and pre-leptotene spermatocytes and is down-regulated upon meiotic entry in both mice and rats (Guo et al., 2003)
	1419012_at	0.24	Zfpm2	Zinc finger protein, also called Fog-2, which is expressed in the newborn mouse testis in gonocytes and disappears after the first week of testicular development (Ketola et al., 2002)
	1457582_at	0.18	Uty	Ubiquitously expressed Y-linked gene encoding a histocompatibility antigen, which is not expressed in differentiating germ cells of the adult testis (Warren et al., 2000)
	1417262_at	0.17	Ptgs2	Prostaglandin-endoperoxide synthase 2, also known as Cox-2. Its expression in rat testis has been primarily localized in spermatogonial cells (Neeraja et al., 2003). It is not expressed in the adult human testis, but strongly up-regulated in testicular cancer (Hase et al., 2003)
	1441854_at	0.13	Pole	DNA polymerase epsilon. Detectable in mitotically proliferating spermatogonia but not in the early stages of meiotic prophase (Kamel et al., 1997)
	1443831_s_at	0.13	Mtl5 (Tesmin)	Testis-specific gene, whose transcription starts to accumulate in 8-day-old mouse germ cells (Sugihara et al., 1999), and is detectable postnatally in all stages of meiotic prophase I except preleptonema and leptonema (Olesen et al., 2004)
Late meiotic and post-meiotic markers	1434229_a_at	0.62	Polb	DNA polymerase beta, a DNA repair enzyme in pachytene spermatocytes and post meiotic male mouse germ cells (Hirose et al., 1989)
	1437693_at	0.62	D1Pas1	Expressed in pachytene spermatocytes and haploid cells, in which it replaces the spermatogonial and early meiotic expression of the homologous Y-linked Dby - Ddzx3 gene (Session et al., 2001)
	1437313_x_at	0.58	Hmgb2	Highly expressed in pachytene spermatocytes and early spermatids. Its disruption causes sterility in male mice (Ronfani et al., 2001)
	1441948_x_at	0.54	Zfand3, Tex27	Also called Tex27. A postmeiotic germ cell marker (de Luis et al., 1999)
	1440500_at	0.53	Map3k10	Also called mixed lineage kinase (Mlk2), expressed in late pachytene spermatocytes and spermatids (Phelan et al., 1999)
	1419772_at	0.15	Mllt10	Myeloid/lymphoid or mixed lineage-leukemia translocation to 10 homolog), highly expressed in the testis in postmeiotic cells (Linder et al., 1998)

[no specific references are given for these genes, since description of their metabolic function can be found in the free databases at the link http://www.informatics.jax.org]. The KL-mediated down-regulation of the expression of several metabolic genes in spermatogonia is an. apparent paradox for the action of a growth factor, since these genes encode for proteins involved in nutrient transport (amino acids, oxygen, glucose), nucleotide and protein biosynthesis and energy production through the Krebs cycle and the respiratory chain. However, it is worthy to remind that starvation from nitrogen and carbon nutrient sources is the environmental signal which induces the switch from the mitotic to the meiotic cell cycle in yeast. Indeed, in *S. cerevisiae*, nutritional signaling pathways converge on transcriptional activation of the Ime1 gene, which encodes a transcription factor that, in turn, triggers initiation of meiosis, by turning off the expression of genes required for the mitotic cell cycle, while activating the expression of other genes which are crucial for execution of the meiotic program (Kassir et al., 2003). Thus, it is tempting to speculate that one of the effects of KL/kit interaction in differentiating spermatogonia is to establish progressively, during their mitotic divisions, a sort of endogenous state of nutritional stress, which might be the ancestrally inherited trigger for transcription of a master transcriptional regulator of meiosis.

Table 3
Metabolism

Functional groups of genes	Target ID	Fold change vs. control	Gene symbol	Description and functional notes
Nucleotide biosynthesis	1439012_a_at	0.64	Dck	Involved in purine and pyrimidine biosynthesis
	1416319_at	0.60	Adk	Involved in purine and pyrimidine biosynthesis
	1435277_x_at	0.32	Nme1	Involved in purine and pyrimidine biosynthesis
	1442353_at	0.30	Itpa	Involved in purine and pyrimidine biosynthesis
Metabolite transport	1434897_a_at	0.62	Slc25a4	Intramitochondrial transporter
	1437052_s_at	0.62	Slc2a3	Glucose transporter
	1454622_at	0.57	Slc38a5	Transporter for both oxygen and amino acids
	1420504_at	0.41	Slc6a14	Amino acid transporter
	1437069_at	0.41	Osbpl8	Oxysterol binding protein-like 8. Involved in lipid transport and steroid metabolism
Energy production	1423159_at	0.60	Dld	Part of the of the energy-transfer system of the respiratory chain
	1451084_at	0.59	Etfdh	Part of the of the energy-transfer system of the respiratory chain
	1422478_a_at	0.58	Acas2	Enzyme which produces acetyl-CoA mainly for the oxidation of acetate
	1423747_a_at	0.53	Pdk1	A pyruvate dehydrogenase kinase isoform known to be repressed under starvation
	1456573_x_at	0.46	Nnt	Part of the of the energy-transfer system of the respiratory chain
	1423621_a_at	0.42	Slc33a1	Acetyl-CoA transporter
	1422500_at	0.42	Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha. A key enzyme which catalyze the allosterically regulated rate-limiting step of the tricarboxylic acid cycle, required to utilize acetate as a carbon source
	1416617_at	0.29	Acas2l	Enzyme which produces acetyl-CoA mainly for the oxidation of acetate
Protein synthesis	1429317_at	0.41	Qrsl1	Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1. Involved in protein biosynthesis

1.4. Growth factors, receptors, nucleic acid binding proteins and signal transducers

This wide category of genes is summarized in Table 4 [references are given only for genes playing a known or potential role in the spermatogenic process; in the other cases, references can be found in the free databases at the link http://www.informatics.jax.org].

