

## Reduced Fertility in Male Mice Deficient in the Zinc Metallopeptidase NL1

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**Members of the M13 family of zinc metalloendopeptidases have been shown to play critical roles in the metabolism of various neuropeptides and peptide hormones, and they have been identified as important therapeutic targets. Recently, a mouse NL1 protein, a novel member of the family, was identified and shown to be expressed mainly in the testis as a secreted protein. To define its physiological role(s), we used a gene targeting strategy to disrupt the endogenous murine *Nli* gene by homologous recombination and generate *Nli* mutant mice. The *Nli*<sup>-/-</sup> mice were viable and developed normally, suggesting that zygotic expression of *Nli* is not required for development. However, *Nli*<sup>-/-</sup> males produced smaller litters than their wild-type siblings, indicating specific male fertility problems. Reduced fertility may be explained by two impaired processes, decreased egg fertilization and perturbed early development of fertilized eggs. These two phenotypes did not result from gross anatomical modifications of the testis or from impaired spermatogenesis. Basic sperm parameters were also normal. Thus, our findings suggest that one of the roles of NL1 in mice is related to sperm function and that NL1 modulates the processes of fertilization and early embryonic development in vivo.**

A wide variety of biologically active peptide hormones, neuropeptides, and regulatory peptides are proteolytically activated or inactivated by zinc metalloproteases (58, 59). The M13 family of zinc metalloproteases attracts much interest because its members are involved in the metabolism of several regulatory peptides of the mammalian nervous, cardiovascular, inflammatory, and immune systems as well as in the mineralization process (8, 56, 57, 59). For these reasons, several members of this family of peptidases have been selected as targets for therapeutic intervention by inhibitors or pharmacological agents.

NL1 (also called SEP or NEP2) is the newest member of the M13 family of zinc metallopeptidases (19, 25, 42), which also includes neprilysin (neutral endopeptidase 24.11; also called NEP and CALLA) (reviewed in reference 11), the endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2) (reviewed in reference 55), PHEX (formerly PEX; a phosphate regulating gene with homologies to endopeptidases on the X chromosome) (reviewed in reference 54), the Kell blood group protein (reviewed in reference 35), and ECEL1/DINE (endothelin-converting enzyme-like 1/damage-induced neuronal endopeptidase) (29, 60, 61). *Nli* cDNA was simultaneously cloned by reverse transcription-PCR technology with degenerate oligonucleotide primers based on conserved sequence similarity

between members of the family and mRNA isolated from mouse and rat testis or from *Ece-1*<sup>-/-</sup> embryos (19, 25, 42). Finally, a clone coding for a putative human homologue has been obtained from a central nervous system cDNA library (4).

As observed for other members of the M13 family of peptidases, the *Nli* cDNA encodes a type II transmembrane glycoprotein encompassing a short N-terminal cytoplasmic tail, a single transmembrane domain, and a large C-terminal extracellular region that contains the zinc binding motif HEXXH, where X is any amino acid. However, in contrast to the other members, NL1 can be expressed as both a membrane-bound and a soluble protein (25). Differential splicing introduces an additional exon that causes the insertion of a furin-like cleavage site at the C-terminal end of the transmembrane domain and the release of the protein into the extracellular space. Data from reverse transcription-PCR experiments suggest that the secreted form of NL1 is mainly found in the testis, whereas the membrane-bound form is expressed in the remaining expressing sites (25).

NL1 degrades a broad variety of small peptides. Oligopeptides shorter than 3 kDa and containing neutral bulky aliphatic or aromatic amino acid residues are preferred substrates. These characteristics are close to those described for neprilysin. In fact, all peptide substrates identified so far for NL1 are also metabolized by neprilysin (48). Furthermore, for many peptides, both enzymes cleave at the same amide bond. As expected, prototypical transition state inhibitors of neprilysin, such as phosphoramidon and thiorphan, and dual neprilysin-angiotensin-converting enzyme inhibitors such as omapatrilat

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TABLE 1. Oligonucleotide sequences used for PCR amplification of genomic DNA

Primer pair	Sense primer	Reverse primer
1	5'-CAGCACCGTACCTCACACCC-3'	5'-CTGGTCTCTGCTCTCAATGCTG-3'
2	5'-TCTTAAGTACCTGCTACACTTCTCC-3'	5'-TGCTATCACACAGCTTGGGGTG-3'
3	5'-TGCACCACCCCAAGCTGTGTGATAGC-3'	5'-TGGGATCACGTGGTGCCTCAGCC-3'
4	5'-TCGAGGAACCCCTGTGAAAATTCTACC-3'	5'-TCGATGAGGACCCGCTGTTGAACTG-3'
5	5'-AGTTCACCAGGCGGGTCTCATCG-3'	5'-AGATGCGACTCCAGTCCAGCACCTCC-3'
6	5'-ACTATGCTTAGGAAAGACCAGAACC-3'	5'-TCATCTGGCAACAGCTCGACTTCC-3'
7	5'-TTGTCTTCTGTGGAAGTCGAGCTGTTCC-3'	5'-TTGACATAGCTGACACACTCTCGCC-3'
8	5'-TGGCGAGAGTGTGTCAGCTATGTCAAC-3'	5'-TCTTCCAAAATGTAGTCAGGGTAGC-3'
9	5'-TGGCTACCTGACTACATTTTGGAAAG-3'	5'-AGGCTCCTTGGGCATTTGTTCTTG-3'
10	5'-ACTTCAGAACCTCAAGAACAATGC-3'	5'-TAGCCCCCAAATTC AAGGAC-3'
11	5'-ATTCTCCAGCCGCCCTTCTTCAGC-3'	5'-ACCTCCGTTGTGCGGCAATGTTCTCC-3'
12	5'-AGAACATTGCCGACAACGGAGG-3'	5'-TCCGTCTTGATGGACTGGACGG-3'
13	5'-AGTTCGCCGTCCAGTCCATCAAGACG-3'	5'-ACCTGCACATCTCTGCCAGCTAC-3'

also inhibit NL1 enzymatic activity (19, 48). NL1 is more closely related to neprilysin, with 55% amino acid identity, than to any other members of the family.

Northern blot analyses with total RNA isolated from a variety of mouse tissues reveal strong expression of the *Nli* gene in the testis, while other tissues are negative (19). By in situ hybridization, *Nli* mRNA is detected in round and elongated spermatids in seminiferous tubules of the mature testis at all spermiogenesis maturational stages, suggesting that the predominant role of NL1 in the mouse may be related to sperm functions (19). Nevertheless, with the more sensitive reverse transcription-PCR technology, weak expression of *Nli* mRNA is also detected in several other tissues, such as heart, spleen, lungs, liver, kidney, intestine, and brain (19, 25). In situ hybridization experiments on mouse sections reveal the presence of *Nli* mRNA in neurons of the ventroposterior regions of the brain as well as in the anterior and intermediate lobes of the pituitary gland (16).

