Post-Print: Interaction between Mouse DNAJB13 and HK1

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Date: 2017-12-21T00:00:00+00:00

Abstract

Objective To investigate the interaction between DNAJB13 and HK1 in mice. Methods The prokaryotic expression vector pGEX-4T-1/Dnajb13 was constructed by double enzyme digestion and ligation, and verified by sequencing; the recombinant plasmid was transformed into competent DH5 cells, and fusion protein GST-DNAJB13 expression was induced with IPTG; proteins were analyzed and identified by SDS-PAGE with Coomassie brilliant blue staining and Western blotting; mouse testicular proteins were extracted, and GST pull-down was performed to detect the interaction between DNAJB13 and HK1. Results The recombinant plasmid pGEX-4T-1-Dnajb13 was successfully constructed, with sequencing results consistent with the standard sequence; Escherichia coli transformed with the recombinant plasmid efficiently expressed the fusion protein upon induction at 37 °C with 1 mmol/L IPTG; GST pull-down yielded positive results, indicating that DNAJB13 interacts with HK1. Conclusion In mouse testis, DNAJB13 interacts with HK1 and may participate in spermatogenesis and sperm motility.

Full Text

Interaction of DNAJB13 with HK1 in Mouse

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Abstract

Objective To investigate whether DNAJB13 interacts with HK1 in mouse. **Methods** The prokaryotic expression vector pGEX-4T-1/Dnajb13 was constructed using double enzyme digestion and ligation, and verified by sequencing. The recombinant plasmid was transformed into competent DH5 cells, and fusion protein GST-DNAJB13 expression was induced with IPTG. Protein expression was analyzed and identified by SDS-PAGE with Coomassie brilliant blue staining and Western blotting. Mouse testicular proteins were extracted, and GST pull-down assay was performed to detect the interaction between DNAJB13 and HK1. **Results** The recombinant plasmid pGEX-4T-1-Dnajb13 was successfully constructed, and sequencing confirmed consistency with the reference sequence. *E. coli* transformed with the recombinant plasmid efficiently expressed the fusion protein when induced with 1 mmol/L IPTG at 37 °C. Positive GST pull-down results demonstrated an interaction between DNAJB13 and HK1. **Conclusion** DNAJB13 interacts with HK1 in mouse testis and may participate in spermatogenesis and sperm motility.

Key words: DNAJB13; recombinant plasmid; fusion protein; HK1; GST pull down; spermatogenesis; sperm motility

Introduction

Idiopathic male infertility, including asthenozoospermia, oligospermia, and teratospermia, represents a class of unexplained male infertility disorders. Statistics indicate that global infertility rates are rising, with male factors accounting for 50% of cases. Among these, 60–75% of patients have no identifiable cause, classified as idiopathic infertility. Genetic factors such as gene mutations or chromosomal abnormalities contribute to 30% of idiopathic infertility cases [1-2].

Spermatogenesis is the process by which spermatogonia develop into mature spermatozoa through a series of developmental stages, regulated by numerous genes and molecules including Sox9, Eif2s3y, miRNAs, and PAIP2A [3-5]. Impaired spermatogenesis leads to abnormal sperm morphology and reduced sperm count. Sperm motility depends on rapid forward propulsion generated by flagellar beating; structural abnormalities of the flagellum or energy metabolism disorders can reduce sperm viability and cause infertility (asthenozoospermia). Related genes include solute carrier 22a14, fibrous sheath protein CABYR, NDUFA13, PP1 2, and PPP1R11 [6-9]. Research into the etiology of idiopathic infertility will provide new guidance for clinical treatment.

In previous work, we cloned DnaJB13/Dnajb13, a novel HSP40 family gene highly expressed in human and mouse testicular tissue. Its mRNA is expressed in various spermatogenic cells, and the protein primarily localizes to the tail of spermatids and mature sperm [10], suggesting functions related to spermiogen-

esis and sperm motility. Recent studies have increasingly focused on DNAJB13 function [11-12], all demonstrating its important role in ciliary and flagellar movement.

