Nuclear envelope remodelling during human spermiogenesis involves somatic B-type lamins and a spermatid-specific B3 lamin isoform

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ABSTRACT: The nuclear lamina (NL) is a filamentous protein meshwork, composed essentially of lamins, situated between the inner nuclear membrane and the chromatin. There is mounting evidence that the NL plays a role in spermatid differentiation during spermiogenesis. The mouse spermatid NL is composed of the ubiquitous lamin B1 and the spermatid-specific lamin B3, an N-terminally truncated isoform of lamin B2. However, nothing is known about the NL in human spermatids. We therefore investigated the expression pattern and localization of A-type lamins (A, C and C2) and B-type lamins (B1, B2 and B3) during human spermiogenesis. Here, we show that a lamin B3 transcript is present in human spermatids and that B-type lamins are the only lamins detectable in human spermatids. We determine that, as shown for their mouse counterparts, human lamin B3, but not lamin B2, induces strong nuclear deformation, when ectopically expressed in HeLa cells. Co-immunofluorescence revealed that, in human spermatids, B-type lamins are present at the nuclear periphery, except in the region covered by the acrosome, and that as the spermatid matures the B-type lamins recede towards the posterior pole. Only lamin B1 remains detectable on 33–47% of ejaculated spermatozoa. On spermatozoa selected for normal head density, however, this fell to <6%, suggesting that loss of the NL signal may be linked to complete sperm nucleus compaction. The similarities revealed between lamin expression during human and rodent spermiogenesis, strengthen evidence that the NL and lamin B3 have conserved functions during the intense remodelling of the mammalian spermatid nucleus.

Key words: nuclear lamina / lamins / spermiogenesis / human

Introduction

The nuclear lamina (NL) is a meshwork of intermediate filament proteins (type V) situated within the nucleoplasm between the chromatin and the inner nuclear membrane, with which the NL is tightly associated. The NL is mainly composed of A-type and B-type lamins and remains located at the nucleoplasmic face of the inner nuclear membrane through its tight interactions with a wide range of transmembrane proteins (Cau et al., 2014). In mammals, there are three major A-type lamins, A, C and a male meiosis-specific isoform C2 and three major B-type lamins, B1, B2 and a spermatid-specific isoform B3. Lamins A and C are expressed in most differentiated cells, and are translated from alternatively spliced transcripts of the LMNA gene, while lamins B1 and B2 are expressed in nearly all cells and are encoded by distinct genes, LMNB1 and LMNB2, respectively (Capell and Collins, 2006; Worman and Bonne, 2007). The lamin isoforms C2 and B3 lack the N-terminal domains of lamin C and B2, respectively, and are expressed during rodent spermatogenesis, through the use of alternative promoters (Furukawa and Hotta, 1993; Furukawa et al., 1994; Nakajima and Abe, 1995).

The filamentous networks formed by lamins are required for normal nuclear structure and physiological development, and play various roles in chromatin organization, nuclear positioning, cell survival, DNA replication and transcription regulation in different cell types (Burke and Stewart, 2013). The best-characterized lamin-binding proteins illustrate this diversity of function: SUN-domain proteins connect the NL to the cytoskeleton and the centrosome, while LEM-domain proteins and the...
lamin B receptor connect the NL to the chromatin (Wilson and Foisner, 2010).

In humans, anomalies of the NL have been identified as the cause of several diseases. Mutations in LMNA encoding A-type lamins are known to underlie the pathogenesis in at least 12 genetic disorders (Worman and Bonne, 2007), including type 2B1 Charcot–Marie–Tooth disease (De Sandre-Giovannoli et al., 2002), Hutchinson–Gilford progeria syndrome (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003) and mandibuloacral dysplasia (Novelli et al., 2002). Duplications of LMNB1 have been identified in adult-onset autosomal dominant leukodystrophy (Padiath et al., 2006), while mutations in LMNB2 have been associated with acquired partial lipodystrophy (Hegele et al., 2006).

Spermatogenesis is a complex process leading to the formation of haploid spermatozoa from diploid spermatogonia. The germ cells develop through three phases: mitotic proliferation as spermatogonia, meiotic divisions as spermatocytes and spermatogenensis when they differentiate from haploid round spermatids into mature spermatozoa. During this final phase, the spermatid nucleus is subjected to a unique remodelling of its chromatin which allows an extreme compaction of the genome and a large reduction in nuclear volume, with a streamlining of its shape from round to elongated and finally pyriform in humans. This remodelling is orchestrated by dynamic interactions between the NE and the manchette, a network of cytoplasmic microtubules surrounding the nucleus (Kierszenbaum and Tres, 2004).

