Identification of the Gene *imds-60* in Mouse Epididymis

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Abstract Sperm maturation, including the acquisition of motility and the full ability to fertilize oocyte, occurs during its transit through the dynamic environment of the epididymis. However, the roles of many genes involved in the process of sperm maturation still remain to be found. Based on an expressed sequence tag named *imds-60*, which was first found in uterus but is highly expressed in epididymis, the full-length cDNA sequence of *imds-60* with a complete open reading frame was obtained in mouse epididymis by GenBank searching, polymerase chain reaction-based procedures, and 5′- and 3′-rapid amplification of cDNA ends. This protein was predicted to have an N-terminal signal peptide and a C-terminal DNase I-like domain with nine transmembrane motifs in the middle part of the protein. Northern blot analysis showed that the mRNA of *imds-60* was highly expressed in epididymis but at a rather lower level in uterus, seminal vesicle gland, and stomach. Further study revealed that the mRNA of *imds-60* is only expressed in corpus and cauda regions of epididymis, not in caput. It is regulated partially by androgen and peaked in male mice aged from 3 weeks to adult. The imds-60 protein might play an important role in cell communication during sperm maturation.

Keywords epididymis; DNase I-like domain; mouse

The mammalian epididymis is important for sperm storage, protection, and maturation [1–3]. Epididymal sperm-coating proteins might exert their effects on the male gamete already in the epididymis or become functional in the female tract. Some sperm decapacitating factors might associate to the cell surface and prevent premature sperm activation, and other epididymal proteins might act directly in the process of gamete recognition and interaction. Although cross-species homology analysis has suggested the conservation as well as possible functions of these proteins and the existence of a sperm maturation process in humans has been reported [4,5], the functions of epididymal proteins in sperm maturation have not been completely understood [6].

Mammalian DNases have been classified as DNase I and DNase II. DNase I is one of the most characterized mammalian endonucleases. However, besides the DNases, several DNase I-like human genes/proteins have been cloned in the last 10 years to characterize their activities, namely, DNase X/Xib [7,8], DNase γ/DNAS1L3/LS-DNase [9–11], and DNAS1L2 [10]. The DNases of the DNase I family are now being recognized and becoming a focus of research for their clinical application in cystic fibrosis, a lethal disease common in white populations [12, 13]. Moreover, the presence of DNase I-like proteins in bull accessory sex gland indicated that these proteins might participate in fertility of bulls [14].

Here we identified a novel gene *imds-60* in mouse epididymis. The gene was first found in uterus but is highly expressed in epididymis, and is similar to *Homo sapiens* hypothetical protein FLJ21511. Moreover, a C-terminal DNase I-like domain exists in the imds-60 protein.

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Materials and Methods

Animals

BALB/c and C57BL/6 mice were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China).

Tissue samples and RNA extraction

Male C57BL/6 mice were killed and different tissue samples were obtained and frozen immediately in liquid nitrogen. The samples of uterus were obtained from female BALB/c and C57BL/6 mice. The epididymis was divided into three parts: caput, corpus, and cauda. Total RNA was isolated by acid phenol extraction [15] or a Trizol reagent kit (Invitrogen, Grand Island, USA).

Sequence identification

By constructing the subtracted cDNA library of mouse implantation-site uterus plus embryo at 4.5 d post-fertilization using the polymerase chain reaction (PCR)-select cDNA subtraction kit (Clontech, Palo Alto, USA), a 313 bp cDNA-expressed sequence tag (EST) sequence (pTADVIMDS-60; GenBank accession No. BG797173) was obtained. Tester double strain (ds)DNA was prepared from the mRNA samples of mouse implantation-site uterus plus embryo at 4.5 d post-fertilization. Driver dsDNA was prepared from the mRNA samples of mouse 4.5 d inter-implantation-site uterus and 5.5 d embryo. By searching the BLAST database (http://www.ncbi.nlm.nih.gov/blast/) using the 313 bp EST, a predicted mouse sequence was found. A pair of primers was designed according to this predicted sequence: 5′-TTCCGGGTGGTCGCAGTCAT-3′ (P1) as the forward primer, and 5′ -TGATGGGATTC-TCATTGCCG-3′ (P2) as the reverse primer (**Fig. 1**).