Although NL1 enzymatic activity and tissue expression has been studied extensively, no physiological function has yet been identified for this protein. To gain more insight into its role(s) in mammals, we disrupted the *Nli* gene by homologous recombination and produced *Nli*-deficient mice. Here, we report the reduced fertility of *Nli*<sup>-/-</sup> mutant male mice.

#### MATERIALS AND METHODS

**Gene targeting.** The genomic organization of the *Nli* gene was characterized by PCR amplification as done previously (6). Briefly, several pairs of overlapping primers (Table 1) spanning the entire *Nli* cDNA sequence were used to amplify mouse 129/SvEv genomic DNA. The resulting PCR products were cloned, and the intron-exon junctions were sequenced with the Sanger dideoxynucleotide chain termination method (50). To construct the targeting vector, two genomic fragments were PCR amplified. The 5.6-kb 5'-end fragment and the 4.1-kb 3'-end fragment were amplified with the primer pairs 5'-AGTGACATCTGTG GACCACACCCCAAGCTG-3' (sense; SalI restriction site in italics) and 5'-ATGCGTCTCCAGTTCAGCACCTC-3' (antisense) and 5'-CCAGGAGAA AAGGCTCGAGATGATGTCACTGC-3' (sense) and 5'-CCAAAATGTAGTC TCGAGAGGGTAGCCAATC-3' (antisense; XhoI restriction site in italics), respectively. The 5'-end fragment was digested with SalI and HindIII (which removes a 729-bp fragment at the 3' end of the amplification product), whereas the 3'-end fragment was digested with XhoI. The genomic fragments were cloned on each side of the neomycin selection cassette in the PGKneo vector. A counterselection herpes simplex virus thymidine kinase cassette was inserted at the 3' end of the homology region of the targeting vector (2, 34).

WW6 embryonic stem (ES) cells were electroporated with 25 µg of SalI-linearized targeting vector DNA as previously described and plated on neomycin-resistant feeder layers (2). After 24 h, selection for G418 (400 µg/ml; Invitro-

gen, Burlington, Ontario, Canada) and ganciclovir (2 µM; Syntex, Palo Alto, Calif.) was applied. ES cell clones recovered from drug selection were screened by Southern analysis. Positive clones were injected into MF1 blastocysts and transferred into pseudopregnant foster mothers to generate chimeras that were tested for germ line transmission as previously described (2, 9, 20, 34).

**Genotyping of ES cells and mice.** Genomic DNA was isolated from ES cells or mouse tail biopsies. Extracts were treated with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation (24). Purified DNA was digested with restriction enzymes as indicated in Fig. 1, fractionated by electrophoresis through 0.8% agarose gels, blotted onto a Hybond-NX membrane (Amersham Biosciences, Baie d'Urfé, Québec, Canada), and hybridized by following the procedures recommended by the supplier for either the 5' *Nli* probe (the first 733 bp of the *Nli* cDNA sequence, nucleotides 332 to 1064 of GenBank accession number AF176569) or the 3' *Nli* probe (the last 1,000 bp of the *Nli* cDNA, nucleotides 1925 to 2925 of GenBank accession number AF176569).

**Northern blot analysis.** Total RNA from testes was isolated with the RNeasy column kit (QIAGEN, Chatsworth, Calif.). Equal amounts (12.5 µg) of total RNA isolated from *Nli*<sup>+/+</sup>, *Nli*<sup>+/-</sup>, and *Nli*<sup>-/-</sup> specimens were electrophoresed on a 1.5% formaldehyde agarose gel, transferred onto a Hybond N+ blotting membrane, and hybridized with the <sup>32</sup>P-labeled 3' cDNA probe described above. The 645-bp β-actin fragment corresponding to nucleotides 333 to 978 of the coding sequence (GenBank accession number NM\_007393) was used as a quantitative control. Samples from three *Nli*<sup>+/+</sup>, four *Nli*<sup>+/-</sup>, and four *Nli*<sup>-/-</sup> adult mice aged between 3 and 6 months were analyzed.

**Histology.** Testes were fixed in 4% paraformaldehyde for 16 h at 4°C and embedded in paraffin wax as previously described (27). Specimens were sectioned at 5 µm and stained with hematoxylin and eosin for general histological examination. Sections from four wild-type and five *Nli*<sup>-/-</sup> males aged from 3 to 7 months were analyzed.

**Sperm motility analysis.** Spermatozoa were collected from the caudal epididymides of six wild-type and six *Nli*<sup>-/-</sup> males in HMB fertilization medium (62) and incubated for 30 min in 5% CO<sub>2</sub>-90% O<sub>2</sub>-air. Motility parameters were measured by computer-assisted analysis with a Ceros Analyzer (software version 12.0F; Hamilton-Thorn Research, Beverly, Mass.). The following parameters were measured: (i) average path velocity (velocity along the average path of the spermatozoon), (ii) straight-line velocity (calculated from the straight-line distance between the start and the end of the observed track), (iii) curvilinear velocity (the total distance divided by time), (iv) lateral head displacement (the deviation of the head from the mean head trajectory), (v) beat cross frequency (beats per second, the number of times the spermatozoon head crosses the head trajectory per seconds), (vi) straightness (calculated as straight-line velocity/average path velocity × 100), (vii) linearity (calculated as straight-line velocity/curved-line velocity × 100). The settings for the Ceros analyzer were 30 frames captured for analysis at 60 Hz (frame rate, 0.50), minimum contrast 30, and minimum cell size 4 pixels; progressive cells were defined as having an average path velocity of >50 µm/s and straightness of >50%, and slow cells were defined as having an average path velocity cutoff of 10 µm/s.

**Blastocyst analysis.** F<sub>1</sub> *Nli*<sup>+/-</sup> females (aged 2 to 5 months) were mated with wild-type or mutant males (aged 2 to 5 months). Three days following the detection of a copulatory plug, females were sacrificed and embryos were recovered from uterine horns in phosphate-buffered saline. Embryos were counted and photographed for microscopic analysis. A total of five wild-type and five *Nli*<sup>-/-</sup> males were mated with nine and 11 females, respectively.