The importance of the NL in the radical cellular differentiation that occurs during spermiogenesis first became evident from the study of two mouse lines mutant for genes encoding proteins linking the NL to the chromatin or the cytoskeleton. Mice inactivated for a testis-specific transcript of Lis1 (Lissencephaly 1) or for the Gmcl1 gene (Germ cell loss 1) show malformations of the spermatid nucleus and aberrant formation of the acrosome and the flagellum (Kimura et al., 2003; Nayernia et al., 2003). The Lis1 gene codes for a cytoplasmic protein required for the link between the NE and the cytoskeleton involving the lamin-binding protein SUN1 (Zhang et al., 2009). The Gmcl1 gene encodes a transcription factor that localizes to the NL through its interaction with the lamin-binding protein LAP2B (Nilil et al., 2001). Recently, a direct link between human infertility and the nuclear envelope has been established through the study of DPF19L2, a gene transcribed predominantly in spermatids. In human, both copies of the DPF19L2 gene are deleted in more than 80% of men with globozoospermia, a rare phenotype characterized by malformed round sperm heads without an acrosome (Couton et al., 2012; Elinati et al., 2012). In the mouse, the knockout of DPF19L2 produced an identical phenotype, and the DPF19L2 protein was shown to localize to the inner nuclear membrane, inferring an essential link between the acrosome and the NL (Pierre et al., 2012).

Despite this, little is known about the structure and function of the NL during human spermiogenesis, with all studies of the NL in mammalian cells. We show that a transcript encoding a lamin B3 isoform is present in human spermatids, and we investigate the effect of human lamin B3 on nuclear structure by ectopic expression in HeLa cells.

Materials and Methods

Patients

Sperm samples were obtained from 13 normospermic men who consulted at our reproduction centre or gamete bank (CECOS—Centre d’Etude et de Conservation des Œufs et du Sperme) in Marseilles: 11 men consulted for couple infertility (10 primary and 1 secondary infertility), two men were fertile sperm donors.

Testicular samples came from three patients: one patient (T3) who underwent orchidectomy in the management of prostate carcinoma (Metzler-Guillemain et al., 2000), and two non-obstructive azoospermic patients with a normal karyotype and no AZF interval deletion detected by standard Y chromosome microdeletion screening (T01 1000120 and T01 1000145). They were selected on the basis of the retrieval of numerous spermatozoa on the testicular biopsy, and were considered as ‘controls’ in a previous study (Streicherberger et al., 2012).

Semen and testicular samples

Semen was collected via masturbation after a period of sexual abstinence of 2–6 days. After 30 min of liquefaction, semen analysis was performed according to the WHO criteria (WHO, 2009), and according to the French David classification for the morphology analysis (Auger and Eustache, 2000). Sperm was diluted in cryoprotectant medium (Spermfreeze; JCD, La Mulatière, France) and transferred into straws (Cryo Bio System, Saint Ouen sur l’Isle, France). Straws were then suspended in vapour-phase nitrogen before being stored in liquid nitrogen until use. All patients gave an informed consent for the conservation of the remnant sperm in the Gernmetheque biobank and their use in studies on human fertility in accordance with the Helsinki Declaration of 1975 on human experimentation. The Germetheque Scientific Committee approved the present study design. All sperm samples came from patients with normal sperm parameters according to WHO criteria (Table I). Fresh spermatozoa were used for the spermatozoa selection procedure.

Patients for whom we studied the testicular samples also gave informed consent for inclusion in the Germetheque biobank. Testicular cells from patient T3 were fixed in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS) before freezing (Metzler-Guillemain et al., 2003). For the two
others, testicular cells were frozen and stored in liquid nitrogen until use (Streichemberger et al., 2012).

### Spermatozoa RNA extraction

Following storage in liquid nitrogen, sample straws were thawed and the sperm were washed twice in 2 ml of PBS, and then suspended in round cell lysis buffer [0.1% (w/v) sodium dodecyl sulphate (SDS), 0.5% (v/v) Triton X-100 in RNase free H₂O]. RNA was extracted with 1 ml of Tripure (Roche) and precipitated with 20 μg of glycogen as carrier. RNAs were treated with 10 units of DNase I, room temperature 10 min, in 1X reverse transcriptase buffer with 10 mM dithiothreitol (DTT) and 20 units of Protect (Roche). The resulting cDNA was diluted to 40 ng/μl with water and 1 μl used in subsequent PCR amplification with 1X reverse transcriptase buffer with 10 mM dithiothreitol (DTT) and 20 units of Protect or RNase inhibitor (Roche).

### RT–PCR and quality control of spermatozoa RNA extracts

Before reverse transcription, RNA was purified on chromaspin 100 (Clontech) or Nucleospin RNA XS columns (Macherey Nagel). Concentration was determined using a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNAs (400 ng) were reverse transcribed in a 20 μl reaction with random nanomers (75 pmols) and Expand Reverse Transcriptase (Roche). The resulting cDNA was diluted to 40 μl with water and 1 μl used in subsequent PCR amplification with Q5 High-Fidelity DNA Polymerase (New England Biolabs).

Control PCRs were carried out with standard Taq polymerase. We checked for the presence of spermatic RNA derived cDNAs by amplifying the protamine 1 transcript with primers o1680/o1681 annealed at 60°C.

At the junction between the first B3 exon and exon 5 of LMNB2, the B3 sequence (Genbank accession KP137589) corresponds to the major transcript.

### Lamin B2 and lamin B3 constructions

In order to express lamin B2 or lamin B3 as N-terminal enhanced green fluorescent protein (eGFP) fusion proteins, we ligated the full coding sequence for each protein into a modified pcDNA4/TO vector (Life Technologies), carrying an eGFP coding region between BamH1 and EcoRI in the multiple cloning site. The coding region of lamin B2 and lamin B3 (the major transcript) were amplified from human testis cDNA with the primer pairs o4518/o4621 (B2) and o4516/o4621 (B3).