An interesting information coming from this set of data is that KL, which is known as a mitogenic and survival factor for differentiating spermatogonia (Rossi et al., 1993; Dolci et al., 2001) up-regulates the expression of ligands and receptors of the Tnf superfamily that might be involved in regulating the number of germ cells entering the first wave of spermatogenesis, which is characterized by an early and massive wave of apoptosis in the spermatocyte compartment (Rodriguez et al., 1997). The induction of Plzf, a known marker of spermatogonial stem cells (Buaas et al., 2004; Costoya et al., 2004), is in apparent conflict with the above described pro-differentiative pattern of gene expression set up by KL treatment of spermatogonia. Indeed, Plzf is expressed in gonocytes, and in undifferentiated spermatogonia, but it is thought not to be expressed in kit positive spermatogonia in the adult testis (Oatley et al., 2006). However, Plzf is co-expressed with kit in a subset of spermatogonial stem cells during the first wave of spermatogenesis (Naughton et al., 2006). Moreover, recent data in our laboratory indicate that one of the mechanisms through which Plzf maintains the stem cell phenotype is the suppression of kit expression (Filipponi et al., 2007). Thus, Plzf induction by KL in Plzf/kit co-expressing spermatogonia during the first wave of spermatogenesis might contribute to the definitive cessation of kit expression in these cells, which is required to establish the pool of spermatogonial stem cells in the adult testis (Naughton et al., 2006). Another possibility is that a transient Plzf induction by KL in differentiating spermatogonia might contribute to the cessation of kit expression which is known to occur at the onset of meiosis (Sorrentino et al., 1991). Finally, among the list of KL down-regulated genes encoding nucleic acid binding proteins, we noticed the gene encoding the RNA binding protein Rod1, which might be involved in the negative control of meiosis. Indeed, the Rod1 homolog gene in the fission yeast *Schizosaccharomyces pombe* [nrd1(+)] negatively regulates the onset of meiotic differentiation (its biological role is to block differentiation by repressing a subset of the Ste11-regulated genes, essential for conjugation and meiosis until the cells reach a critical level of nutrient starvation). Interestingly, mammalian Rod1 can complement yeast nrd1 defects (Yamamoto et al., 1999).

1.5. Concluding remarks

One should consider that, in view of the fact that after 24 h of culture the difference in the percentage of apoptotic cells between KL-treated and. untreated cells is 17% vs. 38% (Dolci et al., 2001), the possibility exists that changes of gene expression levels that we observed could be ascribed at least in part to variation of the relative content of live vs. apoptotic cells in KL-treated and untreated cells. Indeed, we did not perform treatments longer than 24 h, since the high rate of apoptosis in control cells would have impaired the significance of the changes in the pattern of

Table 4
Growth factors, receptors, nucleic acid binding proteins and signal transducers

Functional groups of genes	Target ID	Fold change vs. control	Gene symbol	Description and functional notes
Nucleic acid binding proteins	1457177_at	6.91	Rora	RAR-related orphan receptor alpha. A nuclear receptor activated by stress (hypoxia) and binding cholesterol derivatives (Jakel et al., 2006). Strongly expressed in the mouse testis in cells at the periphery of seminiferous tubules (Steinmayr et al., 1998)
	1427638_at	1.64	Zbtb16 (Plzf)	Transcription factor co-expressed with Oct4 in gonocytes and undifferentiated spermatogonia, whose knock-out causes stem cell depletion in the testis (Buaas et al., 2004; Costoya et al., 2004)
	1449978_at	1.59	Zfy2	Y-linked zinc finger protein
	1420759_s_at	0.60	Zfy1	Y-linked zinc finger protein
	1424084_at	0.35	Rod1	Regulator of differentiation 1. A RNA binding protein, whose homolog in the fission yeast *S. pombe* negatively regulates the onset of meiotic differentiation (Yamamoto et al., 1999)
Growth factors and cytokines	1417936_at	9.19	Ccl9	Chemokine (C–C motif) ligand 9, also called MIP-1 gamma. A cytokine which shares the same receptors with Ccl3 (MIP 1-alpha), which increases in vitro DNA synthesis of type A_1–A_4 spermatogonia and of pre-leptotene spermatocytes but inhibits DNA synthesis in intermediate and type B spermatogonia (Hakovirta et al., 1994)
	1459913_at	2.63	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10, also called Tl2, or Trail, or Apo-2L. Expressed in all human and rat testicular cells (Grataroli et al., 2002), it induces apoptosis in germ cells, specifically spermatocytes, (McKee et al., 2006)
	1459947_at	2.09	Bmp6	An oocyte-secreted growth factor (Hussein et al., 2005)
	1439959_at	0.62	Fgf11	Growth factor of the FGF family
	1419123_a_at	0.62	Pdgfc	Growth factor of the PDGF family
	1437270_a_at	0.47	Clcf1	Cardiotrophin-like cytokine factor 1. A gp-130 ligand of the IL-6 family, which have been proposed as inhibitors of transition into meiosis in the fetal testis trough in vitro studies (Chuma and Nakatsuji, 2001)
Receptors	1446875_at	12.43	Il1rapl2	Interleukin 1 receptor accessory protein-like 2. A X-linked gene also called IL-1R9, IL1R9, TIGIRR-1
	1455689_at	3.12	Fzd10	Frizzled homolog 10. A receptor for ligands of the WNT family of signaling proteins
	1440085_at	2.00	Eda2r	Ectodysplasin A2 isoform receptor, also called Xedar (X linked ectodysplasin receptor). A tumor necrosis factor (Tnf) receptor superfamily member
	1449149_at	1.94	Traf3	Tnf receptor-associated factor 3, a co-receptor of the Tnf family
	1448167_at	0.56	Ifngr1	An interferon gamma receptor
	1429216_at	0.56	Paqr3	Progestin and adipoQ receptor family member III. A membrane progesterone receptor
	1418723_at	0.53	Edg7	Member of family I of the G protein-coupled receptors functioning as a lysophosphatidic acid (LPA) receptor and contributing to Ca2+ mobilization
	1442291_at	0.43	Edg4	Member of family I of the G protein-coupled receptors functioning as a lysophosphatidic acid (LPA) receptor and contributing to Ca2+ mobilization
Signal transducers	1424881_at	2.58	Trib1	Tribbles homolog 1. It is the homolog of a cell cycle regulator which affects the number of germ cell divisions as well as oocyte determination in Drosophila (Mata et al., 2000)
	1417784_at	2.52	Als2	A Rho/Rab/Rac activator which plays an anti-apoptotic role in neuronal cells
	1420539_a_at	2.50	Chrdl2	Chordin-like 2. A regulator of BMP signaling
	1439467_at	2.40	Mtap4	Microtubule-associated protein 4. Involved in the changes in microtubule dynamics or function that are known to accompany differentiation
	1455297_at	2.32	Spin2	Spindlin family, member 2—spindlin-like. A X-linked gene, cloned through a functional retroviral cDNA library-based screen to identify genes that prevent growth factor withdrawal-mediated apoptosis (Fletcher et al., 2002)
	1418332_a_at	1.86	Agtpbp1	ATP/GTP binding protein 1, also called Pcd (Purkinje cell degeneration) or Nna1. A gene essential for normal spermatogenesis (Fernandez-Gonzalez et al., 2002)
	1435647_at	1.70	Ikbkg	Inhibitor of kappaB kinase gamma. An inhibitor of TNF-induced apoptosis
	1460304_a_at	1.55	Ubtf	Upstream binding transcription factor, RNA polymerase I. A transcription factor downregulaed by Gsk3b in myeloid cells
	1423389_at	0.58	Smad7	An inhibitor of signaling from TGF-beta family members
	1423176_at	0.57	Tob1	Transducer of ErbB-2.1. A negative regulator of BMP signaling
	1423350_at	0.49	Socs5	Negative regulator of kit signaling
	1450870_at	0.48	Rala	V-ral simian leukemia viral oncogene homolog A—ras related. An activator of PLD, which is activated in response to mitogenic stimulation
	1425514_at	0.34	Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1. Also called Grb1 or p85 alpha, it is the regulatory subunit of PI3 kinase and the mediator of tyrosine-kinase-mediated PI3K signaling