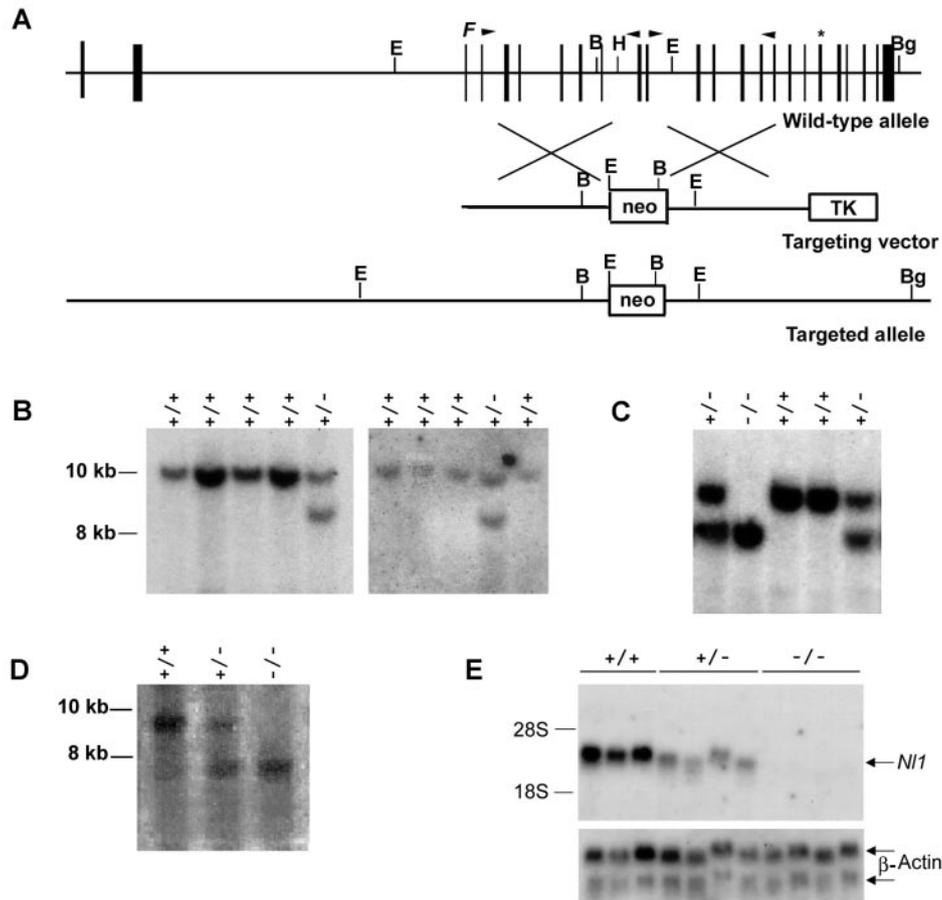


FIG. 1. Disruption of the *NII* gene. (A) Schematic representation of the wild-type mouse *NII* gene, the targeting vector, and the targeted *NII* allele. Vertical black boxes represent exons. The positions of the neomycin resistance gene (*neo*) and the herpes simplex virus thymidine kinase gene (*TK*) are shown. The *neo* cassette is flanked at its 5' and 3' ends by 4.9 and 4.1 kb of *NII* genomic sequence, respectively. *F* indicates the position of the convertase cleavage site, and the asterisk shows the position of the NL1 active site. B, BamHI; Bg, BglII; H, HindIII; E, EcoRI. Arrowheads indicate the positions of oligonucleotide primers used to PCR amplify genomic DNA to generate the vector. (B and C) Southern blot analyses of DNA isolated from ES cell clones (B) and mouse tails (C). Genomic DNA was digested with BglII and BamHI and transferred to nylon membranes, and the blots were hybridized with an *NII* probe located 3' of the targeting vector sequence (described in Materials and Methods). Fragments from the wild-type (+/+; 9.6 kb) and mutated (-/-; 8.1 kb) alleles were detected. (D) Southern blot analysis of genomic DNA digested with EcoRI, transferred to nylon membranes, and hybridized with an *NII* probe located 5' of the targeting vector sequence (described in Materials and Methods). Fragments from the wild-type (+/+; 8.9 kb) and mutated (-/-; 7.3 kb) alleles were detected. (E) Northern blot analysis of total RNA (12.5  $\mu$ g) isolated from the testes of three wild-type, four *NII*<sup>+/-</sup>, and four *NII*<sup>-/-</sup> mice. RNA was hybridized with either the 3'-derived *NII* or *actin* probe.

**In vivo fertilization analysis.** Thirty-two C57BL/6  $\times$  CBA F<sub>1</sub> females aged between 24 and 28 days were superovulated by intraperitoneal injection of 5 U of pregnant mare's serum (Intervet, Whitby, Ontario, Canada) at 1:00 p.m., followed by 10 U of human chorionic gonadotropin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) 46 h later. The evening after the second injection, superovulated females were housed with single *NII*<sup>+/+</sup> or *NII*<sup>-/-</sup> males. The next morning, the males were removed and mating was confirmed by detection of a copulatory plug. At 0.75 day postcoitus, the females were killed and the embryos were collected from the ampulla of the oviduct in M2 medium (24) and digested with 300  $\mu$ g of hyaluronidase (embryo tested; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) per ml. Embryos were first washed twice in M2 medium and then twice in M16 medium before incubation in M16 medium at 37°C in 5% CO<sub>2</sub> for 4 days. Embryo development was monitored daily by microscopic inspection. Litters from the mating of 18 females with five different wild-type males and from 14 females with five different *NII*<sup>-/-</sup> mutant males were recovered. Males of both genotypes were age matched.

## RESULTS

**Generation of *NII* mutant allele in ES cells.** As a first step toward the generation of a targeting vector for *NII* genetic

elimination, the structure of the murine *NII* gene was defined. Based on the high conservation of the genomic organization of the members of the neprilysin family, genomic DNA was PCR amplified with several primer pairs derived from the *NII* cDNA sequence. The resulting PCR products were sequenced, and the intron-exon junctions were determined (Table 2). The mouse *NII* gene is divided into 24 small exons distributed over at least 27 kb of genomic DNA (Fig. 1A). The size of exons varied from 60 to 391 bp, whereas the size of introns ranged from 75 bp to over 8 kb. The ATG initiation start site was located in the second exon and the protein convertase cleavage site was in the third exon, whereas the HEITH motif, the signature of zinc metalloproteinases, was found in exon 19. The third exon is alternatively spliced in the membrane-bound form of NL1 (46). The positions of the intron-exon junctions were mostly conserved between the *NII* and *Neprilysin* genes (12).