### Table I Sperm parameters of patients whose spermatozoa were used for RT–PCR, western blot (WB), immunofluorescence analysis (IF) and selection procedures.

<table>
<thead>
<tr>
<th>Patients</th>
<th>GT number</th>
<th>Sperm concentration (10⁶/ml)</th>
<th>Motility (a + b/c/d)</th>
<th>Vitality (%)</th>
<th>Normal forms (%)</th>
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GT, Germethque biobank; IF, Immunofluorescence with anti-lamin A/C, B1 and B2; WB, western blot for lamin B1 and B2.

aFertile patients. bSecondary infertility.

WHO (2009) criteria.

Normal forms (David criteria; Auger and Eustache, 2000).
Cell culture and transfection

HeLa cells were cultured according to standard procedures. Cells were transfected in two-well Lab-tek chambers using jetPRIME® short-DNA transfection protocol (Polyplus-transfection) and 500 ng of plasmid DNA according to the manufacturer’s instructions. The medium was replaced after 4 h of incubation with the transfection mix.

Spermatozoa protein extraction and western blot analysis

Protein was prepared from 12 × 10⁶ spermatozoa which were separated from round cells as for RNA extraction, and then lysed on ice for 30 min in 100 μl of 0.5% (w/v) SDS, 50 mM Tris—HCl pH 8.0, 5 mM EDTA, 50 mM DTT, 2 × complete proteinase inhibitor cocktail (Roche), followed by 30 s of sonication at high setting in a Bioruptor Standard (Diagenode). Lysate from ~2 million spermatozoa was mixed with 4 × lithium dodecyl sulphate sample loading buffer (Invitrogen) loading buffer with 5% (v/v) β-mercaptoethanol, heated to 95°C for 5 min and subjected to SDS–PAGE on a 7% (w/v) gel. For testsis, a commercially prepared protein lystate was used (Clontech).

Spermatozoa selection

Spermatozoa were selected using two techniques: a discontinuous gradient centrifugation (DGC) and a swim-up procedure. For DGC, we used three layers of Percoll (Amersham Biosciences) diluted in Ferticult (JCD) at 90, 70 and 50% (v/v). After 20 min centrifugation at 250 g, selected spermatozoa were taken from the 90% layer. For swim-up procedure, semen was washed in Ferticult medium, centrifuged 10 min at 300 g. The supernatant was discarded and 200 μl culture medium was carefully applied so as not to dislodge the sperm pellet. The tube was placed at an angle of 45° and the preparation incubated at 37°C under 5% CO₂ for 30 min. Selected motile spermatozoa were recovered from the culture medium.

The primary antibodies used were rabbit anti-Lamin B1 (1:500, ab6048 Abcam) and rabbit anti-Lamin B2/B3, (1:500, ab151735 Abcam). A cross-absorbed horse radish peroxidase conjugated anti-rabbit IgG conjugate (1:20 000, A16104 Life Technologies) was used as secondary antibody and the signal revealed with SuperSignal West Femto Substrate (Thermo Scientific).

Immunocytochemistry

Immunocytochemistry was performed on spread spermatozoa and/or testicular germ cells. Fresh selected or thawed spermatozoa were washed two times in PBS and the pellet resuspended in PBS for spreading onto polylysine coated slides by cytocpin (Shandon). Testicular cells were thawed and also spread onto polylysine slides by cytocpin (Shandon) as previously described (Metzler-Guillemain and Guichaoua, 2000). Slides were fixed 5 min in 2% (w/v) formaldehyde in PBS, rinsed in PBS and permeabilized using 0.3 or 0.5% (v/v) Triton X-100, 3% normal goat serum in PBS for 30 min. Slides were blocked with 1% (w/v) bovine serum albumin, 7% (v/v) normal goat serum in PBS for 1 h. They were then incubated for 1 h with lectins (Lectin PNA Alexa Fluor 488 conjugates, L-21409 Molecular Probes) at a dilution of 1:600 in PBS to mark the acrosome. After washes in PBS under gentle agitation, slides were incubated 1–3 h in a moist chamber at 37°C with primary antibodies directed against lamin: mouse monoclonal anti-Lamin A (1:100, ab8990 Abcam), rabbit polyclonal anti-Lamin A/C (1:100, sc 20681 Santa Cruz), mouse monoclonal anti-Lamin A + C (1:200, ab8994 Abcam), rabbit anti-Lamin B1 (1:100, ab6048 Abcam), rabbit anti-Lamin B2/B3 (1:500, ab151735 Abcam), rabbit anti-Lamin B2 (1:100, AV4636 Sigma). After several washes in PBS, detection was performed using Alexa fluor 488 goat anti-rabbit (Invitrogen). Alexa fluor 488 goat anti-mouse (Invitrogen) or anti-mouse FITC (Jackson laboratory, 515-095-062) at dilutions recommended by manufacturers for 1 h at 37°C. Slides were then rinsed twice in PBS and mounted with 25–75 ng/ml 4(6-diamidino-2-phenylindole) (DAPI) in Vectorshield mounting medium for microscope analysis. The slides were analysed using the Zeiss ApoTome.2 microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam MRm camera. Images were captured and merged with the ZEN software, and were treated using Imagej software.