gene expression. However, if the changes in the pattern of gene expression after a 24 h incubation depended just on the variation in the proportion of live cells between KL-treated and untreated samples, one would expect a widespread variation in gene expression, whereas we found that 80% of expressed genes (i.e. those with a P detection parameter) did not show quantitative variations after KL treatment. Moreover, we had previously performed preliminary duplicate microarray experiments, using different, less representative, Affymetrix gene chips (MG-U74A), after a KL treatment of only 4 h, a time at which no change in the rate of live vs. apoptotic cells in control vs. KL-treated cells is observed (see the third file in the Supplementary data). As expected, using a 1.5-fold change threshold, we could not detect significant changes in the rate of gene expression after such a short incubation time for most of the 12,500 genes represented in these arrays. However, in a retrospective analysis, we have compared these data with the results obtained with the 24 h treatment. When considering the absolute values, we noticed that a 4 h KL treatment induces an increase for some of the genes that we classified as G1/S inhibitors (Btg4, fold change vs.control: 3.44), meiotic markers (Top1: 1.37; Phf7: 1.50; Lhx8: 1.47; Nek1: 2.52) and transcription factors (Rora: 3.44), and a decrease for some of the genes that we classified as signal transducers (Rala: 0.49) and spermatogonial markers (Egr2: 0.72; Lin9: 0.65). Thus, the trend of the changes in the pattern of gene expression which is observed after 24 h of culture is already present after a short-time incubation with KL.

Overall, the present study suggests that the soluble form of KL, beside being a positive regulator of cell divisions and survival in kit expressing type A spermatogonia, also modifies the characteristics of their cell cycle and stimulates their entry into the meiotic program. Even though further studies, extended at the post-transcriptional and proteome level, are required to fully evaluate the effects of KL stimulation in spermatogonia, this transcriptome analysis suggest that KL plays an important and direct role in male germ cell differentiation that leads to the establishment of spermatogenesis.

2. Experimental procedures

2.1. Isolation and culture of spermatogonia

Germ cell populations highly enriched in mitotic spermatogonia were obtained as previously described from testes of 7-day-old mice (Rossi et al., 1993; Pellegrini et al., 2003; Dolci et al., 2001; Rossi et al., 2004). Briefly, germ cell suspensions were obtained by sequential collagenase–hyaluronidase–trypsin digestions of freshly withdrawn testes. A 3 h period of culture in E-MEM additioned with 10% FCS was performed to facilitate adhesion of contaminating somatic cells to the plastic dishes. At the end of this pre-plating treatment, enriched mitotic germ cell suspensions were rinsed from FCS and incubated for 24 h in the presence or absence of 100 ng/ml soluble KL (R&D Systems, Minneapolis, MN), or 100 ng/ml Bmp4 (R&D Systems, Minneapolis, MN), or 0.3 μM ATRA (Sigma Aldrich, Milan, Italy). Purity of 7 dpn spermatogonia was about 90% after the pre-plating treatment, and 95% after 24 h of culture. The homogeneity of the cell populations was assessed through both morphological criteria and by specific immunostaining with antibodies directed against three specific markers of mitotic germ cells, which are not expressed in testicular somatic cells (Smad5, Alk3 and kit), as described previously (Pellegrini et al., 2003).

2.2. RNA extraction, cDNA and cRNA preparation

Total RNA was extracted using TRIzol Reagent (Gibco), followed by clean up on RNeasy mini/midi columns (RNeasy Mini/Midi Kit, Qiagen). For each cell treatment (control, KL, Bmp4 and ATRA) two separate RNA pools were prepared, each of which was obtained from cell preparations from two separate cultures. Biotin-labelled cRNA targets were synthesized starting from 5 μg of total RNA. Double stranded cDNA synthesis was performed with GIBCO SuperScript Custom cDNA Synthesis Kit, and biotin-labelled antisense RNA was transcribed in vitro using Ambion's In Vitro Transcription System, including Bio-11-UTP and Bio-11-CTP (NEN Life Sciences, PerkinElmer Inc., Boston, Massachusetts, USA) in the reaction. All steps of the labelling protocol were performed as suggested by Affymetrix (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

The size and the accuracy of quantitation of targets were checked by agarose gel electrophoresis of 2 μg aliquots, prior to and after fragmentation. After fragmentation, targets were diluted in hybridisation buffer at a concentration of 150 μg/ml.

2.3. DNA microarray hybridization

Hybridization mix for target dilution (100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20) was prepared as indicated by Affymetrix, including pre-mixed biotin-labelled control oligo B2 and bioB, bioC, bioD and cre controls (Affymetrix cat #900299) at a final concentration of 50 pM, 1.5 pM, 5 pM, 25 pM and 100 pM, respectively. Targets were diluted in hybridization buffer at a concentration of 150 μg/ml and denatured at 99 °C prior to introduction into the GeneChip cartridge. A single GeneChipMOE430 v2.0 was then hybridized with each biotin-labelled target. For each of the four experimental conditions tested (control, KL, Bmp4, ATRA), two GeneChipMOE430 v2.0 were used for a total number of 8 GeneChip arrays.