To construct the targeting vector designed to eliminate the

TABLE 2. Exon-intron boundaries of the murine *Nli* gene

Exon no.	Intron 3' acceptor site	Exon			Intron 5' donor site	Intron size (kb)
		5' end	Size (kb)	3' end		
1	Undetermined	Undetermined	>0.141	GCACCAG	GTAGGATT...	1.7
2	...CCCTCCTACACAG	GGTCCCC	0.315	AGCATAG	GTGAGCAAGGA...	>8
3 <sup>a</sup>	...TTTCTCCCCAG	GGAAGCA	0.069	AAACGAG	GTAGCCTTTACC...	0.55
4	...CTGTTCTCCAG	CCCTCAG	0.060	ATAGCAG	GTGAGCTTCTCC...	0.84
5	...CCTTAATACAAAG	CTGCCAG	0.161	CTCAAAG	GTGAAGAGTGG...	0.28
6	...CTGCCACAG	GGGTGCT	0.081	AACCAAA	GTGAGTAGAGCC...	1.24
7	...CCCCTTCCAG	GTGTGAT	0.096	ACCATGG	GTGAGTATCGG...	0.46
8	...AACTCCTCAG	GCCTCAA	0.119	TCATCTAC	GTATGCAATGCCCT...	0.52
9	...GTGTGTTTCCAG	ATAGACC	0.065	CCACAAG	GTAAGCAGGG...	1.18
10	...CCTTTGGCCAG	GTACGGA	0.075	GGCCAAC	GTGAGGCGGGG...	0.07
11	...CCCCACACAG	GCCACAG	0.090	TCTGAAG	GTGAGACCCAGGCA...	1.66
12	...ACCCCTTACAG	GGGTTTA	0.137	CAGCACG	GTGAGCCCAGGG...	0.28
13	...GCTACCTTAG	GACCATG	0.094	CCGCAAG	GTGAGCGCAT...	0.81
14	...AACACACAG	GCGTGT	0.129	GAGCACG	GTGCGTATCCTTGA...	0.51
15	...GCATTCCCCAG	GTCAGAG	0.099	GGAAAAG	GTGGGCAAGCC...	0.36
16	...CCTCCACTCAG	GCCATGA	0.084	CTCCAGT	GTGCGTACCTTCC...	0.39
17	...CTTCTCATAG	TTGACTT	0.104	AGAATCT	GTGAGTAGGATGG...	0.30
18	...ATTACACAG	CTGGATC	0.059	CAGATCG	GTAAGTCCACC...	0.34
19	...GTGGGGGACAG	TCTTTCC	0.120	GATAATG	GTAAGCAGCA...	0.48
20	...TCCTGCACAG	GTGAACG	0.134	CCAGAAT	GTGAGCATGTC...	0.11
21	...TCTTTTTATGCAG	GTGAACG	0.066	ATACAAG	GTGGGCCCTA...	0.46
22	...TGCTCGACAG	GCTTACC	0.096	TGCCAAG	GTAATCCCTAG...	0.27
23	...ATGCATCTCCAG	GTGTGGT	0.077	AGTACAG	GCAAGTGTGC...	0.09
24	...TGGTCTACAG	GGTGCTG	≥0.391	Undetermined		
Consensus	(y) <sub>12</sub> NCAG <sup>b</sup>	N		AG	GT	

<sup>a</sup> Exon 3 is differentially spliced to generate the membrane-bound and secreted forms of NLI.

<sup>b</sup> y, pyrimidine nucleotide; N, any nucleotide.

mouse *Nli* gene in ES cells by homologous recombination, two noncontiguous DNA fragments from the *Nli* gene were PCR amplified and cloned on each side of the PGKneo cassette. A cDNA cassette coding for herpes simplex virus thymidine kinase was included at the 3' end of the targeting vector for negative selection against random integration of the vector. The *Nli* 5'-end (4.9-kb) and 3'-end (4.1-kb) fragments encompass regions that include exons 4 to 9 and exons 11 to 16, respectively. An intervening region of 823 bp was omitted between the 5' and 3' fragments. Therefore, integration of the vector into the *Nli* gene locus by homologous recombination deletes exon 10 and part of exon 11, modifying the open reading frame of the gene.

The vector was electroporated into ES cells, and positive and negative selections were applied to obtain clones that were resistant to G418 and insensitive to ganciclovir. Homologous recombination events were detected by Southern blot analyses. Genomic DNA from all recovered clones was digested with BglII and BamHI restriction enzymes, and membranes were hybridized with a probe generated from the *Nli* cDNA sequence flanking the 3'-end fragment of the targeting vector (Fig. 1B). Digestion of wild-type and *Nli* mutant alleles generated fragments of 9.6 and 8.1 kb, respectively. A targeting event was detected in 2 of 480 ES cell clones analyzed.

**Generation of *Nli* mutant mice and Mendelian distribution of offspring.** Targeting ES cell clones were injected into mouse MF1 blastocysts, and chimeric transmitters were obtained. Chimeric males were bred either with MF1 females or with 129/SvEv females to establish the mutation in an inbred background. All subsequent phenotypic analyses were done with animals from the outbred background. Heterozygous mice de-

veloped normally and showed no obvious abnormalities. To determine the effect of the *Nli* mutation in homozygous animals, heterozygous intercrosses were performed and offspring were analyzed. Progeny from heterozygous intercrosses were genotyped, and *Nli*<sup>-/-</sup> mice were recovered (Fig. 1C). Table 3 shows that the incidence of *Nli*<sup>+/+</sup>, *Nli*<sup>+/-</sup>, and *Nli*<sup>-/-</sup> genotypes corresponds to a Mendelian distribution, as expected for random allele segregation and inheritance.

Mice were further genotyped to test whether integration at the 5' end of the *neo* cassette also occurred by homologous recombination (Fig. 1D). Digestion of genomic DNA with EcoRI enzyme and hybridization of the membrane with a 5' probe generated fragments of 8.9 kb for the wild-type allele and 7.3 kb for the mutated allele in *Nli*<sup>+/-</sup> mice but only the 7.3-kb fragment in *Nli*<sup>-/-</sup> mice. Altogether, these results indicate that the vector was integrated by homologous recombination and exclude any gross rearrangement at either end of the targeting site.

As a means to monitor *Nli* expression in mutant mice, Northern blot analyses were performed with RNA isolated from the testis. As expected, no *Nli* mRNA was detected in

TABLE 3. *Nli* genotypes of the F<sub>2</sub> progeny from F<sub>1</sub> heterozygote intercrosses

Genotype	No. (%) of F <sub>2</sub> mice per genotype	Expected Mendelian values (%)
+/+	20 (20.6)	25
+/-	55 (56.7)	50
-/-	22 (22.7)	25

TABLE 4. Reduced fertility of male *Nli*<sup>-/-</sup> mice

Parents (female × male)	No. of productive matings	Avg litter size (no. of pups)
+/- × +/+	18	8.9
+/- × +/-	20	7.85
+/- × -/-	21	4.7 <sup>a</sup>
+/+ × +/-	9	7.44
-/- × +/-	11	7.45
+/+ × +/+	7	10.1

<sup>a</sup> *P* = 0.0004 for variance between -/- and +/+ males and *P* = 0.0393 for variance between -/- and +/- males (one-way analysis of variance).

*Nli*<sup>-/-</sup> specimens (Fig. 1E). The levels of *Nli* mRNA were reduced by about half in heterozygous mice compared to *Nli* expression in wild-type mice (Fig. 1E). These results clearly demonstrated that no *Nli* expression occurs in *Nli*<sup>-/-</sup> mice, indicating that the mutation generated is a null allele.

