Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2

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SUMMARY

High mobility group 2 protein (Hmgb2) is a member of the HMGB protein family, which includes the ubiquitous Hmgb1 and the embryo-specific Hmgb3. The three proteins are more than 80% identical at the amino acid level and their biochemical properties are indistinguishable. Hmgb1 is an abundant component of all mammalian nuclei and acts as an architectural factor that bends DNA and promotes protein assembly on specific DNA targets. Cells that lack Hmgb1 can survive, although mutant mice die shortly after birth. As Hmgb2 is present in all cultured cells and is abundant in thymus, the preferred source for HMGB proteins, it was considered a ubiquitous variant of Hmgb1. We show that in adult mice Hmgb2 is restricted mainly to lymphoid organs and testes, although it is widely expressed during embryogenesis. Mice that lack Hmgb2 are viable. However, male Hmgb2−/− mice have reduced fertility, that correlates with Sertoli and germ cell degeneration in seminiferous tubules and immotile spermatozoa. Significantly, Hmgb2 is expressed at very high levels in primary spermatocytes, while it is barely detectable in spermatogonia and elongated spermatids. This peculiar pattern of expression and the phenotype of mutants indicate that Hmgb2 has a specialised role in germ cell differentiation.

Key words: Chromatin, High mobility group, Testis, Transcription, Mouse

INTRODUCTION

Hmgb2 is a member of one of the three families of high mobility group (HMG) proteins (for reviews on HMGs, see Bustin, 1999; Bianchi and Beltrame, 2000). The nomenclature of HMGs has been recently revised (see http://www.informatics.jax.org/mgihome/nomen/genefamilies/hmgfamily.shtml), and the family that comprises the ubiquitous Hmgb1 (previously, Hmg1), Hmgb2 (previously, Hmg2) and Hmgb3 (previously, Hmg4 or Hmg2b) is now identified as HMGB. Hmgb1 is the more well-known member of the family. Hmgb1 interacts with the minor groove of DNA, and distorts the DNA segment to which it is bound. It has no sequence specificity, but can be recruited via protein-protein interactions by a variety of sequence-specific DNA binding proteins to provide an ‘architectural’ activity: it facilitates or stabilises the assembly of multiprotein complexes on DNA, both by imposing the correct geometrical path onto the double helix and by locking in other DNA-binding proteins.

Hmgb2 is extremely similar to Hmgb1 (more than 80% amino acid identity). We and others have shown that Hmgb1 and Hmgb2 are completely interchangeable in vitro: they both bind to Hox proteins (Zappavigna et al., 1996), steroid hormone receptors (Boonyaaratanakornkit et al., 1998) and Rag1 recombinase (Aidinis et al., 1999), and they both enhance the transcription and recombination activities of their partner proteins when transiently transfected in mammalian cells. We have carefully compared the ability of Hmgb1 and Hmgb2 to bind nucleosomes in vitro and in vivo and to promote transcriptional transactivation, and concluded that even the difference in the length of their acidic tails has minimal consequences (L. R. and M. E. B., unpublished).

Despite the similarity of Hmgb1 and Hmgb2, evolutionary considerations suggest that their functions do not overlap completely. The nucleotide sequence of Hmgb1 transcripts is extremely conserved in vertebrates, even in untranslated regions; likewise, Hmgb2 transcripts are also highly conserved across species. However, the nucleotide sequences of Hmgb1 and Hmgb2 are loosely related in the coding regions, as expected, but are unrelated elsewhere. Previous experiments hinted that Hmgb2 is required for the progression of the cell cycle: expression is much higher in proliferating cells (Seyedin
and Kistler, 1979) and much lower in fibroblasts from old-age humans (Ly et al., 2000), and incubation with Hmgb2 antisense oligonucleotides inhibits cell division (Yamazaki et al., 1995). To clarify the role of Hmgb2, we studied its expression pattern in the mouse, and generated null mutants.