Hybridizations were performed for 16 h at 45 °C in a rotisserie oven. GeneChip cartridges were washed and stained in the Affymetrix fluidics station following the EukGE-WS2 standard protocol (including Antibody Amplification), including the following steps: (1) (wash) 10 cycles of 2 mixes/cycle with Wash Buffer A (6X SSPE, 0.01% Tween 20) at 25 °C; (2) (wash) 4 cycles of 15 mixes/cycle with Wash Buffer B (100 mM MES, 0.1 M [Na+], 0.01% Tween 20) at 50 °C; (3) staining of the probe array for 10 min in SAPE solution (10 μg/mL SAPE in 100 mM MES, 1 M [Na+], 0.05% Tween 20, 2 mg/mL BSA) at 25 °C; (4) (wash) 10 cycles of 4 mixes/cycle with Wash Buffer A at 25 °C; (5) staining of the probe array for 10 min in antibody solution (Normal Goat IgG 0.1 mg/mL; (6) addition of the biotinylated antibody 3 μg/mL, 100 mM MES, 1 M [Na+], 0.05% Tween 20, 2 mg/mL BSA) at 25 °C; (7) staining of the probe array for 10 min in SAPE solution at 25 °C; (8) (final wash) 15 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C.

2.4. Scanning and bioinformatic analysis

Images were scanned using an Affymetrix GeneChip Scanner3000 7G, using default parameters. The resulting images were analysed using GeneChip Operating Software v1.2 (GCOS1.2).

"Absolute analysis" was performed for each chip with GCOS1.2 software using default parameters, scaling signal intensity global trimmed mean of all images to a value of 500. Report files were extracted for each chip, and performance of labelled targets was evaluated on the basis of several values (scaling factor, background and noise values, % present calls, average signal value, etc.). Further data analysis was performed using both GenePicker, a proprietary software developed by Giacomo

Finocchiaro and Heiko Muller (http://bioinformatics.oxfordjournals.org/cgi/reprint/bth416v1) and GeneSpring GX v.7.3.1 (Agilent Technologies), a commercial software for analysis of microarrays data, with the following criteria.

2.4.1. GenePicker analysis

(1) Samples were compared two by two; signal intensity values from first condition were compared to signal intensity values from second condition, for each of the four conditions; (2) in addition signal intensities from single condition were used for regulated genes selection; (3) starting from the complete list of probesets represented on the GeneChip arrays, a fold change criteria was used to select probesets regulated; (4) transcripts showing both a regulation call from the GCOS algorithm (I or D for increase and decrease) and a fold change higher that the cutoff value were positively selected as being regulated. The cutoff value was set to 1.5. These list of regulated probesets, were furtherly refined by applying a statistical parametric test, with p-value cutoff set to 0.05 without multiple testing correction.

2.4.2. GeneSpring analysis

(1) Starting from the list of all the probesets represented on the GeneChipMOE430 v2.0, an initial selection was performed based on the "Flag" parameter generated by the GCOS software; (2) to avoid the selection of false positives, a reduction of transcripts included in the starting genelist was achieved, accordingly to the "flag" parameter associated with each transcript; (3) probesets showing a "Present" flag in both replicate of at least one experimental condition were included in the genelist, thus reducing the number of probesets (P in 2_2 either conditions); (4) starting from this genelist, each condition was compared to the others using normalized expression values and setting the fold change cutoff to 1.5, providing several genelists of regulated probesets. These list of regulated probesets, were further refined by applying a statistical parametric test, with variances not assumed to be equal (Welch t-test) and p-value cutoff set to 0.05 without multiple testing correction.

2.5. RT-PCR analysis

RT-PCR amplification of selected mRNAs (shown in Fig. 1), was performed by using specific oligonucleotide primers designed on the basis of the sequence of the corresponding Affymetrix target genes. Specificity of the primers was previously controlled through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/). The full list of the primers used follows:

Ccl9: ATGGCTTGGTGGTTAAGAGCACC (Forward); ACCAGA CACATTCCAAAGTCCCA (Reverse). Amplification product 300 bp
Lhx8: GTCTGGAGATAGTTGGCTCGAGT (Forward); GGATGG TAGGCTTTGTAAACTAG (Reverse). Amplification product 357 bp
Rnf141: TGCTGTTGTCTGTCATACCTTAG (Forward); TAGGTA AGCACTCTACCAACTGA (Reverse). Amplification product 382 bp
Top1: GTAACAGCTATAGCACCGGAGTG (Forward); CTCCTC CAAATAAGTCAAGAGTG (Reverse). Amplification product 284 bp
Rora: CTGCAATGTTCTCAGAGCTCCAG (Forward); CCACACC CTCACATAACAGTCCT (Reverse). Amplification product 283 bp
Xrcc3: ATCCAGGCTGGAAGGCACCACAA (Forward); CCTGGT TTAAGGAGTCAGACACT (Reverse). Amplification product 264 bp
Plzf: AGTTCAGCCTCAAGCACCAGTTG (Forward); GTAGAGG TACGTCTTCTCTATCC (Reverse). Amplification product 263 bp
Btg4: AAGCATTTGGCAGATGGTCGTGG (Forward); TGTGCT CACATTCCTGAAGCAGG (Reverse). Amplification product 252 bp
Spin2: GTACATGTATCAGCTTCTGGATG (Forward); ATGAAG TACACAGAAGGCTTGGC (Reverse). Amplification product 207 bp
Dzip1l: GTGAACAATCTAGACAATCCCTC (Forward); AAGAG GCTTCATCCAGATTGCTC (Reverse). Amplification product 222 bp
Gsk3b: TTCCTGCTGACACACGATGGAGC (Forward); TCCATT CCCACTTGGAAAGTATC (Reverse). Amplification product 284 bp
Phip: GTAATTTCCTGATGTTATCTGCA (Forward); TTTCCTTG TTATAAGAGAGATGG (Reverse). Amplification product 190 bp

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.modgep.2007.10.007.

References

Arama, E., Yanai, A., Kilfin, G., Bernstein, A., Motro, B., 1998. Murine NIMA-related kinases are expressed in patterns suggesting distinct functions in gametogenesis and a role in the nervous system. Oncogene 16, 1813–1823.

Archer, J.E., Vega, L.R., Solomon, F., 1995. Rbl2p, a yeast protein that binds to beta-tubulin and participates in microtubule function in vivo. Cell 82, 425–434.

Augustin, A., Spenlehauer, C., Dumond, H., Menissier-De Murcia, J., Piel, M., Schmit, A.C., Apiou, F., Vonesch, J.L., Kock, M., Bornens, M., De Murcia, G., 2003. PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. J. Cell Sci. 116, 1551–1562.

Ayad, N.G., Rankin, S., Murakami, M., Jebanathirajah, J., Gygi, S., Kirschner, M.W., 2003. Tome-1, a trigger of mitotic entry, is degraded during G1 via the APC. Cell 113, 101–113.