Reverse transcription was carried out according to the standard protocol of the Superscript pre-amplification system (Gibco BRL, Grand Island, USA). The PCR was carried out using *Ex Taq* (TaKaRa, Dalian, China) with the following conditions: 94 ºC for 3 min, then 30 cycles of 94 ºC for 1 min, 60 ºC for 50 s, 72 ºC for 1.5 min, and finally 72 ºC for 10 min. The PCR products were analyzed on 1% (*W*/*V*) agarose gels. The target product was cloned into pGEM-T easy vector (Promega, Shanghai, China) and sequenced by Invitrogen.

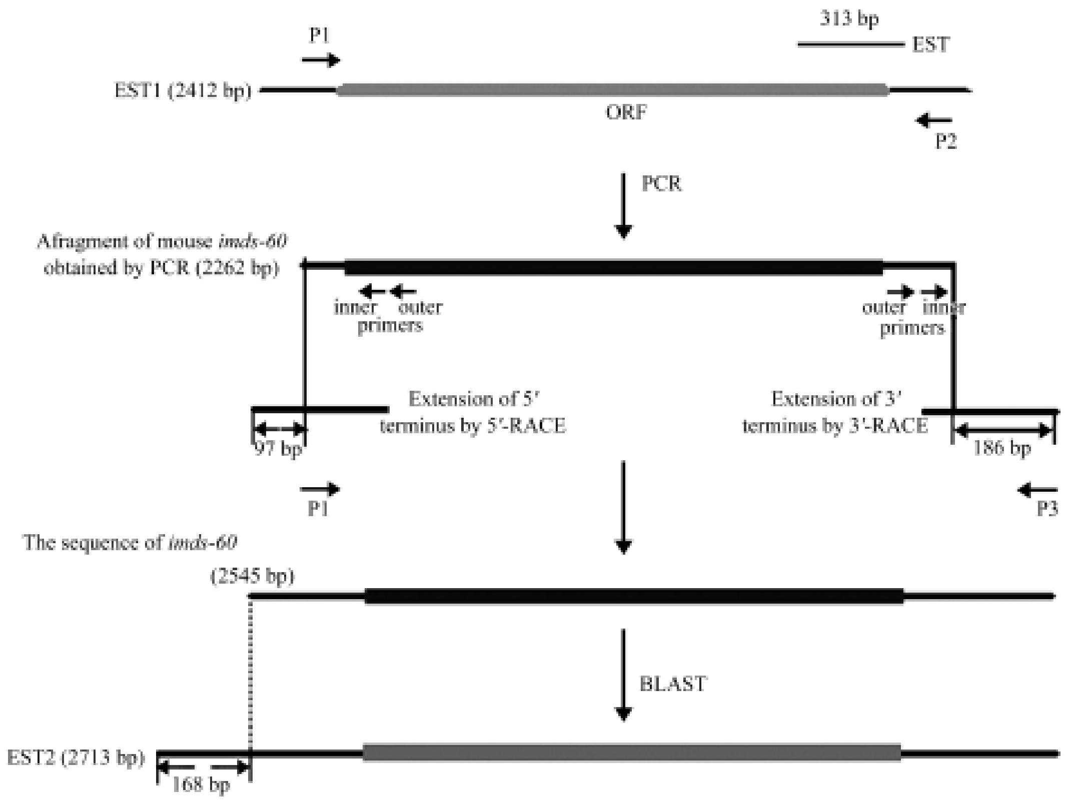

Fig. 1 Diagram of cloning for *imds-60* gene

EST, expressed sequence tag; ORF, open reading frame; P1, forward primer; P2, reverse primer; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

The 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE were carried out according to the protocol of the FirstChoice RLM-RACE kit (Ambion, Austin, USA). The specific outer and inner primers for 5′-RACE were 5′-CAGCCCTGTGAGCTCCAGCGTT-3′ and 5′-GCAGGGGAAAATAATAGATCATGGGGCT-3′, respectively, and the specific outer and inner primers for 3′-RACE were 5′-AAAATGTCACGGGGAAGCCAAAA-3′ and 5′-GTGACATCCGGCAATGAGAATCCCA-3′, respectively.

The open reading frame of the gene was predicted using DNAStar software and the potential structure components of the predicted protein were forecasted with Interproscan (http://www.ebi.ac.uk/InterProScan/), Signal P (http://www.cbs.dtu.dk/services/SignalP/), and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1).