**MATERIALS AND METHODS**

**Cloning of the mouse Hmgb2 gene**

One million λFIXII phage plaques from a 129Sv genomic library (Stratagene) were screened using as probe intron 4. This was amplified by PCR from total mouse genomic DNA using primers designed on the coding sequence of mouse Hmgb2 cDNA (Zwilling et al., 1995) and the intron/exon organization of the human HMGB2 gene (Shirakawa and Yoshida, 1992). PCR consisted of 35 cycles of denaturation (30 seconds at 94°C), annealing (45 seconds at 56°C) and extension (30 seconds at 72°C) on a Hybaid OmniGene instrument, using 20 ng of genomic DNA from 129Sv mice, 30 pmol of each primer INT4for (coding strand) 5'- GGAGAAGATF GAAAGTTGGAGAAGTA TGAAA-3' and INT4rev (non-coding strand) 5'- GGGATGCCGACCAAT- ATCC-3', 0.2 mM dNTPs, 3 μl of 10× Dynazyme buffer and 0.6 units of polymerase (Finnzyme) in a total volume of 30 μl. The pHMG2 plasmid was constructed by cloning into the pBlueScriptII KS(+) vector a genomic 4.8 kb EcoRI fragment, containing the whole mouse Hmgb2 gene under the control of its own promoter.

**Mapping of the mouse Hmgb2 gene**

We identified a length polymorphism in intron 4 that differentiated Mus musculus from *M. splendens*. The DNA from the backcross progeny of (C57BL/6J × *M. splendens*) F1 hybrid female mice mated with C57BL/6J males, designated BSS, was provided by The Jackson Laboratory. Reaction mixtures (50 μl) containing 125 ng of genomic DNA from the BSS progeny, 5 μl of 10× Promega DNA polymerase buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 μM INT4for, 1 μM INT4rev, 1 μCi of [32P]dCTP were heated at 94°C for 5 minutes and at 80°C for 5 more minutes. After this hot start, 2.5 units of AmpliTaq DNA polymerase (Promega) were added, and two cycles of amplification were carried out as follows: 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds. Two more rounds of two cycles each were performed with annealing at 60°C and 58°C, respectively, followed by 24 cycles with annealing at 56°C. The PCR products were loaded on a 6% nondenaturing polyacrylamide gel and electrophoresed at room temperature in TBE. The ensuing Strain Distribution Patterns were analysed with Map Manager 2.6.5 (Manly and Elliott, 1991).

**Northern blots and in situ hybridizations**

Total cellular RNA was extracted from tissues or cells by the guanidine isothiocyanate technique (Chirgwin et al., 1979). For northern blot analysis, 5 μg of total RNA isolated from various adult tissues and embryos was run on a 1.2% agarose/formaldehyde gel, transferred onto a GeneScreen Plus membrane (Dupont) and probed with 32P-labeled Hmgb2 cDNA. Radioactive and non-radioactive in situ hybridizations were performed according to standard methods.

**Generation of Hmgb2−/− mutant mice**

A 5 kb XbaI-XbaI fragment covering the complete Hmgb2 coding sequence was cloned in pBlueScriptKS+ to generate p5kbXbaI. p5kbXbaI was first digested with BstEII and Stul restriction enzymes, and 741 bp from exon 2 to exon 4 were replaced with the ble-lacZ-coding sequence (from plasmid pUT529A, Cayla SA), using adaptors to recreate the BstEII and Stul sites. Then, the XbaI site distal to the gene was deleted, generating pKO. pKO was digested with XbaI and XhoI, and the insert was cloned into the XbaI-XhoI-digested pPNT vector, that contains the herpes simplex virus thymidine kinase gene (tk) under the control of the PGK promoter. The resulting plasmid, pKO22, was linearised and used for transformation.

**Immunohistochemistry**

Testis sections were immunostained with rabbit anti-Hmgb2 antibody from Pharmingen and the Vectastain Elite ABC kit from Vector Laboratories. Staining for β-gal was performed with the β-gal staining set (Boehringer Mannheim).

**In vitro fertilization assays**

Spermatozoa were collected from cauda epididymis and capacitated in 500 μl of M16 medium (Sigma), pre-gassed in 5% CO2 at 37°C. One million spermatozoa were added to oocytes collected from the oviduct of superovulated females and were incubated for 6 hours in a humidified 37°C incubator with 5% CO2. Fertilised eggs were distinguished by the presence of male pronuclei and two polar bodies.