Our proteomic analysis of sperm proteins using co-immunoprecipitation suggested that DNAJB13 may interact with the glycolytic enzyme HK1. To verify this interaction, we cloned the full-length cDNA of Dnajb13 into the pGEX-4T-1 vector to express a GST fusion protein [13-14] and performed GST pull-down assays to detect DNAJB13-interacting proteins, revealing one aspect of its mechanism and providing guidance for clinical work.

Materials and Methods

1.1 Materials

C57 adult male mouse testicular tissue and PCR master mix (referred to as "绿水" in the original) were used. Primers were synthesized by Sangon Biotech. Restriction enzymes EcoRI and XhoI were purchased from NEB. DNAJB13 antibody was from Santa Cruz. pGEM-T vector kit was from Promega. Gel extraction and plasmid mini-prep kits were from TAKARA. Protein marker was from Thermo. 1.5 mL EP tubes were from Axygen. pGEX-4T-1 plasmid was preserved in our laboratory. Competent DH5 cells were from TAKARA. GST pull-down kit was from Promega.

1.2 Methods

1.2.1 Construction and Identification of pGEM-T/Dnajb13 Recombinant Plasmid (TA Cloning) (1) Full-length amplification of Dnajb13 open reading frame: Primers with EcoRI and XhoI restriction sites and protective bases were designed: Dnajb-pGEX-T-F: 5' -CTAGCCGGAATTCATGGGGCTGGATTACTATGC-3' (EcoRI site) and Dnajb-pGEX-T-R: 5'-CTAGCCGCTCGAGTTAGGTCAGCAATGCCTGGCGCA-3' (XhoI site). PCR was performed using mouse testicular cDNA as template in a 10 μL reaction containing 5 μL PCR master mix, 0.1 μL each of 20 μmol/L primers, 1 μL template, and 3.8 μL double-distilled water, with a negative control included. Amplification was performed on an Eppendorf Mastercycler Gradient PCR 仪 under the following conditions: 95 °C for 1 min 30 s, followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min and hold at 4 °C. PCR products were analyzed by 2% agarose gel electrophoresis.

(2) TA cloning: After confirming the correct PCR product size, ligation to pGEM-T Vector was performed using the pGEM T vector kit in a 10 μL system containing 5 μL 2× rapid ligation buffer, 1 μL pGEM T vector, 0.2 μL PCR product, 2.8 μL double-distilled water, and 1 μL T4 DNA ligase.

(3) Transformation of TA cloning vector: The ligation product was transformed into competent *E. coli* DH5 . Ten microliters of competent cells were thawed on ice, mixed with 1 µL ligation product, and incubated on ice for 30 min. Heat shock was performed at 42 °C for 50 s, followed by immediate ice bath for 3–5 min. Pre-warmed AMP-negative LB broth (300 µL) was added, and cells were shaken at 225 r/min for 1 h at 37 °C. For plating, 4 µL IPTG and 40 µL X-Gal were mixed and spread onto solid medium containing 0.1 mg/mL AMP. Then 100–200 µL of bacterial suspension was plated, air-dried for 5–10 min, and incubated overnight inverted.

(4) Identification of TA clones: Single colonies were picked into sterile EP tubes containing 70 µL LB medium (with 0.1 mg/mL AMP) and shaken at 225 r/min for 4 h at 37 °C. Colony PCR was performed in a 10 µL system containing 5 µL PCR master mix, 0.2 µL primer pair, 1 µL bacterial suspension, and 3.8 µL double-distilled water under the same PCR conditions. Positive clones were expanded and plasmids were extracted and sent to BGI for sequencing.

1.2.2 Construction and Identification of pGEX/Dnajb13 Recombinant Expression Plasmid (1) Gel recovery of Dnajb13 and vector fragments: pGEM/Dnajb13 recombinant plasmid and pGEX-4T-1 vector were double-digested with EcoRI and XhoI. The target gene and vector fragments were recovered from the digestion products.