Northern blot analysis

The samples of various mouse tissues were pooled from six adult (56 d old) C57BL/6 mice. Northern blot was carried out as described previously [16]. An α-^{32}P-labeled *imds-60* cDNA sequence (2262 bp) was used as a probe for hybridization. The membrane was subject to autoradiography at –80 ºC overnight. RNA expression levels were quantified using a Gel-Pro Analyzer 3.0 (Microsoft, Redmond, USA) and normalized by 18S ribosomal RNA.

Developmental change and androgen regulation analysis

Adult (56 d old) male C57BL/6 mice were castrated by surgery. Northern blot for detecting the expression pattern in androgen manipulation was carried out as described previously. The RNA was also pooled from five mice [17].

Developmental change analysis was carried out by Northern blot. The RNA samples were prepared from pooled epididymis (six mice/group), with different ages, that is, at week 1, 2, 3, 4, and 8, and at month 4 and 12 after birth.

Results

Cloning of *imds-60* cDNA sequence

A 313 bp cDNA EST was found from the subtracted cDNA library of mouse implantation-site uterus plus embryo at 4.5 d post-fertilization. The results of Northern blot analysis indicated it was highly expressed in epididymis with this 313 bp cDNA as probe (data not shown). A predicted mouse sequence (GenBank accession No. NM_145560; 2412 bp) was found after a BLAST search using this EST as a query (**Fig. 1**). A pair of primers (P1 and P2) was designed based on the predicted sequence and used in the reverse transcription-PCR with mouse epididymis total RNA as the template. The PCR product (2262 bp in size), regarded as the putative *imds-60*, was obtained and cloned. Sequence alignment showed that the *imds-60* EST fragment shared 99.6% identity with the predicted sequence and low identity with that of any other genes. This was further confirmed by PCR and Northern blot analysis under high stringency conditions (hybridization and washing at 65 ºC) with the 2262 bp fragment as a probe. A weak signal in mouse uterus and a rather higher signal in mouse epididymis were detected (**Fig. 2**). Moreover, the *imds-60* expression level in epididymis was 21.4-fold higher than that in uterus.

To obtain the full-length cDNA of *imds-60*, 5′- and 3′-

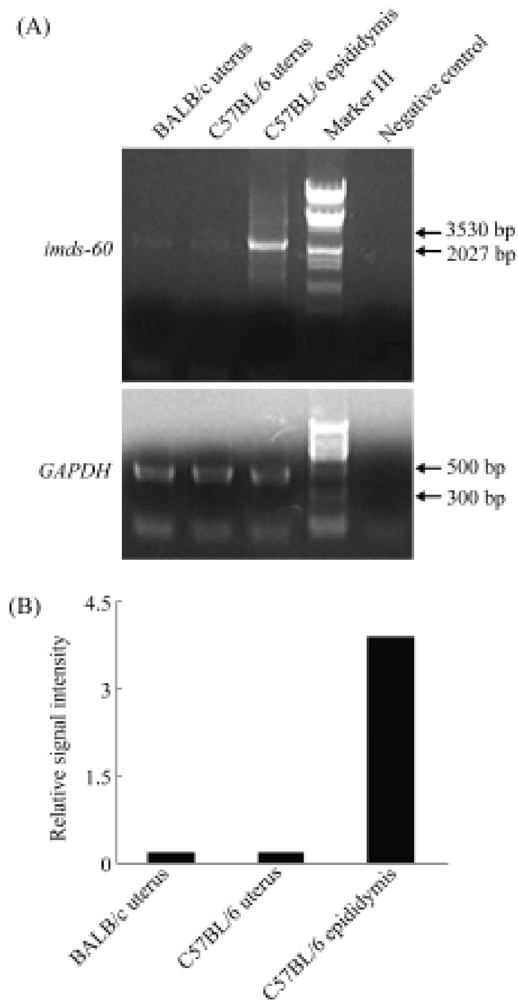

Fig. 2 Mouse *imds-60* was detected in uterus and epididymis
(A) Polymerase chain reaction detection of *imds-60*. The reaction mixture without any RNA was used as the negative control. (B) The relative expression levels expressed as *imds-60*/glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in uterus and epididymis.