***Nli*<sup>-/-</sup> male mice show reduced fertility.** Mutant *Nli*<sup>-/-</sup> mice did not exhibit obvious physical or behavioral abnormalities. Therefore, since NL1 is predominantly expressed in the round and elongated spermatids, we first tested whether disruption of the *Nli* gene impairs male fertility. The fertility of *Nli*<sup>+/+</sup>, *Nli*<sup>+/-</sup>, and *Nli*<sup>-/-</sup> male and female mice was compared with different combinations of intercrosses (Table 4). First, heterozygous *Nli*<sup>+/-</sup> females were crossed with wild-type, heterozygous, or homozygous mutant males. Wild-type males produced an average of 8.9 pups per litter, while *Nli*<sup>-/-</sup> males generated only 4.7 pups per litter. Intercrosses with *Nli*<sup>+/-</sup> males produced an intermediate average of 7.4 pups per litter. The differences observed in litter sizes produced by *Nli*<sup>-/-</sup> males compared to those produced by wild-type and *Nli*<sup>+/-</sup> males were statistically significant (Table 4). About 20% of the *Nli*<sup>-/-</sup> males did not produce any progeny when mated with *Nli*<sup>+/-</sup> females, even if they were housed together for several months. These males were not included in the calculation of the average litter size in Table 4. These results suggest that the loss of *Nli* function results in male hypofertility.

We also tested female fertility. Wild-type, heterozygous, and homozygous mutant females were bred with heterozygous *Nli*<sup>+/-</sup> males. The average litter size obtained for matings between *Nli*<sup>+/-</sup> males and *Nli*<sup>-/-</sup> or *Nli*<sup>+/+</sup> females was 7.4 and 7.5, respectively (Table 4), which was equivalent to the values for heterozygous male and female intercrosses, indicating that fertility is not affected in female mice. The small difference in the litter size between wild-type breedings (10.1) and crosses between wild-type males and *Nli*<sup>+/-</sup> females (8.9) was not statistically significant (*P* = 0.9348).

**Testis development and spermatozoa are normal.** To determine whether the reduced fertility of *Nli*<sup>-/-</sup> mice resulted from a spermatogenic defect, a microscopic analysis of the seminiferous tubules was conducted. Testis sections from four wild-type and five *Nli*<sup>-/-</sup> males showing various stages of the spermatogenic cycle were stained with hematoxylin and eosin. No major difference was observed, and the morphology of the tubules as well as the relative numbers of spermatogonia, spermatocytes, and round and elongated spermatids were similar

between control and *Nli*<sup>-/-</sup> mice (Fig. 2). The histological characteristics of testes from mutant males that were determined to be infertile from the absence of progeny were also normal. Overall, the testis morphology of *Nli*<sup>-/-</sup> mice was similar to that of wild-type littermates.

We also compared the motility parameters of spermatozoa collected from wild-type and *Nli*<sup>-/-</sup> males. Although most of the motility parameters of *Nli*<sup>-/-</sup> spermatozoa were slightly lower than those of *Nli*<sup>+/+</sup> spermatozoa, the difference was not statistically significant (Table 5).

***Nli* mutation reduces both fertilizing capacity and early embryonic development.** No morphological anomaly appears to be responsible for the reduced litter size phenotype observed with *Nli*<sup>-/-</sup> males. To determine if defects in sperm fertilization ability and/or early embryonic development underlie the phenotype, outbred F<sub>1</sub> *Nli*<sup>+/-</sup> females were mated with either wild-type or *Nli*<sup>-/-</sup> males, and embryos were collected at 3.5 days postcoitum. Microscopic examinations revealed that 74% of the embryos issued from crosses with *Nli*<sup>+/+</sup> males were in the late morula or blastocyst stage, compared to 33% of those fertilized by *Nli*<sup>-/-</sup> males (Table 6; Fig. 3). This significant difference (chi square test, *P* < 0.0001) was very close to that observed in the litter size, suggesting that early development is perturbed when the sperm originates from *Nli*<sup>-/-</sup> mice.

To determine whether fertilization was normal and define at which embryonic stage development was impaired, a time course experiment was performed. Superovulated C57BL/6 × CBA F<sub>1</sub> virgin females were mated with either wild-type or *Nli*<sup>-/-</sup> males. Embryos were collected around 0.75 day postcoitum and their development was pursued in culture for 4 days. Embryo development was monitored by daily microscopic observations. Around 1.0 day postcoitum, 62% of the embryos collected from matings with wild-type males were fertilized and had divided. In contrast, only 38% of the embryos obtained from matings with *Nli*<sup>-/-</sup> males had reached cell division (Fig. 4). These results indicated that the fertilization rate was reduced by 39% when the sperm originated from *Nli*<sup>-/-</sup> males. However, 2 days after fertilization, the *Nli*<sup>-/-</sup> sperm fertilization ability was only decreased by 15% compared to wild-type sperm, suggesting that, in addition to fertilization defects, the absence of NL1 causes a delay in the first cell division(s). At 3.5 and 4.5 days postcoitum, the number of morulae and blastocysts generated following fertilization with *Nli*<sup>-/-</sup> sperm was 30% lower than that obtained with wild-type sperm. These results suggest that the *Nli* mutation reduces the ability of fertilized eggs to develop normally. Differences in the distribution of embryos resulting from *Nli*<sup>-/-</sup> and *Nli*<sup>+/+</sup> males mated with *Nli*<sup>+/-</sup> females were statistically significant at each day of development (Fig. 4). We cannot rule out the additional possibility that early processes of preimplantation development may also be perturbed by the mutation. Altogether, these anomalies explain the reduced litter size observed in matings with *Nli*<sup>-/-</sup> males, since the differences closely reflect the variation detected in the litter size.

## DISCUSSION

In this study, we disrupted the *Nli* gene by homologous recombination and generated *Nli*<sup>-/-</sup> mice. The mutation pre-