(2) Ligation of digested products: Dnajb13 and pGEX-4T-1 digested products were ligated at a molar ratio of 3:1 in a 10 µL system containing 1 µL 10× ligase buffer, 5 µL target gene fragment, 3.2 µL vector, 0.3 µL T4 DNA ligase, and 0.5 µL double-distilled water at 16 °C.

(3) Identification of recombinant plasmid: Transformation and identification of the recombinant plasmid were performed as described above.

1.2.3 Fusion Protein Expression and Identification [15] (1) Expression: Bacteria containing the sequence-verified recombinant plasmid were cultured overnight, then diluted 1:20 into 4 mL LB medium containing 100 µg/mL ampicillin and grown at 37 °C with shaking at 225 r/min to A600 nm of 0.6–0.8. Gradient experiments were performed for IPTG induction time and concentration. For concentration gradient, IPTG concentrations of 1, 10, 40, 70, and 100 mmol/L were used to induce expression for 4 h; 1 mL bacterial culture was centrifuged, lysed, and mixed with loading buffer, then boiled for 5 min. For time gradient, 1 mmol/L IPTG was used to induce expression for 1–4 h; at each time point, 1 mL culture was centrifuged at 12,000 r/min for 1 min, resuspended in 15 µL cell lysis buffer and 15 µL loading buffer, and boiled for 5 min. Expression was detected by 10% SDS-PAGE.

(2) Western blotting identification of fusion protein: Goat anti-DNAJB13 antibody was used for Western blotting. After SDS-PAGE, proteins were semi-dry transferred to PVDF membrane, blocked with 5% skim milk in

PBST for >1 h, and incubated with primary DNAJB13 antibody (F-20, 1:100) at 4 °C overnight with gentle shaking. The membrane was washed 3 times with 1×PBST (10 min each), incubated with secondary anti-goat antibody (1:2000) at room temperature for 1 h with gentle shaking, washed again 3 times, and detected with ECL chemiluminescence reagent.

1.2.4 GST Pull-Down Assay (1) Preparation of GST-DNAJB13 fusion protein (bait) and GST protein (negative control): pGEX-4T-1/Dnajb13 recombinant plasmid and empty vector were transformed into competent DH5 cells, induced with 1 mmol/L IPTG for 4 h. Ten milliliters of bacterial culture was centrifuged to collect cells, resuspended in 400 µL MagneGST™ Cell Lysis Reagent, and sonicated on ice (1 min on, 45 s off) for 30 min. Lysates were centrifuged at 12,000 r/min for 20 min at 4 °C, and supernatants were collected.

(2) Mouse testicular protein extraction: (i) All steps were performed on ice. Two C57 mouse testes were homogenized in 1 mL ice-cold RIPA buffer using an electric homogenizer, incubated on ice for 20 min, then centrifuged at 12,000 r/min for 20 min at 4 °C. (ii) Protein concentration was determined by BCA assay.

(3) GST pull-down [16-17]: Equilibration: Twenty microliters of magnetic beads were placed in a 1.5 mL EP tube, washed twice with 250 µL binding/wash buffer, and beads were collected using a magnet. **Binding:** Forty microliters binding/wash buffer, 200 µL GST-DNAJB13 fusion protein or GST protein, and 60 µL 5% BSA were added and incubated with gentle shaking at room temperature for 30 min. **Washing:** Beads were washed 3 times after magnetic collection. **Capture:** One hundred fifty microliters mouse testicular protein, 60 µL 5% BSA, and 70 µL buffer were added to the beads and incubated with gentle shaking at room temperature for 1 h. After brief vortexing, supernatant was reserved for Western blot analysis. **Elution:** Beads were washed 5 times with 400 µL buffer, and finally 20 µL loading buffer was added.