**Transfection assays**

Mouse embryonic fibroblasts (MEF) primary cultures were established from the skin of Hmgb2−/−, +/- and +/+ embryos at E16. 3T3 cells and MEFs were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM), supplemented by 10% foetal bovine serum (GIBCO), 100 IU/ml of penicillin and 100 μg/ml streptomycin in 5% CO2 in air humidified atmosphere. MEFs were transiently transfected by calcium phosphate co-precipitation in 6 cm dishes. Cells were transfected with combinations of 6 μg of PRE-2-TK-LUC reporter plasmid, 3 μg of AR-expressing construct (Boonyaaratanakornkit et al., 1998), 2.5 μg of pHM2G, 1 μg of pRSV-CAT as an internal control and pBlueScript. When necessary, 0.1 μM testosterone (dissolved in EtOH) was added after 48 hours. Cells were harvested 72 hours after transfection, lysed and assayed for luciferase and β-gal expression on a PlateLumino 96-well luminometer (Stratec Biomedical Systems AG). All transfections were carried out in triplicate and in at least three separate experiments, using at least two different DNA preparations of each expression construct.

**RESULTS**

**Structure of the mouse Hmgb2 gene**

We first cloned the Hmgb2 gene from a 129Sv mouse genomic library. In contrast to the high abundance of Hmgb1-related pseudogenes (Ferrari et al., 1994), we found 16 nonidentical but overlapping Hmgb2 clones, but no Hmgb2-related pseudogenes. The complete sequence of the mouse Hmgb2 gene was deposited in GenBank (Accession Number AF267733; Fig. 1). The gene contains 5 exons (Fig. 2A); the first exon, and part of the second, are not translated. Such organization is typical of all genes of the Hmgb family (Ferrari et al., 1994), we found 16 nonidentical pseudogenes. The complete sequence of the mouse Hmgb2 gene was deposited in GenBank (Accession Number AF267733; Fig. 1). The gene contains 5 exons (Fig. 2A); the first exon, and part of the second, are not translated. Such organization is typical of all genes of the Hmgb family (Ferrari et al., 1994; Ferrari et al., 1996; Shirakawa and Yoshida, 1992; Vaccari et al., 1998). A length polymorphism within intron 4 that distinguished *M. musculus* from *M. splendens* allowed us to map the Hmgb2 gene close to the centromere of mouse chromosome 8 (Fig. 2C). This region is syntenic to the human chromosome arm 4q where the human *HMB2* maps (Wanschura et al., 1996).

We identified two transcription start sites by RNase protection on total RNA from embryonic fibroblasts: the main one corresponds to the 5′ terminus already reported for Hmgb2 cDNA (Zwilling et al., 1995), and the alternative one is 5 bp upstream (Fig. 2B). Transcription terminates at a single site, as (1) a single Hmgb2 transcript is found in all mouse tissues and cell lines (Fig. 3B), and (2) no sequences extending further to the 3′ side are present in complete cDNAs or mouse EST.
Fig. 1. Sequence of the Hmgb2 gene. Exon sequences are shown in UPPERCASE, introns and non-transcribed sequences in lowercase. exon 1 is indicated from the major, downstream transcription start site. The first base (G) of the minor upstream start site. The first base (G) of the minor upstream start site. The first base (G) of the minor upstream start site. The first base (G) of the minor upstream start site. The first base (G) of the minor upstream start site. The first base (G) of the minor upstream start site.
databanks. The 5’ untranslated (UTR) region, which is fairly long, and the 3’ UTR are remarkably conserved (about 90% identical) between human and mouse.

The sequence upstream of the transcription initiation site contains no TATA box, while CCAAT sequences are present at position −340, −295, −165 and −103. The comparison to the corresponding human sequence revealed a region of high similarity extending to −425 (Fig. 2A). This conserved sequence is capable of driving the expression of Hmgb2 or reporter genes in transfection assays (results not shown).

Expression pattern of mouse Hmgb2
Contrary to the ubiquitous Hmgb1 protein, we found that Hmgb2 has a limited presence in adult organs. For example, Hmgb2 is prominently present in thymus and testes, while it is undetectable in westerns of total extracts of brain (Fig. 3A). Northern blots confirm the limited expression of Hmgb2 in adults, but indicate a high expression during embryogenesis (Fig. 3B). Moreover, all mouse cell lines contain a high level of Hmgb2 transcripts, with a clear difference between the proliferating and growth-arrested states (Fig. 3B, 3T3 cells and results not shown for other cell lines).