RACEs were carried out using the process shown in **Fig. 1**. An extension of 266 bp was obtained toward the 5′-terminus by 5′-RACE, with the 169 bp fragment as a template. And by 3′-RACE, an extension of 300 bp toward the 3′-terminus was obtained with the 114 bp fragment as a template. Finally, the cDNA sequence of *imds-60* was obtained by PCR with primers designed on the terminus of the 3′-RACE product and the 5′-terminal of the 2262 sequence (P3 and P1) because of the high ratio of CG in the terminus of 5′-RACE. As shown in **Fig. 3**, the full-

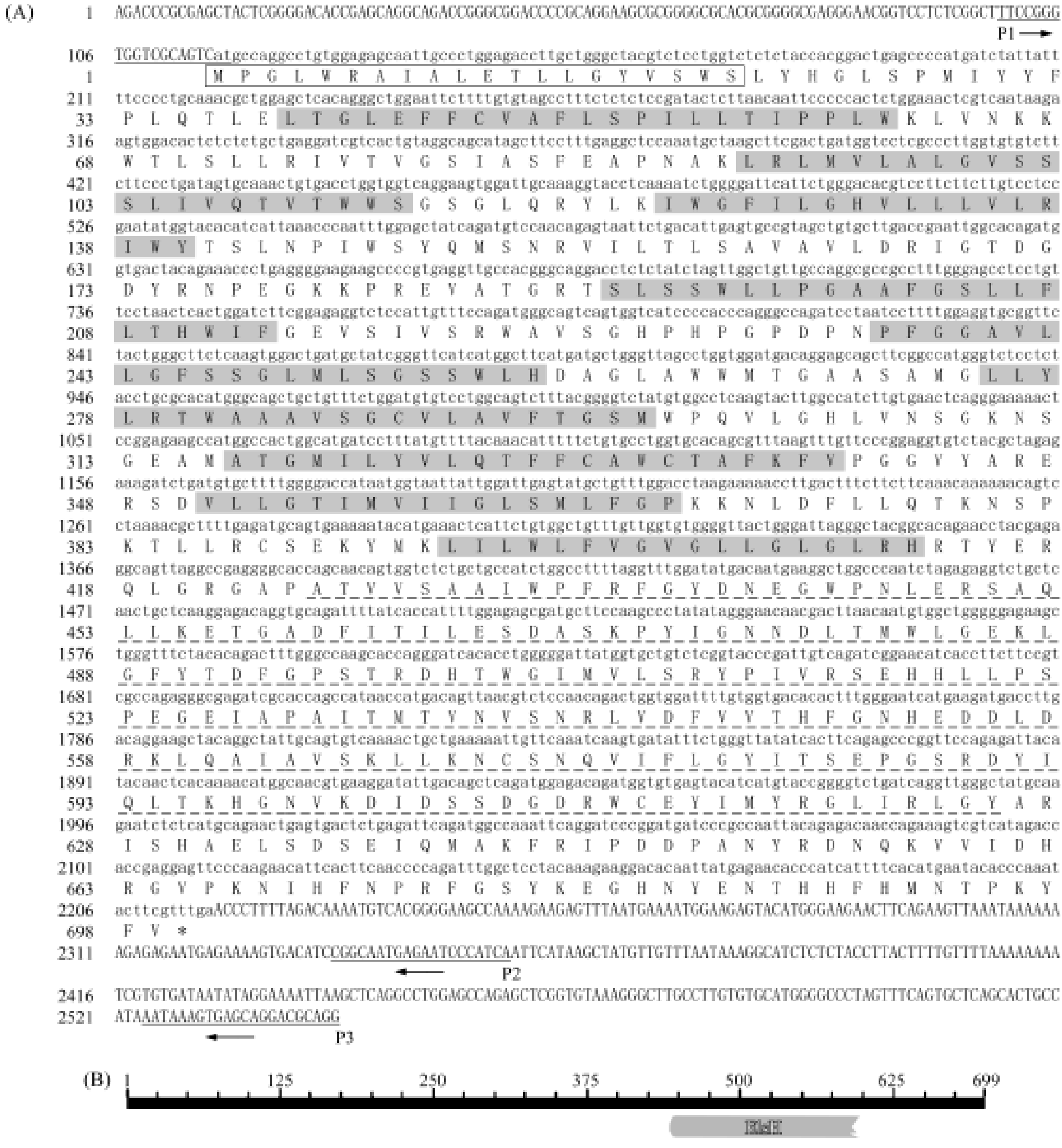

Fig. 3 Nucleotide, amino acid sequence and conversed domains of imds-60

(A) The underlined areas are the sequences of primers P1, P2, and P3 (solid underline), and the DNase I-like domain predicted by Interproscan at http://www.ebi.ac.uk/InterProScan/ (broken underline). The signal peptide is boxed and the nine transmembrane regions are shaded. (B) The putative domain of metal-dependent hydrolase (ElsH) in the imds-60 protein.