Results

B-type but not A-type lamin transcripts are present in human spermatozoa

Human spermatozoa are known to retain RNA, and can be used to identify transcripts present in spermatids (Sendler et al., 2013). In order to study the expression of the known human lamins during spermiogenesis, we therefore initially performed RT–PCR on RNA extracted from purified spermatozoa from five normospermic men: two fertile donors and three from infertile couples (Fig. 1). We detected a transcript for lamin B1 in the spermatozoa RNA of all five men, but, no transcript for lamin A or lamin C. Lamin C2 primers did not amplify a product for four samples, and only very weakly for sample 1, a fertile donor, suggesting that C2 expression or retention in spermatids could be variable, but that C2 is mostly absent from spermatids. We also detected a transcript from the LMNB2 gene in all five samples with primer pairs from the 3’ end of the coding region. Since the LMNB2 primer pairs used would...
amplify transcripts encoding either lamin B2 or lamin B3, we next investigated whether this transcript encodes lamin B2, or a lamin B3 isoform homologous to that found in rodent spermatids (Schutz et al., 2005a).

**A spermatid transcript encoding a human lamin B3 isoform**

In the mouse, lamin B2 is absent from spermatids, while its isoform lamin B3 is present only in spermatids (Schutz et al., 2005a). Lamin B3 is expressed from the Lmnb2 gene using an alternative spermatid-specific promoter that, together with the first B3 exon, is situated within intron 4 of Lmnb2. As a result, lamin B3 lacks the 225 amino acids from the B2 N-terminus, which are replaced by a unique non-helical domain of 84 amino acids encoded by the first exon of the B3 transcript. The existence of this transcript, or of the lamin B3 isoform in human spermatids, is uncertain because there are no sequences at the human LMNB2 locus that could encode a peptide homologous to the mouse lamin B3 N-terminus. Nevertheless, there are four 5′-ESTs from testis transcripts that begin within intron 4 (DB457056, HY047891, HY034239 and HY007444). Although such ESTs have been presented as evidence for a human lamin B3 (von Moeller et al., 2010), no experimental data has been presented describing their expression during human spermatogenesis.

We reasoned that if these transcripts truly encode a human lamin B3 then they would, like their mouse counterpart, be expressed in spermatids. We therefore performed RT–PCR on spermatozoa RNA from five normospermic men, using primers specific for either the B2 or the B3 transcript (Fig. 2). The B3 primers, in intron 4 and exon 5 of LMNB2, amplified two fragments, which we isolated separately, following agarose gel electrophoresis, and sequenced. The longer fragment, the minor form, has the same structure as the testis EST DB457056 and differs from the shorter fragment, the major form (ESTs HY007444, HY047891, HY034239), by the use of an alternative splice donor site that adds 39 bases to the 3′ end of the first exon of the lamin B3 transcript. We detected both B3 transcripts in testis but not in brain, intestine, muscle or thymus, and only the minor transcript in prostate. In contrast, we detected the B2 transcript in all six tissues tested. We obtained a strong amplification of the major lamin B3 transcript from five spermatozoa, but we obtained no amplification of the B2 transcript from three extracts and a weak amplification from samples 3 and 5 (Fig. 2).

For three of the 5′ ESTs, the 3′ EST from the same testis cDNA is present in the databases (Fig. 2A). This provides the structure of the 3′ UTR of the lamin B3 transcript, and shows that it uses an alternative polyadenylation site that results in a very short 3′ UTR of around 70 nucleotides. The lamin B3 transcript identified in the mouse (Furukawa and Hotta, 1993) has a similar short 3′ UTR. Comparison of mouse and human sequences reveals 28/29 bases situated near the polyadenylation site that have 86% nucleotide identity, suggesting that this short 3′ UTR is a conserved feature with a function during spermatogenesis. In contrast, the lamin B2 transcript has a 3′ UTR of 2744 bp.

To investigate the use of this alternative polyadenylation signal in LMNB2 transcripts in spermatids, we performed two RT–PCR tests on spermatozoa RNA using a reverse primer specific for sequences on either side of the lamin B3 transcript polyadenylation site (Fig. 2C). We amplified a product from all samples with o4691, situated within the 3′ UTR of the short B3 transcript, but with o4717, situated beyond the 3′ end of the short B3 transcript, no amplification was observed from three samples, with a strong product for sample 3, and a faint product for sample 4. Interestingly, sample 3 was also faintly positive with the lamin B2 transcript-specific PCR (Fig. 3B), suggesting that the lamin B2 transcript was present in his spermatids. These results show that the short 3′ UTR is present in spermatids consistent with it being part of the lamin B3 transcript. They also suggest that the lamin B2 transcript can be present at variable levels in human spermatozoa.

We conclude that the vast majority of LMNB2 transcripts in human spermatids have a short alternative 3′ UTR and encode lamin B3, an N-terminally truncated isoform of the somatic lamin B2.