We then analysed in detail the pattern of expression of Hmgb2 during embryonic development. RT-PCR indicated that the gene is expressed in blastocysts (not shown). In situ hybridisation showed that in embryos at E10.5 the gene is expressed widely, and especially in all sites of fast cell proliferation (not shown).

At E12.5 (Fig. 4A), regionalization of the expression of Hmgb2 is already evident. The strongest signals are in the ventricular zones (VZ) of brain, consisting exclusively of proliferating neuroepithelial cells, and in the spinal cord (SC, Fig. 4A,B).

At E17 (Fig. 4C) Hmgb2 expression is localized in brain to ventricular zones (VZ), to differentiated cortex (C), and to the mesencephalon (M). In the anterior cerebral cortex, Hmgb2 signal is present in the two parts that compose the transitional field (TF) – the sub-ventricular zone (SV), whose nature remains poorly known, and the intermediate zone (IZ), to which differentiating cortical cells translocate before migrating to outer regions (Fig. 5). The neural retina also expresses Hmgb2 abundantly through its thickness (Fig. 4D). Expression is also very high in thymus and lung, high in kidney cortex and multilobular fat tissue, and lower but significant in liver, in the intestinal epithelium and in hair follicles (Fig. 4C).

At postnatal day 4 (P4) Hmgb2 in the CNS is exclusively expressed in the external granular layer (EGL) of the cerebellum (Fig. 4E) and in the hippocampus (not shown). Later (P17), expression is detectable in the hippocampus (H) and in the internal granular layer of the cerebellum (IGL, postmitotic cells that migrated from P4 EGL) (Fig. 4F). Immunohistochemical analysis revealed that Purkinje cells express Hmgb2 at high level as well (not shown). In adult mice, Hmgb2 expression is not detectable at all in the CNS, even in areas that showed high expression in earlier stages (not shown).

Thus, Hmgb2 is not ubiquitously expressed, contrary to Hmgb1. Expression broadly correlates with active cell proliferation, as previously noted (Seyedin and Kistler, 1979). However, there are significant exceptions: for example, Hmgb2 is expressed in the cortical plate and internal granular layer of the cerebellum (postmitotic) as well as in the ventricular zone (proliferative).

Hmgb2-null mutant mice are viable
To further investigate the physiological role of Hmgb2, we deleted Hmgb2 by conventional gene targeting (Fig. 6A). The Hmgb2-coding sequence was replaced by homologous recombination in ES cells with the b- lacZ-coding sequence, starting from the ATG translation start site in exon 2 (see Materials and Methods). We obtained 103 zeocyn-resistant clones, two of which were homologous recombinants. Both clones gave rise to chimeric males that transmitted the Hmgb2-
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null allele through the germline (Fig. 6B). Heterozygous mice were phenotypically normal. The pattern of β-gal activity in different tissues (not shown) confirmed the distribution of Hmgb2 transcripts, and in particular their complete absence in adult brain.

About one quarter of F2 offspring were Hmgb2−/− homozygotes, with no Hmgb2 expression either at transcript or protein level (Fig. 6C, and results not shown). These mice appeared healthy; in particular, their lifespan was not shorter. Therefore, Hmgb2 is not required for the cell cycle, and the low level of Hmgb2 transcripts in fibroblasts from old-age humans (Ly et al., 2000) is not directly responsible for the limited division potential of these cells.

As Hmgb2 is expressed at high levels in certain areas of the brain and cerebellum during their development, Hmgb2−/− homozygotes were subjected to behavioural tests (D. P. Wolfer, personal communication). No difference from wild-types was found in the Morris water maze test, that is sensitive to hippocampal lesions (Steward and Morris, 1993), and in the two-way active avoidance test, a measure of emotional responses (Clincke and Werbrouck, 1993). These results suggest that Hmgb2−/− homozygotes have no gross alteration of cognitive functions or coordination.

In adults, Hmgb2 is highly expressed in lymphoid tissues. Moreover, both Hmgb1 and Hmgb2 interact with the Rag1/2 recombinase and enhance V(D)J recombination in vitro and in vivo (Agrawal and Schatz, 1997; Aidinis et al., 1999; Kwon et al., 1998; van Gent et al., 1997). Nonetheless, Hmgb2−/− homozygotes had a normal serum concentration of immunoglobulins, a normal number of peripheral B and T cells, and a normal thymus with respect to size, cellularity and histological architecture (not shown).