length *imds-60* sequence was 2545 bp with an open reading frame of 2100 bp coding a peptide containing 699 amino acids, from which the predicted molecular mass and isoelectric point were approximately 78.17885 kDa and 8.95, respectively. The result of BLAST Assembled Genomes in the National Center for Biotechnology Information (NCBI) mouse genomic BLAST database showed that the gene *imds-60* was located at chromosome 5 (location: 5C3.3).

The protein encoded by *imds-60* contained one signal peptide (amino acid 1–20), a DNase I-like domain (amino acid 425–625), and nine transmembrane regions [**Fig. 3 (A)**], according to the bioinformatics prediction. The result of another prediction, a conserved domain BLAST search in NCBI, showed that the sequence of the imds-60 protein contained a putative domain of metal-dependent hydrolase (ElsH) [**Fig. 3(B)**]. Moreover, the result of the NCBI BLAST search showed there were three highly homologous but unknown proteins, *Rattus norvegicus* RGD1310958 (predicted, 91%), *H. sapiens* hypothetical protein LOC80157 (82%), and *Canis familiaris* hypothetical LOC482143 (81%) (**Fig. 4**).

Tissue distribution of *imds-60* mRNA

The tissue distribution of mouse *imds-60* mRNA detected by Northern blot is shown in **Fig. 5(A)**. A strong signal was detected in epididymis. Signals also existed in seminal vesicle and stomach, but no signal was observed in the rest of the tested tissues. The *imds-60* expression level in epididymis was 10.6-fold higher than that in stomach [**Fig. 5(B)**]. Furthermore, Northern blot and PCR results showed that the expression of *imds-60* appeared with a