**Localization of lamins during human spermiogenesis**

To determine the expression pattern and cellular localization of lamin proteins during human spermiogenesis, we performed immunofluorescence analysis using antibodies specific for lamins in association with fluorescently labelled lectin to visualize the acrosome in spermatids of all stages of spermiogenesis and in mature spermatozoa (Lee and Damjanov, 1985). We did not detect a lamin A/C signal during human spermiogenesis, on either spermatids or mature spermatozoa, although the NL of somatic testicular cells was labelled (data not shown). The lamin B1 signal was present at the nuclear periphery of all human round spermatids, homogeneous in the area that is not covered by the acrosome. During spermiad maturation, the lamin B1 signal was observed to progressively slide back to the posterior pole of the nucleus as the acrosome spread. In testicular spermatozoa, lamin B1 was located behind the acrosome, at the posterior pole of the nuclei (Fig. 3). The lamin B1 signal was visible in mature ejaculated spermatozoa at the posterior pole of the nucleus in 30.8–43.1% of spermatozoa.

Using an antibody directed against a C-terminal region of lamin B2 (ab 151735) that is predicted to be common to B2 and B3, a faint signal was detectable at the nuclear periphery of human round and elongated spermatids in only one patient (T3). As for lamin B1, the B2/B3 signal was not visible in the area covered by the acrosome, retreated during spermiad maturation and was visible, but very weak, at the posterior pole of the testicular spermatozoa (Fig. 4). Using a different antibody (Sigma AV46356), specific for the N-terminal region of lamin B2, we obtained a very faint lamin B2 signal on spermatids and testicular spermatozoa in the same patient, but the percentage of each cell type labelled was reduced compared with the lamin B2/B3 antibody (Fig. 3B). The three anti-lamin B antibodies gave a strong perinuclear signal in human Sertoli cells (data not shown). The increased labelling observed with the anti-lamin B2/B3 antibody compared with the anti-lamin B2 antibody, taken together with the extremely low level of lamin B2 transcripts in the spermatozoa of at least four of the five men tested here, is in favour of the presence of the lamin B3 protein in human spermatids, but a specific antibody will be required to confirm this. We did not detect a lamin B2 or B3 signal in human mature ejaculated spermatozoa.

**Detection of lamins in purified spermatozoa by western blot analysis**

In order to further investigate the B-type lamins retained in the ejaculated spermatozoa, we performed western blot analysis of protein extracts of purified spermatozoa from three normospermic men using antibodies against the lamins B1, B2/B3 or B2. We detected lamin B1 only (Fig. 5).
The lamin B3 transcript, but not that of lamin B2, predominates in human testis and is present in spermatids. (A) Schematic representation of the lamin B2 and B3 isoforms relative to the LMNB2 gene. The approximate positions of primers used for the specific detection of transcripts for each isoform are shown relative to the exons of the LMNB2 gene. Available testis ESTs derived from putative lamin B3 transcripts are schematised. These reveal the alternative 3′ UTR used by lamin B3 transcripts and the structure of the minor transcript created by the use of an alternative splice donor site in the first B3 intron that extends the coding region of the first B3 exon by 39 in-frame bases, resulting in a predicted lamin B3 isoform with 13 amino acids inserted between the N-terminal domain and the rod domain. (B) RT–PCR analysis of lamin B2 and lamin B3 transcripts in spermatozoa and six tissues: brain, small intestine, prostate, thymus, testis and skeletal muscle. (C) RT–PCR analysis of the 3′ UTR of LMNB2 transcripts present in spermatozoa. The primers o4505/o4691 (upper panel) amplify all LMNB2 transcripts, while o4505/o4717 amplify only transcripts that have a 3′ UTR that extends beyond the 3′ end of lamin B3 transcripts present in Genbank. (B and C) PCR products were migrated on 3% agarose gels. Spermatozoa RNAs were extracted from samples from individuals 1–5 in Table I. Indicated to the right of each gel are the protein or proteins encoded by the transcripts detected, the gene from which the transcripts originate and the primer pair used. The expected sizes of amplicons are shown to the left of the gels. The ladder is the 1 kb plus (Life technologies), and the 300 bp band is indicated with a (+).
This finding is consistent with our IF results, where B1 was the only lamin to be detected on ejaculated spermatozoa.

By western blot analysis of the whole testis lysate with the anti-lamin B2/B3 antibody, we did not detect a band at the size expected for the lamin B3 isoform (53 kDa). We also failed to detect a testis-specific band of 53 kDa on a multiple tissue western blot analysis with 40 μg of each protein lysate (data not shown). This does not necessarily mean that lamin B3 is not expressed, but it does show that, if present, lamin B3 levels are much lower than lamin B2, in whole testis. This is consistent with the expectation that lamin B3 is only expressed in spermatids while lamin B2 is expressed in all the somatic cells of the testis. In the mouse, lamin B3 was not detected with an antibody detecting both lamin B2 and B3, it was only detected with a specific antibody (Vester et al., 1993; Schutz et al., 2005a). A specific anti-lamin B3 antibody will be required to obtain definitive proof for the presence of lamin B3 protein in human spermatids.