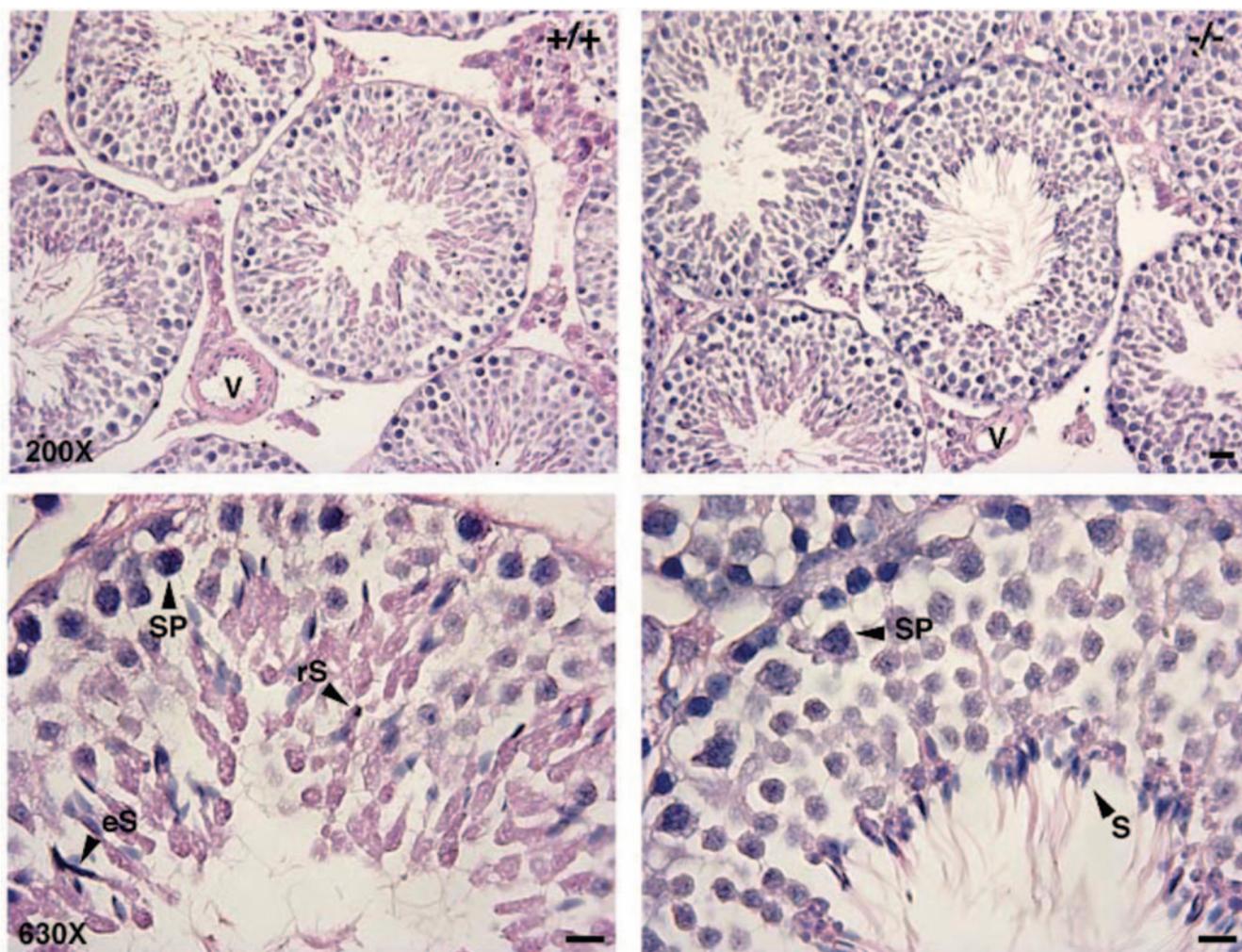


FIG. 2. Histological analysis of testes from *Nll*<sup>-/-</sup> and *Nll*<sup>+/+</sup> mice. Hematoxylin and eosin staining of thin sections from control (+/+) and *Nll* mutant (-/-) mice was performed. Arrowheads indicate round spermatid (rS), elongated spermatids (eS), pachytene spermatocytes (SP), and spermatozoa (S). V, blood vessel. Bar, 10  $\mu$ m.

vents the synthesis of both the transmembrane and secreted forms of NL1 protein. Although our anti-NL1 antibodies did not allow us to directly test whether the protein was present or not in the mutant mice, we demonstrated that *Nll* mRNA was not expressed in testes from *Nll*<sup>-/-</sup> mice, supporting the notion that the protein is not produced. This result is in concordance with the fact that the designed targeting vector adds a new polyadenylation site after exon 9, interrupting transcription of the *Nll* gene. Matings of heterozygous mice produced the expected Mendelian ratio of wild-type, heterozygous, and homozygous mutant mice, and *Nll*<sup>-/-</sup> mice were viable and anatomically normal. Thus, zygotic expression of *Nll* does not seem to be required for mouse development. The data are consistent with our recent *in situ* hybridization studies showing that *Nll* mRNA is not expressed in embryonic day 12 to 17 mouse embryos (7). Targeted disruption of other members of the M13 family has been reported. While elimination of the *Nephrilysin* gene results in only a mild phenotype, disruption of the *ECE-1*, *ECEL1*, or *Phex* gene causes severe developmental defects (33, 49, 51, 63, 64).

We showed that NL1 plays a significant role in male fertility but is not essential for the fertility of females. This result is consistent with the expression profile of *Nll* mRNA in mouse tissues, which is strong in germ cells of the mature testis but weak in ovaries (25). The expression of the fertility impairment among *Nll*<sup>-/-</sup> males was variable. For instance, approximately 20% of the *Nll*<sup>-/-</sup> outbred males did not produce offspring, in contrast, about 10% of the *Nll*<sup>-/-</sup> outbred males had litters approximating wild-type numbers. The variable phenotype in *Nll*<sup>-/-</sup> males is not unprecedented and most probably reflects the segregation of genetic modifiers on the mosaic genetic background, as was shown for at least two other mutations in sperm (39, 43). For instance, male mice bearing mutations in the *spmn-1* transcription factor gene or in the *smcp* mitochondrial protein gene are sterile only if these mutations are maintained in the 129/Sv genetic background (15, 43). Moreover, males deficient in the sperm protein SED1 show a similar variation in litter size (ranging from no pups to litter sizes approaching normal) in an outbred background (15). Testing this hypothesis, however, must await the characterization of

TABLE 5. Comparison of sperm from wild-type and *Nli*<sup>-/-</sup> mice<sup>a</sup>

Parameter	Mean value ±SD for mice with genotype:		P
	+/+	-/-	
Motile sperm (%)	87.2 ± 6.66	78.3 ± 10.01	0.1184
Progressively motile (%)	40.5 ± 3.2	33.2 ± 3.2	0.1368
Avg path velocity (μm/s)	100.1 ± 4.6	86.8 ± 7.6	0.1642
Straight-line velocity (μm/s)	64.2 ± 3.6	55.5 ± 4.3	0.155
Curved-line velocity (μm/s)	186.9 ± 7.2	161.3 ± 12.9	0.1147
Lateral head displacement (μm)	9.8 ± 0.09	9.4 ± 0.2	0.0903
Beat cross frequency (beat/s)	24.3 ± 0.9	25.1 ± 1.2	0.6081
Straightness	58.8 ± 0.7	60.2 ± 0.8	0.2368
Linearity	32.7 ± 0.8	33.0 ± 0.4	0.7342
Total count	1109 ± 198	828 ± 181	0.3203

<sup>a</sup> Seven mice of each genotype were studied; six fields per mouse were analyzed. The parameters examined are defined in Materials and Methods.

the *Nli*<sup>-/-</sup> male fertility phenotype in different inbred genetic backgrounds.