Ballow, D., Meistrich, M.L., Matzuk, M., Rajkovic, A., 2006. Sohlh1 is essential for spermatogonial differentiation. Dev. Biol. 294, 161–167.

Bellani, M.A., Romanienko, P.J., Cairatti, D.A., Camerini-Otero, R.D., 2005. SPO11 is required for sex-body formation, and Spo11 heterozygosity rescues the prophase arrest of Atm−/− spermatocytes. J. Cell Sci. 118, 3233–3245.

Bleuyard, J.Y., White, C.I., 2004. The Arabidopsis homologue of Xrcc3 plays an essential role in meiosis. EMBO J. 23, 439–449.

Blume-Jensen, P., Jiang, G., Hyman, R., Lee, K.F., O'Gorman, S., Hunter, T., 2000. Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility. Nat. Genet. 24, 157–162.

Bothos, J., Tuttle, R.L., Ottey, M., Luca, F.C., Halazonetis, T.D., 2005. Human LATS1 is a mitotic exit network kinase. Cancer Res. 65, 6568–6575.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M.J., Rossant, J., Hamada, H., Koopman, P., 2006. Retinoid signaling determines germ cell fate in mice. Science 312, 596–600.

Buaas, F.W., Kirsh, A.L., Sharma, M., McLean, D.J., Morris, J.L., Griswold, M.D., de Rooij, D.G., Braun, R.E., 2004. Plzf is required in adult male germ cells for stem cell self-renewal. Nat. Genet. 36, 647–652.

Buanne, P., Corrente, G., Micheli, L., Palena, A., Lavia, P., Spadafora, C., Lakshmana, M.K., Rinaldi, A., Banfi, S., Quarto, M., Bulfone, A., Tirone, F., 2000. Cloning of PC3B, a novel member of the PC3/BTG/TOB family of growth inhibitory genes, highly expressed in the olfactory epithelium. Genomics 68, 253–263.

Cartwright, R., Tambini, C.E., Simpson, P.J., Thacker, J., 1998. The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family. Nucleic Acids Res. 26, 3084–3089.

Chen, Y.T., Alpen, B., Ono, T., Gure, A.O., Scanlan, M.A., Biggs 3rd, W.H., Arden, K., Nakayama, E., Old, L.J., 2003. Identification and characterization of mouse SSX genes: a multigene family on the X chromosome with restricted cancer/testis expression. Genomics 82, 628–636.

Chuma, S., Nakatsuji, N., 2001. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. Dev. Biol. 229, 468–479.

Cobb, J., Reddy, R.K., Park, C., Handel, M.A., 1997. Analysis of expression and function of topoisomerase I and II during meiosis in male mice. Mol. Reprod. Dev. 46, 489–498.

Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J., Pandolfi, P.P., 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat. Genet. 36, 653–659.

Curry, B.J., Holt, J.E., McLaughlin, E.A., Aitken, R.J., 2006. Characterization of structure and expression of the Dzip1 gene in the rat and mouse. Genomics 87, 275–285.

de Luis, O., Lopez-Fernandez, L.A., del Mazo, J., 1999. Tex27, a gene containing a zinc-finger domain, is up-regulated during the haploid stages of spermatogenesis. Exp. Cell Res. 249, 320–326.

Ding, H., Schertzer, M., Wu, X., Gertsenstein, M., Selig, S., Kammori, M., Pourvali, R., Poon, S., Vulto, I., Chavez, E., Tam, P.P., Nagy, A., Lansdorp, P.M., 2004. Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. Cell 117, 873–886.

Dolci, S., Pellegrini, M., Di Agostino, S., Geremia, R., Rossi, P., 2001. Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. J. Biol. Chem. 276, 40225–40233.

Dosanjh, M.K., Collins, D.W., Fan, W., Lennon, G.G., Albala, J.S., Shen, Z., Schild, D., 1998. Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes. Nucleic Acids Res. 26, 1179–1184.

Dufresne, A.M., Smith, R.J., 2005. The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling. Endocrinology 146, 4399–4409.

Fanarraga, M.L., Parraga, M., Aloria, K., del Mazo, J., Avila, J., Zabala, J.C., 1999. Regulated expression of p14 (cofactor A) during spermatogenesis. Cell Motil. Cytoskeleton 43, 243–254.

Farhang-Fallah, J., Randhawa, V.K., Nimnual, A., Klip, A., Bar-Sagi, D., Rozakis-Adcock, M., 2002. The pleckstrin homology (PH) domain-interacting protein couples the insulin receptor substrate 1 PH domain to insulin signaling pathways leading to mitogenesis and GLUT4 translocation. Mol. Cell. Biol. 22, 7325–7336.

Fernandez-Gonzalez, A., La Spada, A.R., Treadaway, J., Higdon, J.C., Harris, B.S., Sidman, R.L., Morgan, J.I., Zuo, J., 2002. Purkinje cell degeneration (pcd) phenotypes caused by mutations in the axotomy-induced gene, Nna1. Science 295, 1904–1906.

Filipponi, D., Hobbs, R.M., Ottolenghi, S., Rossi, P., Jannini, E.A., Pandolfi, P.P., Dolci, S., 2007. Repression of kit expression by Plzf in germ cells. Mol. Cell. Biol. 30 (Epub ahead of print).

Fletcher, B.S., Dragstedt, C., Notterpek, L., Nolan, G.P., 2002. Functional cloning of SPIN-2, a nuclear anti-apoptotic protein with roles in cell cycle progression. Leukemia 16, 1507–1518.

Fukushige, S., Kondo, E., Gu, Z., Suzuki, H., Horii, A., 2006. RET finger protein enhances MBD2- and MBD4-dependent transcriptional repression. Biochem. Biophys. Res. Commun. 351, 85–92.

Galili, N., Nayak, S., Epstein, J.A., Buck, C.A., 2000. Rnf4, a RING protein expressed in the developing nervous and reproductive systems, interacts with Gscl, a gene within the DiGeorge critical region. Dev. Dyn. 218, 102–111.

Garkavtsev, I., Kazarov, A., Gudkov, A., Riabowol, K., 1996. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat. Genet. 14, 415–420.

Grataroli, R., Vindrieux, D., Gougeon, A., Benahmed, M., 2002. Expression of tumor necrosis factor-alpha-related apoptosis-inducing ligand and its receptors in rat testis during development. Biol. Reprod. 66, 1707–1715.