**Lamin B1 on selected human spermatozoa**

In mice, lamin B1 was not detected in mature spermatozoa by immunofluorescence. We therefore asked whether the retention of an immunofluorescent lamin B1 signal on human spermatozoa might be a marker of spermatozoon quality. To address this question, we compared lamin B1 labelling of spermatozoa before and after standard selection protocols for either normal head density (DGC) or motility (swim-up). Two sperm samples, R 011400037 and R 011400041, were analysed, and, prior to selection, a lamin B1 signal was detected on 47 and 33% of ejaculated spermatozoa, respectively (n = 250). After DGC, the percentage of lamin B1 labelled spermatozoa was very significantly lower for both samples at 6 and 2%, respectively (n = 250, P < 0.0001; Table II). In contrast, after the swim-up selection procedure, the incidence of lamin B1 labelling was only moderately reduced on selected spermatozoa, 29 and 23%, respectively (n = 250, P = 0.017 for sample R 011400037; Table II).

Our results indicate that the loss of the lamin B1 immunofluorescent signal from human spermatozoa may be linked to the completion of sperm head compaction and thus could serve as a marker of spermatozoa quality.

**Deformation of HeLa nuclei in cells transfected with human lamin B3 isoform**

In a study focused on the NL of amphibian germ cells (von Moeller et al., 2010), the ectopic expression of a human lamin B3 isoform in Xenopus oocytes was shown to cause an accumulation of short structures on the NL, but evidence of nuclear deformation in early Xenopus embryos or Cos-7 cells expressing human lamin B3 was cited as unpublished data only. The lamin B3 isoform used was overexpressed using a cDNA (5′ EST BM563418) derived from an mRNA expressed in brain and not in testis. The full sequence of this brain cDNA is not in the public databases, and therefore, although its partial sequence tag is identical to the other 5′ tags derived from the major testis transcript, it is formally possible that the complete brain cDNA has a structure distinct from the major B3 transcript expressed in spermatids. Overall we concluded that the effects of the human spermatid lamin B3, on the structure of the mammalian nucleus, required confirmation.

To investigate if human lamin B3 can be directed to the nuclear envelope, and to evaluate its effect on nuclear envelope structure, HeLa cells were transfected with two constructs expressing either B2 or B3 fused at their N-terminus to eGFP: eGFP-B2 or eGFP-B3 fusion proteins,
respectively. For eGFP-B3 the coding region of the major transcript was used. In transfected cells, each fusion protein localized predominately to the nuclear periphery. However, the fusion proteins had very different effects on nuclear morphology. We observed that 9.6% of cells expressing the eGFP-B2 fusion showed some deformation of their nucleus \((n = 512)\), mostly a slightly curved aspect, but all other cells remained ovoid in shape. In contrast, 93% of the cells expressing the eGFP-B3 fusion showed strong deformations of their nucleus \((n = 520)\), which appeared multi-lobulated, distorting into a C-shape or an S-shape (Fig. 6A).

These results show that the presence of the human lamin B3 isoform has the ability to destabilize the nuclear envelope. We asked if this deformability could be related to a disorganization of the lamina by the displacement of B1. We show that in eGFP-B3 transfected HeLa cells, and in eGFP-B2 transfected cells, lamin B1 is present at the nuclear periphery, but the peripheral B1 signal appears weaker in eGFP-B3 than in eGFP-B2 transfected cells (Fig. 6B). We conclude that the ectopic expression of lamin B3 does not induce a fundamental reorganization of the NL. Nevertheless the weaker peripheral B1 signal observed, indicates that lamin B3 may displace some lamin B1 from the nuclear envelope, although lamin B1 could appear weaker because it is spread over an increased surface area in the undulating B3-deformed nuclear envelop.

**Discussion**

**NL is similar in human and rodent spermatids**

Our results show that the NL of human spermatids lacks A-type lamins, and is composed of the B-type lamins B1, B2 and probably B3. A similar composition of the NL has been described in rodent spermatids, although no trace of lamin B2 was found in any post-natal germ cells (Vester et al., 1993; Schutz et al., 2005a). Although we lack definitive proof that the lamin B3 protein is present in spermatids, we show for the first time that a transcript encoding a protein with the same structure as the rodent lamin B3 protein is expressed in human spermatids, while the transcript encoding lamin B2 is mostly absent, or present at a very low concentration. Furthermore, we demonstrate that the human lamin B3 protein shares the capacity of its rodent counterpart to induce a generalized deformation of the nuclear envelope, when overexpressed in HeLa cells (Schutz et al., 2005b). We also show that, exactly as reported in rodents, the B-type NL of human spermatids is absent from the nuclear envelope directly under the developing acrosome and that it recedes towards the posterior pole as the spermatids progress through spermiogenesis (Vester et al., 1993; Schutz et al., 2005a; Pierre et al., 2012). The striking similarities between the structure and behaviour of the rodent and human NL provide strong evidence that the


**Figure 4** Immunolocalization of lamin B2 on human spermatids and testicular spermatozoa from patient T3. (A and B) Staining pattern of lamin B2 (A: anti-Lamin B2 specific, green and B: anti-Lamin B2/B3, green) in successive steps of human spermiogenesis. Spermatids are identified using lectin PNA (red) and counterstained with DAPI (blue). Exposures for the two antibodies were normalised on Sertoli cell labelling. (C) Percentages of germ cells stained with anti B2 specific (blue) and anti B2/B3 (red) antibodies on post-meiotic germ cells. Scale bar = 10 \(\mu\)m.
shared features, outlined above, are of functional significance during mammalian spermiogenesis.