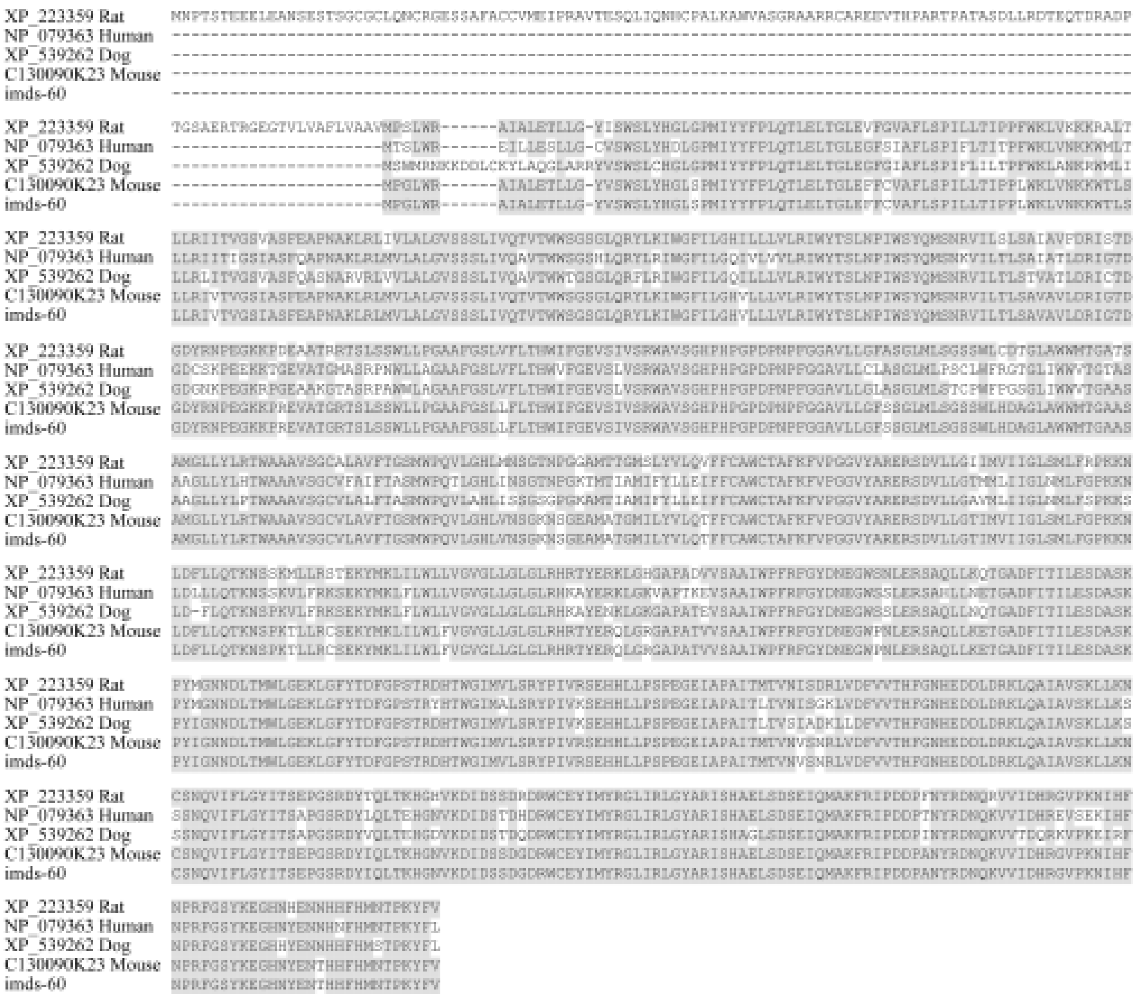

Fig. 4 Comparison of the predicted amino acid sequence of five hypothetical proteins

Comparison was done among *Rattus norvegicus* RGD1310958, *Homo sapiens* hypothetical protein LOC80157, *Canis familiaris* hypothetical LOC482143, C130090K23Rik (mouse) and imds-60. The homologous amino acid sequences are shown in gray. Five hypothetical proteins share high sequence similarity.