Hmgb2−/− males have reduced fertility and defective spermatogenesis

A further site of high Hmgb2 expression in adult mice is testes. We thus examined the functionality of Hmgb2−/− testes: 24 females plugged by nine adult Hmgb2−/− males originated only 41 pups in nine litters. By comparison, 24 females plugged by wild-type sibs of the −/− males originated 235 pups in 24 litters. The fertility of Hmgb2−/− homozygote females was not different from that of wild-type females.

We noticed no difference in mean size of testes between Hmgb2−/− homozygotes and wild-types (when matched for age). However, in about a third of young homozygotes, the testes had not descended from the inguinal ring to the scrotum; the defect was not detected in more mature males. Moreover, even in Hmgb2−/− homozygotes where the testis had descended normally in the scrotum, after P30 there were readily detectable anomalies in the histological organization of seminiferous tubules (Fig. 7A). The defects correlated in severity with the reduction in fertility, and became more

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serious with advancing age. In a fraction of tubule sections, ranging from a few percent to the majority, we noticed a loss of the regular periphery-to-lumen succession of spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa, the presence of gaps among cells, and degenerated Sertoli cells with large vacuoles (Fig. 7B). Large multinucleate round cells were often present, sometimes in the lumen of the tubule. These phenotypes are typical of mice with reduced fertility, like those lacking the estrogen receptor (Lubahn et al., 1993), overexpressing androgen binding protein (Esteban et al., 1997) or triple-knockout for the Tyro3, Axl and Mer receptors (Lu et al., 1999). In addition, even in sections without visible histological disruption, we detected (by TUNEL staining) a mean of four \textit{Hmgb2}−/− cells undergoing apoptosis, against one cell or less in sections from wild-type males (Fig. 7C,D; \textit{P}<0.01).

The observed disruptions in spermatogenesis can be caused by failure of the supporting Sertoli cells, or by cell-autonomous defects in germ cells. To distinguish between these possibilities, we visualized \textit{Hmgb2} expression in the testes of heterozygote males, using immunocytochemistry with anti-\textit{Hmgb2} antibodies (Fig. 7E), in situ hybridization with a cDNA probe (Fig. 7F) and staining for β-gal activity (Fig. 7G). No \textit{Hmgb2} protein was detected in myoid, Leydig and Sertoli cells, and in spermatogonia. \textit{Hmgb2} transcript and protein were prominently present in primary and secondary spermatocytes, but were absent in elongated spermatids and spermatozoa (Fig. 7E,F). In partial contrast, very strong staining with X-gal was present in spermatocytes, but also in spermatids and spermatozoa (Fig. 7G), suggesting that \textit{Hmgb2} (and not β-gal) is specifically degraded after the completion of meiosis.

\textit{Hmgb2}-less testis specimens were also analysed by electron microscopy. Sertoli cells (even the ones without large vacuoles) were separated by gaps from nearby cells (Fig. 8B). Some spermatids had the flagellar midpiece embedded inside the
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Cytoplasm (Fig. 8D); in a high proportion, nuclei had an irregular margin, and the acrosome was not tightly apposed, but separated by intervening cytoplasm (Fig. 8C). These defects were not homogeneously distributed: some areas of testis from mutant animals contained less than one abnormality per field, whereas in nearby areas most cells were abnormal. By comparison, however, in normal testes we found only one abnormal flagellum midpiece in several tens of fields observed.

Despite the abnormalities described above, spermatozoa are produced by Hmgb2−/− males and can be recovered both from the epididymis and from the uterus of mated females. More than 95% of mutant spermatozoa were not motile, and had shorter tails and a slightly more rounded head (compare Fig. 8E, wild type, with Fig. 8F, mutant). However, both −/− and +/+ spermatozoa were functional in in vitro fertilisation assays: in both cases about 50% of the eggs were fertilised.