**NL1 modulates two different processes related to fertility.** Our results suggest a role for NL1 in two developmental processes, egg fertilization and early embryo development. First, spermatozoa from *Nli*<sup>-/-</sup> mice appeared to be less efficient than those produced by *Nli*<sup>+/+</sup> mice for egg fertilization despite normal sperm motility parameters and no major morphological defect of the testis. The fact that NL1 is mostly expressed as a secreted protein in spermatids is consistent with a role in a step that occurs outside of the testis. Accordingly, a neprilysin-like activity has been detected in the sperm of several species (3, 13, 23, 52), although such an activity has not been confirmed by others (10, 17). Generation of specific inhibitors and/or antibodies will be required to determine whether this activity is due to the presence of NL1. It is thus possible that the *Nli* phenotype is linked to defects in sperm maturation or capacitation or sperm-related processes during fertilization.

Several proteases are known to modulate the different steps of sperm maturation that lead to fertilization (31). Some of these are released during the acrosome reaction and play important roles in the fertilization process itself (31, 53). Consistently, several bioactive peptides are either coexpressed with NL1 in testicular germ cells, packaged in the sperm acrosome, and/or released during the acrosome reaction (1, 5, 22, 28). Among them, the enkephalins, which have been shown to be stored in the acrosome of human, hamster, and sheep spermatozoa and released following the acrosome reaction (28), decrease mouse fertility when overexpressed in the testis of transgenic mice (41). These results support the notion that the

levels of neuropeptides are critical for proper fertilization processes. Since some of these bioactive peptides are known substrates of NL1 in vitro, the *Nli* phenotype could be partly explained by a lack of their regulation in vivo.

Second, a large proportion of eggs did not develop normally when fertilized with the *Nli*<sup>-/-</sup> mutant spermatozoa. We must emphasize that defects in embryo development are not likely to be due to the lack of zygotic *Nli* expression, since we did not observe selective elimination of *Nli*<sup>-/-</sup> offspring. The phenotype appears to be associated with the absence of NL1 in sperm. A growing body of evidences supports an important role(s) for spermatozoa and/or its components in early embryonic development (44, 45, 65). Therefore, through its action on active peptides, it is possible that NL1 may regulate sperm-dependent spatial patterning of the early mouse embryo, a sperm-activated block to polyspermy, and/or proteolytic activation of morphogens.

**NL1 is downstream of the protein convertase PC4.** Cleavage and release into the extracellular fluids of the secreted testicular form of NL1 is accomplished by a member(s) of the prohormone convertase family (19). In the testis, the nature of the prohormone convertase that cleaves NL1 remains to be defined. However, a good candidate is the prohormone convertase PC4 because it is abundantly and specifically expressed in the spermatocytes and round spermatids of rodents (38). Moreover, PC4 homozygous mutant males show reduced fertility in the absence of any evidence of spermatogenic abnormality (36), similar to what we observed in *Nli*-deficient mice. Interestingly, the reduced fertility of *Pc4*<sup>-/-</sup> males is due to a combination of both reduced efficiency in egg fertilization and abnormal embryo development after fertilization. Therefore, it is possible that some of the phenotypes attributed to *Pc4* gene elimination are directly related to the failure of *Pc4*-deficient mice to properly process NL1, the lack of secreted NL1 protein in *Pc4*<sup>-/-</sup> mice causing the phenotypes. Analysis of NL1 maturation in *Pc4*<sup>-/-</sup> mice would help in answering this question.

The *Pc4* phenotype is broader than that observed in *Nli*<sup>-/-</sup> mice, suggesting that NL1 acts downstream of PC4. For instance, the hypofertility observed in males is more important in *Pc4* mutants than in *Nli* mutant mice. Second, *Pc4*<sup>-/-</sup> females are less fertile, while the fertility of *Nli*<sup>-/-</sup> females appears normal. Finally, in intercrosses of heterozygous mutant mice, the recovery of *Pc4*<sup>-/-</sup> mutant animals was reduced, conversely to what was observed for transmission of the *Nli* mutation. These observations indicate that NL1 is not the only substrate for PC4 and that the absence of maturation of another bioactive polypeptide(s) may be responsible for the additional phenotypes detected in *Pc4* mutants. Alternatively, the differences can be explained by variations in the genetic back-

TABLE 6. Fertilizing capacity of *Nli*<sup>-/-</sup> males and early embryo development

Genotype	No. <sup>a</sup>	No. of eggs harvested	No. (%) of embryos at 3.5 days after mating at development stage <sup>b</sup> :						
			1	2	3	4	M	B	Dead
+/+	9	88	1 (1%)	2 (2%)	1 (1%)	4 (5%)	22 (25%)	43 (49%)	15 (17%)
-/-	11	80	3 (4%)	3 (4%)	3 (5%)	0	14 (17%)	13 (16%)	44 (54%)

<sup>a</sup> Total number of mated *Nli*<sup>+/+</sup> females.

<sup>b</sup> Each column represents the number of embryos for each developing stage: 1, 2, 3, 4 cells, morulae (M), and blastocyst (B) collected at 3.5 days postconception. Chi square test, *P* < 0.0001.

ground in which the two studies were conducted (C57BL/6J versus outbred).

**Putative functional compensation by other peptidases.** The relatively mild phenotype observed after genetic elimination of the *NII* gene suggests that other peptidases may compensate for the loss of NL1. A strong candidate is neprilysin, since NL1 and neprilysin have overlapping sites of expression and similar enzymatic properties and degrade the same set of peptides (32). It may not be surprising then that mutant mice lacking *Neprilysin* function are viable and appear developmentally normal except for some minor effects on lymphoid development. Therefore, it is possible that the NL1 and neprilysin enzymes have redundant functions and that the expression of one enzyme can compensate for the absence of the other in mutant animals. The production of compound *NII Neprilysin* mutants should test this hypothesis.

Although neprilysin has been shown to be expressed in the testis, *Neprilysin*<sup>-/-</sup> mice have no reported fertility problems. Since neprilysin is membrane bound, it might compensate for the lack of some of the membrane-bound NL1-related functions in the testis of *NII*<sup>-/-</sup> mice, but it might not rescue the phenotypes associated with loss of the secreted form of NL1

protein. In other tissues, NL1 and neprilysin are both believed to be membrane-bound proteins. In the brain, their sites of expression are rather complementary, although many regions expressed both enzymes (7, 16). It will be interesting to study the effect of NL1 deficiency on brain function. For example, injection of thiorphan in the rat brain region that controls nociception causes analgesia resulting from enhanced extracellular concentration of enkephalins (47). This effect was believed to be due to the pharmacological inhibition of neprilysin activity. However, *Neprilysin* mutant animals were unexpectedly reported to exhibit hyperalgesia in the hot plate and tail withdrawal tests (18). This suggests that neprilysin may not be involved in this function and/or that other enzymes closely related to neprilysin, such as NL1, may be involved in the analgesic effect of thiorphan.