(4) Western blot analysis: Positive control (mouse testicular protein), experimental and negative control pull-down fractions, and supernatant fractions were analyzed by SDS-PAGE, transferred to PVDF membrane at 130 mA for 30 min, blocked with 5% skim milk in PBST for >1 h, and incubated with primary HK1 antibody (1:1000) at 4 °C overnight with gentle shaking. After washing 3 times with 1×PBST (10 min each), the membrane was incubated with secondary donkey anti-rabbit antibody (1:5000) at room temperature for 1 h, washed again 3 times, and detected with ECL chemiluminescence reagent.

Results

2.1 Construction and Identification of pGEM-T/Dnajb13 Recombinant Plasmid (TA Cloning)

2.1.1 Full-length PCR Amplification of Mouse Dnajb13 PCR products were analyzed by 2% agarose gel electrophoresis, showing a band at 951 bp, consistent with the expected size [Figure 1: see original paper].

2.1.2 Identification of pGEM-T/Dnajb13 Recombinant Plasmid Clones Eleven single colonies of pGEM-T/Dnajb13-transformed *E. coli* were selected for colony PCR. Four clones showed positive bands of the correct size. Sequencing by BGI confirmed that clones Dnajb13-8 and 9 were completely correct [Figure 2: see original paper].

2.2 Construction and Identification of pGEX/Dnajb13 Recombinant Expression Plasmid

The pGEX-4T-1 vector contains a GST tag. pGEM/Dnajb13 recombinant plasmid and pGEX-4T-1 were double-digested with EcoRI and XhoI. Digested products were analyzed by 1% agarose gel electrophoresis, and vector and target gene fragments were recovered. Ligation products were transformed into competent cells, and colony PCR identified two positive clones of the correct size. Sequencing by BGI confirmed consistency with the expected sequence [Figure 3: see original paper].

2.3 Induced Expression and Identification of Fusion Protein

Concentration gradient: IPTG concentrations of 0, 1, 10, 40, 70, and 100 mmol/L were used to induce expression for 4 h. Results showed low-level expression of GST-DNAJB13 without IPTG induction, with no significant difference in fusion protein expression across the concentration gradient [Figure 4: see original paper].

Time gradient: Using a fixed IPTG concentration of 1 mmol/L, induction times of 1, 2, 3, and 4 h were tested. Results showed low-level expression without induction, with fusion protein expression increasing significantly over time [Figure 5: see original paper].

2.4 Western Blot Identification of Fusion Protein

Western blotting using GST antibody detected expression of both fusion protein and tag protein in lysates from recombinant plasmid- and empty vector-transformed bacteria. The fusion protein was 61 kDa and the tag protein was 26 kDa, both matching expected sizes [Figure 6: see original paper].

2.5 Western Blot Detection of GST Pull-Down Results

In the GST pull-down assay, GST-DNAJB13 specifically bound to GSH on magnetic beads as bait protein. After incubation with total testicular proteins and elution, Western blotting with HK1 antibody detected the precipitated proteins. Positive HK1 signal in the GST-DNAJB13 group indicated interaction between DNAJB13 and HK1. Positive HK1 signals in both experimental and negative control elution fractions confirmed HK1 abundance [Figure 7: see original paper].