Some of the testis abnormalities described above, and in particular the delayed descent in the scrotum, might be due to defective responses to male hormones. Both Hmgb1 and Hmgb2 interact physically with the androgen receptor and facilitate its binding to cognate DNA target sites (Boonyaaratanakornkit et al., 1998). Primary mouse embryonic fibroblasts (MEF) derived from Hmgb2−/− and +/+ mice differ reproducibly in their transcriptional response to androgens, but the difference is limited within a factor of two (Fig. 9). We do not know whether this small difference is representative of the transcriptional responses of cells within living mice, and whether the difference may have physiological consequences.

**DISCUSSION**

Hmgb1 and Hmgb2 are very similar at the amino acid level (>80% identity), and are indistinguishable in their biochemical properties, in the range of known interactors, and in previously known biological functions. However, we show here that Hmgb2 has a restricted pattern of expression, suggesting that it is required only in specific cells and specific conditions. Moreover, mice lacking Hmgb2 are healthy and have a normal lifespan, whereas mice that lack Hmgb1 die before reproductive age (Calogero et al., 1999). These data disprove the hypothesis that Hmgb2 is required for the progression of the cell cycle (Yamazaki et al., 1995), and suggest that the decline of Hmgb2 expression in human fibroblasts obtained from old individuals (Ly et al., 2000) is not a cause of senescence per se.

The simplest interpretation of the data summarized above is that Hmgb1 and Hmgb2 are indeed functionally equivalent, and a minimum total amount of Hmgb1 plus Hmgb2 is required at all times. The lack of Hmgb1 is phenotypically more noticeable because Hmgb1 is usually much more abundant than Hmgb2. The specialised pattern of expression of Hmgb2 might simply indicate that the total Hmgb1 + Hmgb2 amount required by specific cells or in specific developmental conditions is variable rather than constant. The total Hmgb pool is then modulated by varying the concentration of Hmgb2, while the expression of Hmgb1 is
relatively constant at all times. It significant to note, however, that the deletion of \( Hmgb1 \) has no effect on the expression level of \( Hmgb2 \), and vice versa; there appears to be no feedback control over the quantity of Hmgb proteins expressed.

The hypothesis of perfect biochemical interchangeability of Hmgb1 and Hmgb2 can provide a rationale for the survival of \( Hmgb1^{-/-} \) mice up until birth, but not much longer (Calogero et al., 1999). Hmgb2 is expressed widely in embryos, but is present at very low levels in most adult tissues, and the combined absence of Hmgb1 and Hmgb2 might lead to cellular degeneration.

However, the absolute conservation of Hmgb2 in diverse mammalian species does not fit well with the hypothesis of complete Hmgb1 and Hmgb2 interchangeability. \( Hmgb \) genes derive by duplication from a single ancestor gene (possibly at the base of the chordate lineage), and have diverged. However, Hmgb1 is essentially identical in all mammals, and so is Hmgb2. If the two proteins were perfectly interchangeable in mammals, there would be no reason for the relative immutability of each individual protein. In this context, it is satisfying to find that Hmgb2 has a non-redundant biological function in testis, that is revealed by a specific phenotype in \( Hmgb2 \) null mutants, and thus cannot be perfectly equivalent to Hmgb1.

\( Hmgb2^{-/-} \) males have reduced fertility, which correlates with germ cell loss, defects in spermatids and immotility of spermatozoa. Mice produce a vast excess of spermatozoa, and a fivefold reduction in offspring is highly significant. In the testis, Hmgb2 is particularly abundant. Incidentally, this might explain the high effectiveness of cisplatin, an anti-tumour drug that binds covalently to DNA, for the treatment of testicular cancers: both Hmgb1 and 2 bind specifically to cisplatin adducts, and hinder their repair (Huang et al., 1994). The tight regulation of Hmgb2 expression during spermatogenesis is very peculiar: the protein starts to be synthesised quite abruptly at spermatocyte stage, and both transcription and the absolute amount of protein drop as abruptly at the spermatid stage. Hmgb1 and Hmgb2 are stable proteins, with turnover times of several days, and the disappearance of Hmgb2 suggests a targeted destruction.

The correlation between Hmgb2 levels and cell proliferation might suggest that spermatocytes suffer from a defect in DNA replication, and meiotic arrest; however, meiotic arrest should not disrupt cell growth, but causes lethal hypoglycaemia in newborn mice. Nat. Genet. 22, 276-280.