Other zinc proteases are known to be involved in processes related to fertility (10, 13, 14, 40). Any of these peptidases could play a role in the partial rescue of the NL1 phenotype. For example, the testicular form of the angiotensin-converting enzyme could be a good candidate because it is specifically expressed in developing spermatids, and angiotensin-converting enzyme mutant mice show reduced fertility (21). Further-

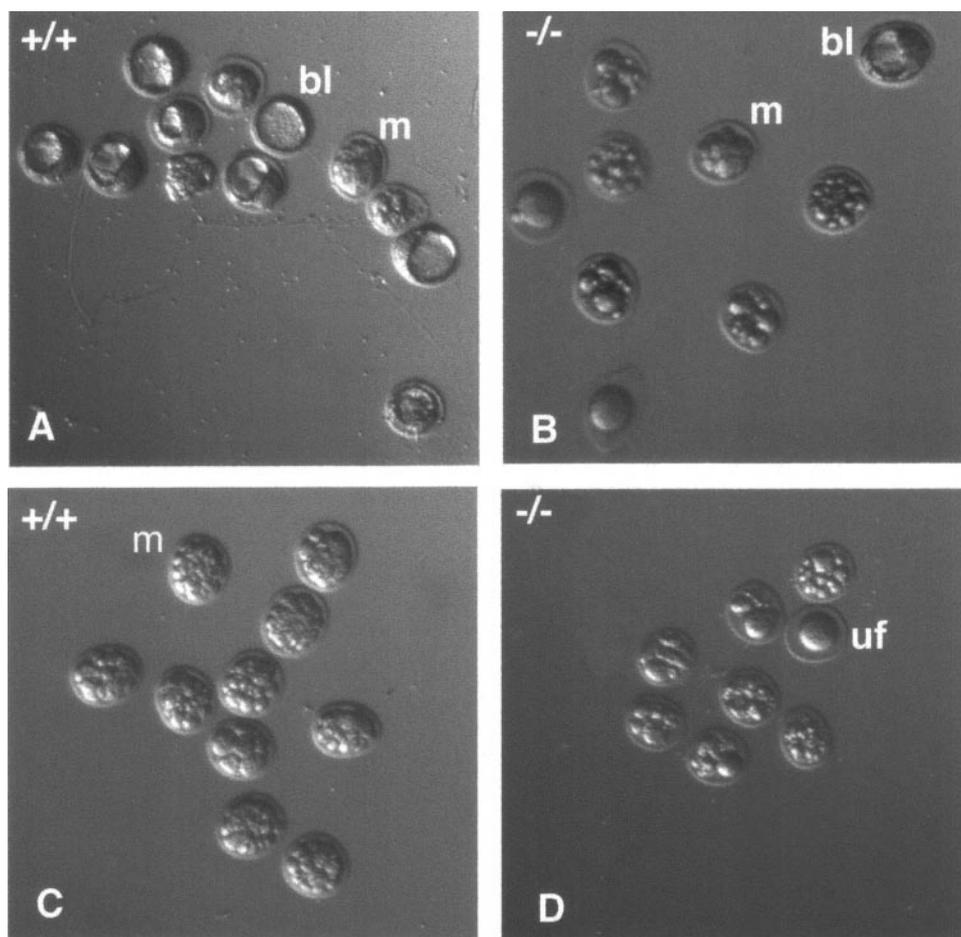


FIG. 3. Embryo recovery at day 3.5 of gestation. Four representative litters from *NII*<sup>+/-</sup> females fertilized by *NII*<sup>+/+</sup> (A and C) or *NII*<sup>-/-</sup> (B and D) males are shown. Morulae (m), blastocyst (bl), and unfertilized (uf) eggs are indicated. A larger proportion of embryos were well developed when fertilized by *NII*<sup>+/+</sup> males compared to those fertilized by *NII*<sup>-/-</sup> males. A total of five wild-type or mutant males were mated with 9 and 11 females, respectively. Embryos were collected at 3.5 days postconception. Magnification, 50 $\times$ .

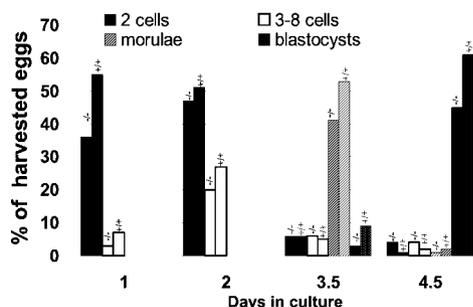


FIG. 4. In vivo fertilization analysis. C57BL/6  $\times$  CBA F<sub>1</sub> females were superovulated and mated with *Nli*<sup>+/+</sup> or *Nli*<sup>-/-</sup> males. Embryos were collected at day 1 postconception and cultured in vitro for 4 days. Embryo development was monitored by daily microscopic analysis. Bars labeled -/- indicate eggs fertilized by *Nli*<sup>-/-</sup> males, while bars labeled +/+ correspond to eggs fertilized by wild-type males. Chi square ( $\chi^2$ ) and Jonkheere-Terpstra (JT) analyses revealed that the distribution of developing embryos was statistically different at each day (day 1:  $\chi^2$ ,  $P < 0.0001$ , and JT,  $P < 0.0001$ ; day 2,  $\chi^2$ ,  $P < 0.0024$ , and JT,  $P < 0.0003$ ; day 3.5,  $\chi^2$ ,  $P < 0.0001$ , and JT,  $P < 0.0001$ ; day 4.5,  $\chi^2$ ,  $P < 0.0001$ , and JT,  $P < 0.0001$ ).

more, NL1 and angiotensin-converting enzyme have overlapping substrate specificities and hydrolyze several common peptides in vitro. Other peptidases of the M13 family of metalloproteases such as Phex, ECE-1, ECE-2, and ECEL1 have also been detected in the testis (26, 30, 37, 60), but their role in male fertility has not yet been established.

The production of *Nli* null mice constitutes an important step in the understanding of the physiological importance of NL1 in mammals. NL1 deficiency has been proven to impair normal fertilization processes. Further investigation will be necessary to precisely determine the molecular basis of the fertility defect, at the level both of the fertilization processes and of the early steps in embryonic development. Moreover, these basic investigations could be clinically relevant because the human homolog of NL1, MMEL2, has recently been identified (4). Therefore, mutations in this gene may be responsible for some of the fertility problems found in humans. Future studies evaluating the importance of MMEL2 in human reproduction processes will contribute to our understanding of the multiple factors involved in male fertility.

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