Gray, S.G., Iglesias, A.H., Lizcano, F., Villanueva, R., Camelo, S., Jingu, H., The, B.T., Koibuchi, N., Chin, W.W., Kokkotou, E., Dangond, F., 2005. Functional characterization of JMJD2A, a histone deacetylase- and retinoblastoma-binding protein. J. Biol. Chem. 280, 28507–28518.

Guo, R., Yu, Z., Guan, J., Ge, Y., Ma, J., Li, S., Wang, S., Xue, S., Han, D., 2004. Stage-specific and tissue-specific expression characteristics of differentially expressed genes during mouse spermatogenesis. Mol. Reprod. Dev. 67, 264–272.

Guo, T.B., Chan, K.C., Hakovirta, H., Xiao, Y., Toppari, J., Mitchell, A.P., Salameh, W.A., 2003. Evidence for a role of glycogen synthase kinase-3 beta in rodent spermatogenesis. J. Androl. 24, 332–342.

Hakovirta, H., Vierula, M., Wolpe, S.D., Parvinen, M., 1994. MIP-1 alpha is a regulator of mitotic and meiotic DNA synthesis during spermatogenesis. Mol. Cell. Endocrinol. 99, 119–124.

Hamra, F.K., Schultz, N., Chapman, K.M., Grellhesl, D.M., Cronkhite, J.T., Hammer, R.E., Garbers, D.L., 2004. Defining the spermatogonial stem cell. Dev. Biol. 269, 393–410.

Haraguchi, K., Hayashi, T., Jimbo, T., Yamamoto, T., Akiyama, T., 2006. Role of the kinesin-2 family protein, KIF3, during mitosis. J. Biol. Chem. 281, 4094–4099.

Hase, T., Yoshimura, R., Matsuyama, M., Kawahito, Y., Wada, S., Tsuchida, K., Sano, H., Nakatani, T., 2003. Cyclooxygenase-1 and -2 in human testicular tumours. Eur. J. Cancer 39, 2043–2049.

He, L., Liu, J., Collins, I., Sanford, S., O'Connell, B., Benham, C.J., Levens, D., 2000. Loss of FBP function arrests cellular proliferation and extinguishes c-myc expression. EMBO J. 19, 1034–1044.

Hirose, F., Hotta, Y., Yamaguchi, M., Matsukage, A., 1989. Difference in the expression level of DNA polymerase beta among mouse tissues: high expression in the pachytene spermatocyte. Exp. Cell Res. 181, 169–180.

Hosono, K., Sasaki, T., Minoshima, S., Shimizu, N., 2004. Identification and characterization of a novel gene family YPEL in a wide spectrum of eukaryotic species. Gene 340, 31–43.

Hurlin, P.J., Queva, C., Koskinen, P.J., Steingrimsson, E., Ayer, D.E., Copeland, N.G., Jenkins, N.A., Eisenman, R.N., 1995. Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. 14, 5646–5659.

Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., Gilchrist, R.B., 2005. Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. J. Cell Sci. 118, 5257–5268.

Jakel, H., Fruchart-Najib, J., Fruchart, J.C., 2006. Retinoic acid receptor-related orphan receptor alpha as a therapeutic target in the treatment of dyslipidemia and atherosclerosis. Drug News Perspect. 19, 91–97.

Kamel, D., Mackey, Z.B., Sjoblom, T., Walter, C.A., McCarrey, J.R., Uitto, L., Palosaari, H., Lahdetie, J., Tomkinson, A.E., Syvaoja, J.E., 1997. Role of deoxyribonucleic acid polymerase epsilon in spermatogenesis in mice. Biol. Reprod. 57, 1367–1374.

Kamma, H., Portman, D.S., Dreyfuss, G., 1995. Cell type-specific expression of hnRNP proteins. Exp. Cell Res. 221, 187–196.

Kassir, Y., Adir, N., Boger-Nadjar, E., Raviv, N.G., Rubin-Bejerano, I., Sagee, S., Shenhar, G., 2003. Transcriptional regulation of meiosis in budding yeast. Int. Rev. Cytol. 224, 111–171.

Ketola, I., Anttonen, M., Vaskivuo, T., Tapanainen, J.S., Toppari, J., Heikinheimo, M., 2002. Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. Eur. J. Endocrinol. 147, 397–406.

Kissel, H., Timokhina, I., Hardy, M.P., Rothschild, G., Tajima, Y., Soares, V., Angeles, M., Whitlow, S.R., Manova, K., Besmer, P., 2000. Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. EMBO J. 19, 1312–1326.

Koubova, J., Menke, D.B., Zhou, Q., Capel, B., Griswold, M.D., Page, D.C., 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc. Natl. Acad. Sci. U SA 103, 2474–2479.

Latham, K.E., Litvin, J., Orth, J.M., Patel, B., Mettus, R., Reddy, E.P., 1996. Temporal patterns of A-myb and B-myb gene expression during testis development. Oncogene 13, 1161–1168.

Lee, M.H., Park, H., Shim, G., Lee, J., Koo, H.S., 2001. Regulation of gene expression, cellular localization, and in vivo function of Caenorhabditis elegans DNA topoisomerase I. Genes Cells 6, 303–312.

Letwin, K., Mizzen, L., Motro, B., Ben-David, Y., Bernstein, A., Pawson, T., 1992. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J. 11, 3521–3531.

Li, B., Nair, M., Mackay, D.R., Bilanchone, V., Hu, M., Fallahi, M., Song, H., Dai, Q., Cohen, P.E., Dai, X., 2005. Ovol1 regulates meiotic pachytene progression during spermatogenesis by repressing Id2 expression. Development 132, 1463–1473.

Linder, B., Jones, L.K., Chaplin, T., Mohd-Sarip, A., Heinlein, U.A., Young, B.D., Saha, V., 1998. Expression pattern and cellular distribution of the murine homologue of AF10. Biochim. Biophys. Acta 1443, 285–296.

Liu, Y., Tarsounas, M., O'regan, P., West, S.C., 2007. Role of RAD51C and XRCC3 in genetic recombination and DNA repair. J. Biol. Chem. 282, 1973–1979.

Maratou, K., Forster, T., Costa, Y., Taggart, M., Speed, R.M., Ireland, J., Teague, P., Roy, D., Cooke, H.J., 2004. Expression profiling of the developing testis in wild-type and Dazl knockout mice. Mol. Reprod. Dev. 67, 26–54.

Mata, J., Curado, S., Ephrussi, A., Rorth, P., 2000. Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis. Cell 101, 511–522.