**Lamin B3 and nuclear remodelling during human spermiogenesis**

It has previously been shown in rodents that the spermatid NL contains the spermatid-specific lamin B3 (Schutz et al., 2005a, b). In the mouse, lamin B3 lacks the 225 N-terminal amino acids of lamin B2, comprising the short head domain and a large part of the alpha-helical rod domain, which is substituted by a unique non-helical domain of 84 amino acids (Furukawa and Hotta, 1993). In the human, the B3 spermatid transcript described here has the same N-terminal portion of lamin B2 replaced by a domain of 91 amino acids that shows very low amino acid identity with its mouse counterpart (12 amino acid identities out of 91). This low degree of conservation implies that the B3-specific non-helical N-terminal domain might not have an important function. In keeping with this, it has been shown that it is the absence of the lamin B2 alpha-helical domains from lamin B3 and not the presence of the lamin B3-specific N-terminal head domain that destabilises the nuclear periphery in monkey kidney fibroblast COS-7 cells (Schutz et al., 2005b), although contradictory findings come from an earlier study in which a mouse lamin B3 protein with the B3-specific N-terminus replaced by a haemagglutinin tag, did not provoke nuclear deformation in murine fibroblastic COP5 cells (Furukawa and Hotta, 1993). Further work is therefore required to clarify how lamin B3 contributes to nuclear remodelling.

To the best of our knowledge, the present report is the first to show that a lamin B3 transcript is expressed in human spermatids, or indeed in the spermatids of any mammalian species other than rodents.

**The NL of human spermatids**

Until our study, nothing was known about the composition of the NL during human spermiogenesis. Only one study had investigated the NL during spermatogenesis, reporting the presence of A-type lamins in spermatocytes, and B1 and B2 lamins in spermatogonia, but providing no data for spermatids (Machiels et al., 1997). Our results show that the human spermatid NL is devoid of A-type lamins, and that the B-type lamins are gradually removed from the nuclear envelope, a process that begins at the anterior pole in round spermatids and proceeds to the posterior pole. Interestingly, the human peripheral blood granulocyte, with its highly plastic polymorphic nucleus, is characterized by an absence of lamin A/C, B2 and by low levels of B1 (Olins et al., 2008). Reduced amounts of lamins may therefore be a cellular mechanism for diminishing mechanical resistance of the nucleus. During human spermiogenesis, the spermatid nucleus is strongly re-shaped, particularly at the anterior pole. The lack of lamin A/C, and the loss of B-type lamins during spermiogenesis, may allow a ‘softening’ of the nuclear envelope that enables its deformation. Moreover, the lamin B3 isoform whose putative transcript, we show to be present in human spermatids, has the capacity to destabilise the nuclear structure, and has been suggested to be an additional factor involved in attaining a malleable spermatid nucleus (Schutz et al., 2005b). The incorporation of lamin B3 into the polymerising lamin filaments may produce a less rigid lamina network, which could favour both its remodelling and its anterior-to-posterior dismantlement.

According to our results, the NL of human spermatids may contain very low levels of lamin B2. In rodents, lamin B2 was not detected in spermatids, or indeed in any post-natal germ cell (Vester et al., 1993), although lamin B2 has been reported to be present in human spermatogonia (Machiels et al., 1997). Our RT–PCR analysis of spermatozoa RNA shows that lamin B2 transcripts are produced at extremely low levels (two men) or are undetectable (three men) (Fig. 2). We nevertheless did detect a lamin B2 signal with a specific antibody in the spermatid nucleus of the T3 subject by immunofluorescence, but a semen sample was nevertheless did detect a lamin B2 signal with a specific antibody in the spermatids of the T3 subject by immunofluorescence, but a semen sample was not available to us to check for the lamin B2 transcript. It is possible that the presence of lamin B2 in spermatids could vary between individuals, as has been suggested for other proteins (de Vries et al., 2013). The analysis of more biopsies should resolve this issue, although the near impossibility of obtaining both spermatozoa and a testicular biopsy for a homospermic man will make it difficult to know whether the lamin B2 found in

**Table II** Percentages of spermatozoa labelled by anti-lamin B1 from patients 12 and 13, before and after the DGC or Swim-up selection protocol.

<table>
<thead>
<tr>
<th>Patient</th>
<th>GT number</th>
<th>Fresh sample %</th>
<th>DGC %</th>
<th>Swim-up %</th>
</tr>
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<tr>
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<td>R01400037</td>
<td>47</td>
<td>6**</td>
<td>29*</td>
</tr>
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<td>13</td>
<td>R01400041</td>
<td>33</td>
<td>2**</td>
<td>23</td>
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</tbody>
</table>

GT, Germetetheque biobank.