Discussion

Idiopathic infertility is a current research focus, with the spermatogenesis-related gene Dnajb13 attracting increasing attention from scholars worldwide. Its role in ciliary and flagellar movement has been extensively confirmed. In 2004, Liu et al. [18] identified Dnajb13 as a novel HSP40 family gene highly expressed in mouse testis. Human and mouse Dnajb13 share high homology, with an open reading frame of 951 bp encoding 316 amino acids. The DNAJB13 protein consists of a DNAJ domain and a C-terminal domain [19]. Guan et al. [20-22] localized DNAJB13 to the radial spokes of the mouse "9+2" axonemal structure by immunoelectron microscopy. In 2014, Li et al. [10] showed that Dnajb13 mRNA expression begins one week after mouse birth and increases sharply after three weeks. Western blotting and immunohistochemistry revealed that DNAJB13 localizes to the cytoplasm of spermatids and the tail of mature sperm, with immunoelectron microscopy further localizing it to the fibrous sheath of the midpiece. Asami et al. [11] considered it a lethal gene; CRISPR/Cas9-mediated knockout of Dnajb13 in mice caused hydrocephalus and death before four weeks, with sperm showing tailless, short-tailed, and immotile phenotypes. El Khouri et al. [12] identified point mutations and truncating mutations in Dnajb13 in three patients with primary ciliary dyskinesia, which caused partial loss of central microtubules in cilia, showing a 9+0 structure, and significantly reduced DNAJB13 protein expression in sperm flagella and nasal cilia. These studies on protein expression and localization suggest its function is related to sperm motility. However, current research remains at the level of localization and functional studies, with specific molecular mechanisms still unclear.

Our previous co-immunoprecipitation and mass spectrometry analysis of sperm proteins suggested that HK1, a glycolytic enzyme, might be an interacting protein of DNAJB13. To verify this, we successfully constructed the pGEX-4T-1-Dnajb13 recombinant plasmid with sequencing results matching expectations. *E. coli* transformed with the recombinant plasmid efficiently expressed the fusion protein when induced with 1 mmol/L IPTG at 37 °C. GST pull-down assays confirmed the interaction between DNAJB13 and HK1.

Hexokinase is the first enzyme in the glycolytic pathway, using ATP to convert

glucose to glucose-6-phosphate (G6P). Immunoblotting shows high HK1S expression in sperm, and immunohistochemistry confirms its primary localization to the sperm flagellum, suggesting involvement in sperm motility. Hironmoy et al. [23] performed Q-PCR analysis of differentially expressed genes in mice of different ages, showing significantly increased expression of HK1 and NME5 from day 5 to day 60, indicating these genes are associated with spermatogenesis. Nakamura et al. [24] found that the spermatogenic cell-specific hexokinase HK1S has a distinct 5' untranslated region containing a spermatogenic cell-specific domain (SSR) that replaces the PBD domain responsible for HK1' s specific binding to the mitochondrial outer membrane. Noriko Nakamura et al. [25] identified through yeast two-hybrid analysis that HK1 binds to PFKM via SSR in the principal piece, which in turn binds to the fibrous sheath component GSTM5 through a testis-specific domain (TSR). These studies on HK1 localization and function demonstrate its involvement in sperm formation and motility and reveal potential mechanisms. Our finding that DNAJB13 interacts with HK1 suggests that DNAJB13 may participate in sperm formation and motility by influencing sperm energy metabolism.

Sperm motility involves three aspects: structural integrity, ion channels, and energy metabolism. The first two are the foundation for the latter, which serves as the motor for sperm movement. Any factor affecting energy metabolism will reduce sperm viability. DNAJB13 localizes to the fibrous sheath of the sperm flagellum, consistent with the localization of HK1 and GSTM5. GST pull-down experiments demonstrated interaction between DNAJB13 and HK1. In summary, DNAJB13 may interact with HK1 to form a DNAJB13/HK1/GSTM5 complex localized to the fibrous sheath of the sperm tail, participating in sperm glycolysis and thereby affecting sperm motility. When DNAJB13 is mutated or absent, assembly of HK1/GSTM5 in the tail may be impaired, leading to defects in tail formation and energy supply, resulting in tailless, short-tailed, or immotile sperm. Other cilia-rich tissues may also develop ciliary motility disorders causing pneumonia or impaired oviduct transport. Any factor causing structural, metabolic, or signal transduction defects will lead to sperm motility disorders. This study reveals a potential mechanism of DNAJB13 action, though the binding sites, specific mechanisms, and whether it affects sperm tail structure and signal transduction require further investigation.

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Edited by: Jing Yuan

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