McKee, C.M., Ye, Y., Richburg, J.H., 2006. Testicular germ cell sensitivity to TRAIL-induced apoptosis is dependent upon p53 expression and is synergistically enhanced by DR5 agonistic antibody treatment. Apoptosis 11, 2237–2250.

Meinhardt, A., O'Bryan, M.K., McFarlane, J.R., Loveland, K.L., Mallidis, C., Foulds, L.M., Phillips, D.J., de Kretser, D.M., 1998. Localization of follistatin in the rat testis. J. Reprod. Fertil. 112, 233–241.

Meng, X., Lindahl, M., Hyvonen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J.G., Westphal, H., Saarma, M., Sariola, H., 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287, 1489–1493.

Monesi, V., 1962. Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. J. Cell Biol. 14, 1–18.

Mootha, V.K., Lindgren, C.M., Eriksson, K.F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstrale, M., Laurila, E., Houstis, N., Daly, M.J., Patterson, N., Mesirov, J.P., Golub, T.R., Tamayo, P., Spiegelman, B., Lander, E.S., Hirschhorn, J.N., Altshuler, D., Groop, L.C., 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34, 267–273.

Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A., Milbrandt, J., 2006. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol. Reprod. 74, 314–321.

Neeraja, S., Sreenath, A.S., Reddy, P.R., Reddanna, P., 2003. Expression of cyclooxygenase-2 in rat testis. Reprod. Biomed. Online 6, 302–309.

Oatley, J.M., Avarbock, M.R., Telaranta, A.I., Fearon, D.T., Brinster, R.L., 2006. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc. Natl. Acad. Sci. USA 103, 9524–9529.

Olesen, C., Moller, M., Byskov, A.G., 2004. Tesmin transcription is regulated differently during male and female meiosis. Mol. Reprod. Dev. 67, 116–126.

O'Reilly, P.G., Wagner, S., Franks, D.J., Cailliau, K., Browaeys, E., Dissous, C., Sabourin, L.A., 2005. The Ste20-like kinase SLK is required for cell cycle progression through G2. J. Biol. Chem. 280, 42383–42390.

Packer, A.I., Besmer, P., Bachvarova, R.F., 1995. Kit ligand mediates survival of type A spermatogonia and dividing spermatocytes in postnatal mouse testes. Mol. Reprod. Dev. 42, 303–310.

Pangas, S.A., Choi, Y., Ballow, D.J., Zhao, Y., Westphal, H., Matzuk, M.M., Rajkovic, A., 2006. Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. Proc. Natl. Acad. Sci. USA 103, 8090–8095.

Payton, M., Coats, S., 2002. Cyclin E2, the cycle continues. Int. J. Biochem. Cell Biol. 34, 315–320.

Pellegrini, M., Grimaldi, P., Rossi, P., Geremia, R., Dolci, S., 2003. Developmental expression of Bmp4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of Bmp4 in spermatogonia differentiation. J. Cell Sci. 116, 3363–3372.

Phelan, D.R., Loveland, K.L., Devereux, L., Dorow, D.S., 1999. Expression of mixed lineage kinase 2 in germ cells of the testis. Mol. Reprod. Dev. 52, 135–140.

Pilkinton, M., Sandoval, R., Song, J., Ness, S.A., Colamonici, O.R., 2007. Mip/LIN-9 regulates the expression of B-Myb and the induction of cyclin A, cyclin B, and CDK1. J. Biol. Chem. 282, 168–175.

Prabhu, S.M., Meistrich, M.L., McLaughlin, E.A., Roman, S.D., Warne, S., Mendis, S., Itman, C., Loveland, K.L., 2006. Expression of c-Kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. Reproduction 131, 489–499.

Puglisi, R., Montanari, M., Chiarella, P., Stefanini, M., Boitani, C., 2004. Regulatory role of BMP2 and BMP7 in spermatogonia and Sertoli cell proliferation in the immature mouse. Eur. J. Endocrinol. 151, 511–520.

Qiu, W., Zhang, S., Xiao, C., Xu, W., Ma, Y., Liu, Y., Wu, Q., 2003. Molecular cloning and characterization of a mouse spermatogenesis-related ring finger gene znf230. Biochem. Biophys. Res. Commun. 306, 347–353.

Rao, D.S., Chang, J.C., Kumar, P.D., Mizukami, I., Smithson, G.M., Bradley, S.V., Parlow, A.F., Ross, T.S., 2001. Huntingtin interacting protein 1 Is a clathrin coat binding protein required for differentiation of late spermatogenic progenitors. Mol. Cell. Biol. 21, 7796–7806.

Rodriguez, I., Ody, C., Araki, K., Garcia, I., Vassalli, P., 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J. 16, 2262–2270.

Ronfani, L., Ferraguti, M., Croci, L., Ovitt, C.E., Scholer, H.R., Consalez, G.G., Bianchi, M.E., 2001. Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. Development 128, 1265–1273.

Rossi, P., Dolci, S., Albanesi, C., Grimaldi, P., Ricca, R., Geremia, R., 1993. Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. Dev. Biol. 155, 68–74.

Rossi, P., Dolci, S., Sette, C., Capoluoghi, F., Pellegrini, M., Loiarro, M., Di Agostino, S., Baronetto, M.P., Grimaldi, P., Merico, D., Martegani, E., Geremia, R., 2004. Analysis of the gene expression profile of mouse male meiotic germ cells. Gene Expr. Patterns 4, 267–281.

Rubin-Bejerano, I., Sagee, S., Friedman, O., Pnueli, L., Kassir, Y., 2004. The in vivo activity of Ime1, the key transcriptional activator of meiosis-specific genes in *Saccharomyces cerevisiae*, is inhibited by the cyclic AMP/protein kinase A signal pathway through the glycogen synthase kinase 3-beta homolog Rim11. Mol. Cell. Biol. 24, 6967–6979.

Session, D.R., Lee, G.S., Wolgemuth, D.J., 2001. Characterization of D1Pas1, a mouse autosomal homologue of the human AZFa region DBY, as a nuclear protein in spermatogenic cells. Fertil. Steril. 76, 804–811.

Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R., Rossi, P., 1997. Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. Development 124, 2267–2274.

Sette, C., Dolci, S., Geremia, R., Rossi, P., 2000. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. Int. J. Dev. Biol. 44, 599–608.

Shao, X., Xue, J., van der Hoorn, F.A., 2001. Testicular protein Spag5 has similarity to mitotic spindle protein Deepest and binds outer dense fiber protein Odf1. Mol. Reprod. Dev. 59, 410–416.