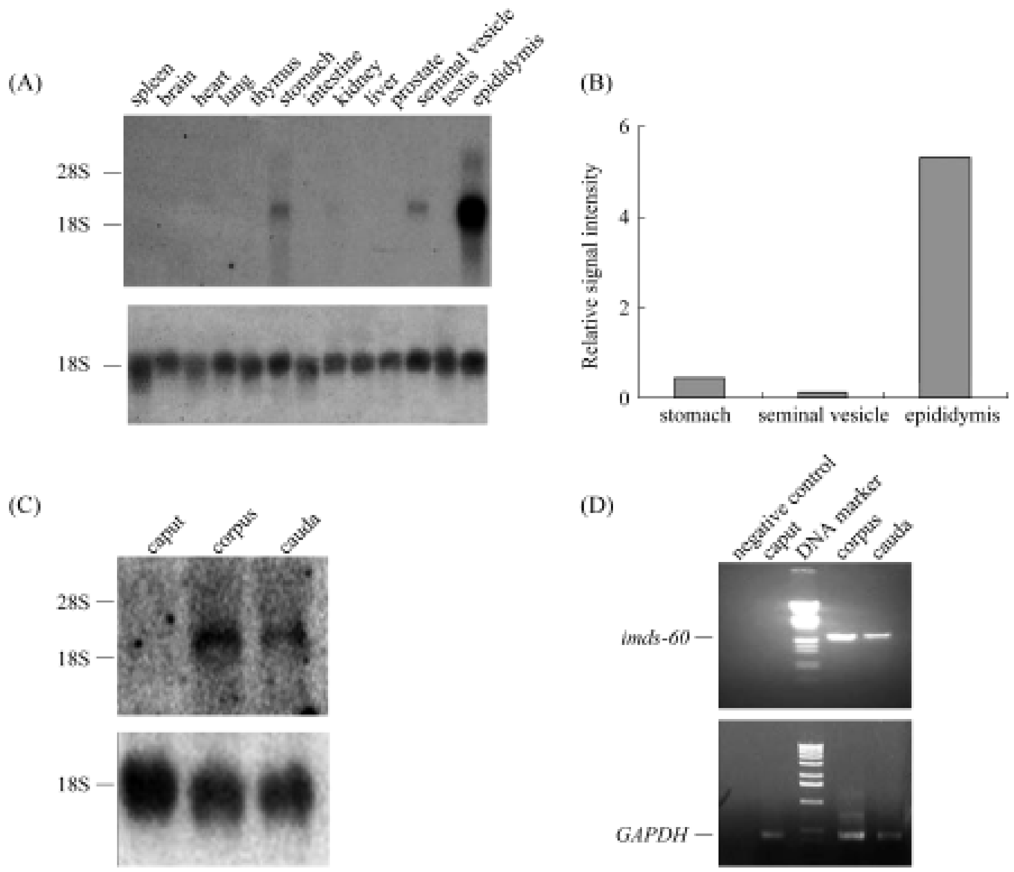

Fig. 5 Tissue distribution of *imds-60* mRNA

(A) Total RNAs from 13 mouse tissues were tested. (B) The relative hybridization intensity expressed as *imds-60*/18S ribosomal RNA. Region-specific expression in epididymis detected by Northern blot (C) and polymerase chain reaction (D). The 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as the loading controls. Negative control, the reaction mixture without any RNA.

region-specific pattern in epididymis and it was only expressed in corpus and cauda [**Fig. 5(C,D)**].

Expression of *imds-60* mRNA is androgen-dependent and peaked at sexually mature period

The effects of androgen on *imds-60* expression were investigated in a castration mouse model. There was a 50% decrease in the *imds-60* expression level on the first day of postcastration and over 60% decrease on day 5 after surgery (**Fig. 6**). Testosterone treatment in the 7 d castrated animal resulted in a gradual increase in the *imds-60* mRNA level. On day 1 of testosterone treatment, *imds-60* increased to 72%, and on day 5 the expression was restored to the normal level.

The variation of *imds-60* expression on different developmental stages is shown in **Fig. 7** as detected by Northern blot. The expression of *imds-60* peaked at 3 weeks, highlighting the sexually mature period (**Fig. 7**).

Discussion

Imds-60 is a novel gene whose characterization has not been reported. Here, according to the 313 bp fragment, the 2545 bp mRNA sequence of *imds-60* was exactly the same as the mouse *C130090K23Rik* gene sequence, except that our sequence had a truncated 5′ untranslated region sequence caused by the uncompleted 5′-RACE or possible isoform. The alignment of the two hypothetical proteins coded by these two genes also showed complete identity indicating that the correct sequence of *imds-60* was obtained.

The prediction by Interproscan showed a DNase I-like domain exists in the C-terminal of the imds-60 protein. The result of conserved domain BLAST research showed that the imds-60 protein sequence also contained a putative domain ElsH, which was potentially related to four other

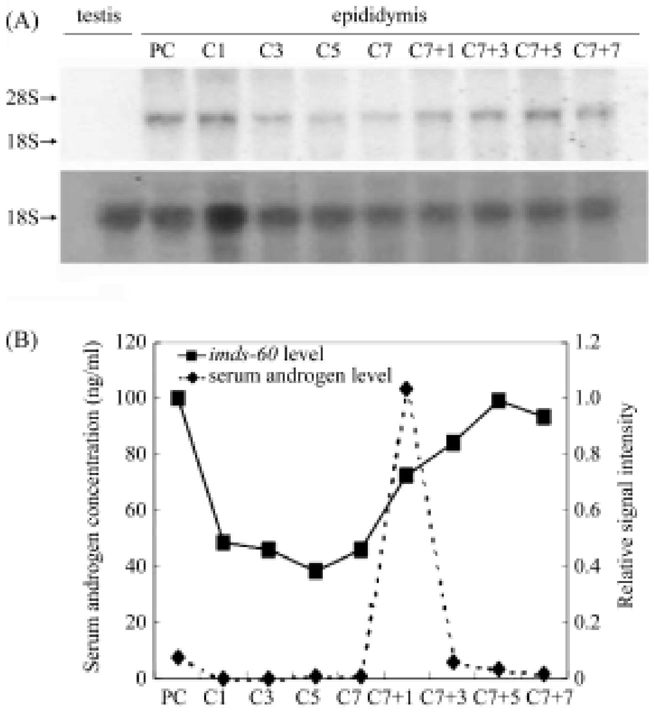

Fig. 6 Expression profiles of *imds-60* in mouse epididymis treated with or without androgen

(A) Northern blot analysis of adult mice epididymis RNA from precastration (PC), bilaterally castrated for 1, 3, 5, and 7 d (C1, C3, C5, and C7); and 1, 3, 5, and 7 d after a single injection of testosterone propionate applied to the 7 d castrated mice (C7+1, C7+3, C7+5, and C7+7). There were five mice in each group. Sample from testis served as a negative control. (B) The relative expression levels of transcripts (hybridization density of *imds-60* mRNA/18S ribosomal RNA).