**Figure 5** Protein expression of B1 and B2 lamins by western blot analysis of human spermatozoa lysates. Results are shown for human testis (10 μg) and three spermatozoa samples (2 × 10⁶ spermatozoa) corresponding to individuals 5–7 in Table I. For each antibody, the results presented are from the same membrane, and all conditions, exposure and image treatment are identical for both panels. The anti α-tubulin antibody was used on the protein extracts as a loading control.
human spermatids is the result of neo-expression in spermatids or the persistence of lamin B2 from earlier germ cell stages.

**NL polarization during human spermiogenesis**

Our results demonstrate that, in human, the localization of lamin B1 and B2/B3 changes during the spermatid maturation process. Both B1 and B2/B3 signals appear at the nuclear periphery in round spermatids, except in the area covered by the acrosome. As spermiogenesis progresses, the signals of the B-type lamins slide back and are progressively restricted to the posterior pole of the elongated spermatids. The same dynamic profile was described for mouse lamin B1 and B3 in round and elongated spermatids, with the B3 signal undetectable at later stages (Schutz *et al.*, 2005a). Unlike in mouse, where no B1 or B3 is detected on the mature epididymal spermatozoa (Pierre *et al.*, 2012), in human, the B1 signal remains detectable in ~30–50% of mature ejaculated spermatozoa. This suggests either that the specific posterior localization of B1 has a functional significance for human spermatozoa, or that NL evacuation is less efficient during human spermiogenesis than it is in the mouse. Our demonstration that retention of B1 is reduced to <6% on spermatozoa selected on the basis of normal head density strongly supports the latter possibility, and suggests that retention of B1 may be a marker of poor sperm quality.

In human spermatids the onset of chromatin remodelling has been shown to occur concomitantly with acrosome and perinuclear theca development. Moreover, a gradual decrease of nucleosomes and several histone post-translational modifications start at the apical pole of the nucleus in human round spermatids in a region situated directly under the spreading acrosome (De Vries *et al.*, 2012). Considered together with our results, and those from rodents, showing that the anterior limit of the receding NL appears to be adjacent to the posterior limit of the advancing acrosome, these observations strongly suggest a parallel kinetics between chromatin remodelling and the restructuring of the NL. Moreover, our finding that lamin B1 labelling of ejaculated spermatozoa decreases from 30–50% to <6% following selection (DGC) for spermatozoa with normal head density, provides further independent evidence that chromatin remodelling and NL dismantlement are synchronized during spermiogenesis.

The posterior pole of the spermatozoon nucleus is also characterized by its proximity to the sperm centriole and the implantation fossa, where the flagellum is anchored to the nucleus. It has been reported that lamin B1 serves the fundamental role within the somatic cell nuclear envelope of anchoring the nucleus to the cytoskeleton (Ji *et al.*, 2007), and that NE-associated proteins involved in LINC complexes are pivotal determinants of cell architecture and polarization (Schneider *et al.*, 2011).

**Figure 6** Distribution of eGFP-lamin B2 and eGFP-lamin B3 ectopically expressed in HeLa cells. (A) Expression of eGFP-lamin B2 and eGFP-lamin B3 (green) in HeLa cell nuclei (counterstained with DAPI, blue), showing that the eGFP-lamin B3, but not the eGFP-lamin B2, induces strong nuclear deformation. For lamin B3, the coding region used corresponds to that of the major transcript. (B) Immunolocalization of lamin B1 on eGFP-lamin B2 and eGFP-lamin B3 transfected HeLa cells. Scale bar = 20 μm.
A study of LINC complexes during mouse spermiogenesis revealed that the formation of the mammalian sperm head involves assembly and polarization of spermiogenesis-specific LINC complexes that probably connect the spermiad nucleus to the surrounding cytoskeleton (Gob et al., 2010). This suggests that, in mammals, lamin B1 or B3 are likely to be involved in the process of positioning and anchoring the flagellum to the spermiad nucleus through interactions with as yet unknown proteins. Our analysis of spermatooza selected for motility, and thus normal flagellar development (swim-up), revealed only a weak correlation between lamin B1 labelling and motility, indicating that the complete dismantlement of the NL is not a prerequisite for normal flagellar function. Nevertheless, the importance of lamin B1 or B3 for the different functional aspects of the mammalian spermatoozon remains to be determined.

In conclusion, our results strongly reinforce the hypothesis that the nuclear shape modifications that occur during spermiogenesis are linked to the remodelling of the exclusively B-type lamin NL in mammals. The specific role of the lamin B3 isoform remains to be determined in mammals. Further experiments are now needed to clarify to what degree the B-type lamins contribute to the quality of the mature spermatoozon.

Supplementary data
Supplementary Material is available at http://molehr.oxfordjournals.org/ online.

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Authors’ roles
R.E. performed most of the experimental work, and was involved in the manuscript writing. V.A. and J.-M.G. played a role in the patient recruitment. G.L. organized and supervised cell transfection and RT–PCR experiments. M.J.M. performed western blot experiments. M.P. was in charge of spermatooza selection experiments. C.M.G. played a role in the patient recruitment and was particularly involved in the study conception and the manuscript writing with M.J.M. and N.L. C.M.-G. took direct responsibility for the manuscript with M.J.M. All authors contributed to the writing and revision of the manuscript.

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Conflict of interest
None declared.

References