Shiseki, M., Nagashima, M., Pedeux, R.M., Kitahama-Shiseki, M., Miura, K., Okamura, S., Onogi, H., Higashimoto, Y., Appella, E., Yokota, J., Harris, C.C., 2003. p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. Cancer Res. 63, 2373–2378.

Sorrentino, V., Giorgi, M., Geremia, R., Besmer, P., Rossi, P., 1991. Expression of the c-kit proto-oncogene in the murine male germ cells. Oncogene 6, 149–151.

Steinmayr, M., Andre, E., Conquet, F., Rondi-Reig, L., Delhaye-Bouchaud, N., Auclair, N., Daniel, H., Crepel, F., Mariani, J., Sotelo, C., Becker-Andre, M., 1998. staggerer phenotype in retinoid-related orphan receptor alpha-deficient mice. Proc. Natl. Acad. Sci. USA 95, 3960–3965.

Sugaya, K., Hongo, E., Tsuji, H., 2005. A temperature-sensitive mutation in the WD repeat-containing protein Smu1 is related to maintenance of chromosome integrity. Exp. Cell Res. 306, 242–251.

Sugihara, T., Wadhwa, R., Kaul, S.C., Mitsui, Y., 1999. A novel testis-specific metallothionein-like protein, tesmin, is an early marker of male germ cell differentiation. Genomics 57, 130–136.

Szczepny, A., Hime, G.R., Loveland, K.L., 2006. Expression of hedgehog signalling components in adult mouse testis. Dev. Dyn. 235, 3063–3070.

Takizawa, C.G., Morgan, D.O., 2000. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr. Opin. Cell Biol. 12, 658–665.

Tatsuta, T., Model, K., Langer, T., 2005. Formation of membrane-bound ring complexes by prohibitins in mitochondria. Mol. Biol. Cell 16, 248–259.

Tsuruta, J.K., O'Brien, D.A., 1995. Sertoli cell-spermatogenic cell interaction: the insulin-like growth factor-II/cation-independent mannose 6-phosphate receptor mediates changes in spermatogenic cell gene expression in mice. Biol. Reprod. 53, 1454–1464.

Upadhya, P., Birkenmeier, E.H., Birkenmeier, C.S., Barker, J.E., 2000. Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc. Natl. Acad. Sci. USA 97, 217–221.

Urano, A., Endoh, M., Wada, T., Morikawa, Y., Itoh, M., Kataoka, Y., Taki, T., Akazawa, H., Nakajima, H., Komuro, I., Yoshida, N., Hayashi, Y., Handa, H., Kitamura, T., Nosaka, T., 2005. Infertility with defective spermiogenesis in mice lacking AF5q31, the target of chromosomal translocation in human infant leukemia. Mol. Cell. Biol. 25, 6834–6845.

van Pelt, A.M., de Rooij, D.G., 1991. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology 128, 697–704.

Vincent, S., Segretain, D., Nishikawa, S., Nishikawa, S.I., Sage, J., Cuzin, F., Rassoulzadegan, M., 1998. Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. Development 125, 4585–4593.

Wang, P.J., McCarrey, J.R., Yang, F., Page, D.C., 2001. An abundance of X-linked genes expressed in spermatogonia. Nat. Genet. 27, 422–426.

Wang, Y., Culty, M., 2007. Identification and distribution of a novel platelet-derived growth factor receptor beta variant: effect of retinoic acid and involvement in cell differentiation. Endocrinology 148, 2233–2250.

Warren, E.H., Gavin, M.A., Simpson, E., Chandler, P., Page, D.C., Disteche, C., Stankey, K.A., Greenberg, P.D., Riddell, S.R., 2000. The human UTY gene encodes a novel HLA-B8-restricted H-Y antigen. J. Immunol. 164, 2807–2814.

Woodruff, T.K., Borree, J., Attie, K.M., Cox, E.T., Rice, G.C., Mather, J.P., 1992. Stage-specific binding of inhibin and activin to subpopulations of rat germ cells. Endocrinology 130, 871–881.

Wu, C.L., Kirley, S.D., Xiao, H., Chuang, Y., Chung, D.C., Zukerberg, L.R., 2001. Cables enhances cdk2 tyrosine 15 phosphorylation by Wee1, inhibits cell growth, and is lost in many human colon and squamous cancers. Cancer Res. 61, 7325–7332.

Xiao, G.H., Gallagher, R., Shetler, J., Skele, K., Altomare, D.A., Pestell, R.G., Jhanwar, S., Testa, J.R., 2005. The NF2 tumor suppressor gene product, merlin, inhibits cell proliferation and cell cycle progression by repressing cyclin D1 expression. Mol. Cell. Biol. 25, 2384–2394.

Xiao, J., Xu, M., Li, J., Chang Chan, H., Lin, M., Zhu, H., Zhang, W., Zhou, Z., Zhao, B., Sha, J., 2002. NYD-SP6, a novel gene potentially involved in regulating testicular development/spermatogenesis. Biochem. Biophys. Res. Commun. 291, 101–110.

Yamamoto, H., Tsukahara, K., Kanaoka, Y., Jinno, S., Okayama, H., 1999. Isolation of a mammalian homologue of a fission yeast differentiation regulator. Mol. Cell. Biol. 19, 3641–3829.

Yang, H.M., Liu, G., Nie, Z.Y., Nie, D.S., Deng, Y., Lu, G.X., 2005. Molecular cloning of a novel rat gene Tsarg1, a member of the DnaJ/HSP40 protein family. DNA Seq. 16, 166–172.

Zhai, L., Graves, P.R., Robinson, L.C., Italiano, M., Culbertson, M.R., Rowles, J., Cobb, M.H., DePaoli-Roach, A.A., Roach, P.J., 1995. Casein kinase I gamma subfamily. Molecular cloning, expression, and characterization of three mammalian isoforms and complementation of defects in the *Saccharomyces cerevisiae* YCK genes. J. Biol. Chem. 270, 12717–12724.

Zhang, N., Yeh, H.J., Zhong, R., Li, Y.S., Deuel, T.F., 1999. A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice. Proc. Natl. Acad. Sci. USA 96, 6734–6738.

Zhang, S., Qiu, W., Wu, H., Zhang, G., Huang, M., Xiao, C., Yang, J., Kamp, C., Huang, X., Huellen, K., Yue, Y., Pan, A., Lebo, R., Milunsky, A., Vogt, P.H., 2001. The shorter zinc finger protein ZNF230 gene message is transcribed in fertile male testes and may be related to human spermatogenesis. Biochem. J. 359, 721–727.