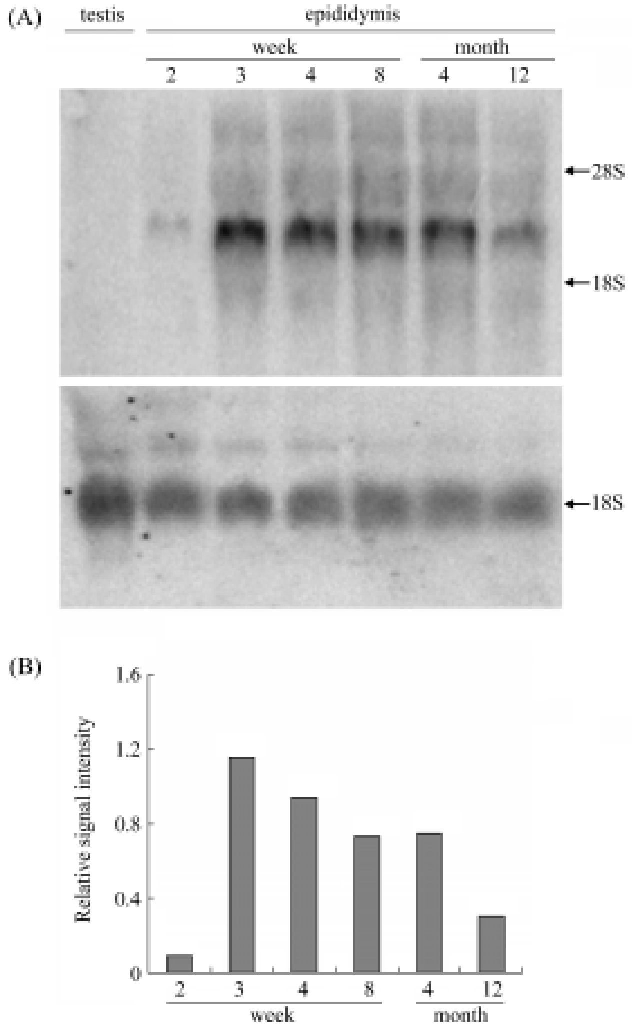

Fig. 7 Developmental change analysis of *imds-60* mRNA

(A) Mice of 2, 3, 4, and 8 weeks, and 4 and 12 months of age were killed. The variation of *imds-60* expression on different developmental stages was detected by Northern blot. Testis served as a negative control. (B) Quantitative comparison of the *imds-60* transcripts expressed at different ages in mouse epididymis.

conserved domains, namely, the endonuclease/exonuclease/phosphatase family including Mg^{2+}-dependent endonuclease, in good agreement with the function of DNase I-like protein. Most phosphoesterases [18–20] take part in intracellular signal transduction.

The fertility-associated antigen, found in seminal vesicle and prostate gland homogenates, was identified as a DNase I-like protein, showing the presence of DNase I-like protein in bull accessory sex glands and forming the groundwork for the identification of a candidate genetic marker for fertility of bulls [14]. Thus, our work on imds-60 containing a 200 amino acid DNase I-like domain with high-level expression in epididymis would provide more potent evidence for the presence of DNase I-like protein in male reproductive tract.

Human DNase I is a secreted protein [9]. However, that the fertility-associated antigen was extracted from membranes indicated [14] it was on the membranes. And the nine transmembrane regions contained in the protein would anchor the imds-60 protein in membranes of epithelia or spermatozoa to carry out its functions, such as cell communication.

The result of testosterone injection suggested that *imds-60* expression was androgen-dependent, in agreement with the variation of *imds-60* expression on different developmental stages. However, the *imds-60* expression peak at 3 weeks indicated that the expression might be affected by some other factors and is involved in epididymal development.

In summary, we have primarily studied *imds-60* expression in epididymis. However, more work, such as a protein-level study, should be done to get a better understanding of its physiological function.

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