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## Identification and characterization of the BGR-like gene with a potential role in human testicular development/spermatogenesis

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### Abstract

**Aim:** To investigate the roles of the BGR-like gene in testicular development/spermatogenesis. **Methods:** A human testis cDNA microarray was hybridized with probes from human adult testes and embryo testes. The differentially expressed clones were sequenced and analyzed. Expression of the BGR-like gene was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). **Results:** A new gene exhibiting 50-fold difference in expression level between adult and fetal human testes was cloned and named the BGR-like gene. The cDNA consisted of 2500 nucleotides and had an open reading frame of 1437 nucleotides encoding a putative protein of 497 amino acid residues. Homologous comparison showed that the BGR-like gene was a new alternative splicing variant of the BGR gene and had sequence homology with the bubblegum gene of human, mouse, rat and *Drosophilia*. Protein motif analysis of the BGR-like gene revealed that it contained a conserved adenosine monophosphate (AMP)-binding domain and a fatty acyl-CoA synthetase signature motif which existed in all acyl-CoA synthetases. The BGR-like gene transcript was imperceptibly expressed in human fetal testes, highly in human adult testes and moderately in elderly testes and human Leydig cells. RT-PCR-based tissue distribution experiments showed that the BGR-like gene transcript was not detected in some azoospermic testes. **Conclusion:** The BGR-like gene may play an important role in spermatogenesis/testicular development and may be correlated with male infertility. *(Asian J Androl 2005 Mar; 7: 21–32)* 

Keywords: BGR-like gene; gene expression; sequence analysis; spermatogenesis; human testis

### 1 Introduction

Spermatogenesis is a complex multi-step process that involves the initial proliferation of spermatogonia, followed by a phase of differentiation generating tetraploid spermatocytes. These cells proceed through two successive meiotic divisions to form haploid round spermatids, which undergo extensive morphological restructuring resulting in elongated sperm. Each of these steps requires a particular combination of gene expression. Some of these genes are testes-specific and cell-typespecific; some are expressed as specific, alternatively spliced transcripts; and some exhibit a quantitative, specific expression pattern. To understand this process, the most straightforward strategy is to identify and characterize development-related or differentiation-specific genes and to investigate its functions during spermatogenesis.

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In humans, approximately 20 % of infertile males display a severe or complete loss of mature spermatozoa, called oligozoospermia and azoospermia, respectively [1]. It is now recognized that a significant number of these cases have underlying genetic etiologies, including chromosome abnormalities, microdeletions of the Y chromosome, meiotic abnormalities and mutations [2]. Mutated or absent genes expressed at different stages of spermatogenesis may lead to spermatogenic arrest and infertility.

Recently, we compared gene expression profiles between adult and fetal testes by hybridizing cDNA probes prepared from adult and fetal testes to membranes fabricated with gene clones derived from the human testis library [3]. A global map of genes related to human testicular development/spermatogenesis was built up (http:// www.njmu.edu.cn/shenzhi/index-sztj.htm). In this map, 266 full length clones were highly expressed in adult human testes and 120 clone in fetal human testes. Among them, 111 clones were previously unidentified. Study of these differentially expressed genes may contribute to a better understanding of normal and abnormal spermatogenesis [4, 5].

Acyl-CoA synthetases (ACS) occupy a central position in fatty acid metabolism. Acyl-CoA, produced by ACS from fatty acid, ATP, and CoA, is a key intermediate in two major metabolic pathways: degradation of fatty acids via the β-oxidation system; and synthesis of cellular lipids that include triglycerides, phospholipids, and cholesterol esters [6, 7]. Over the last decade, several types of ACS have been identified [8–13]. A unifying feature of all ACS is the presence of an AMP-binding domain signature and a fatty acyl-CoA synthetase signature motif [fluorescence-activated cell sorter (FACS) signature motif ] [13]. Based on the amino acid sequence homologies, Steinberg et al. defined six distinct families of ACS [13]. Among them, members of the bubblegum family have been reported to be involved in the pathogenesis of the inherited neurodegenerative and endocrine disorder X-linked adrenoleukodystrophy (X-ALD), which mainly affected the brain, adrenal gland and testes [12–15]. In this paper, we report the identification of the complete sequence of a novel gene named bubblegumrelated-like (BGR-like) gene, which is highly expressed in human testes and contains a putative AMP-binding domain and an FACS signature motif. The developmentdependent, testes-specific, Leydig cells expression and its deletion in some azoospermic testes indicated that BGR-like gene may relate to testicular development/spermatogenesis and idiopathic azoospermia. To the best of our knowledge, this is the first report on a bubblegum related (BGR) gene exclusively expressed in human testes.

### 2 Materials and methods

### 2.1 Samples

Informed consent was received from either the participants or their kin, and the ethics committee of Nanjing Medical University granted research approval prior to sample collection. Human testes from four adult males (37-43-year-old) and one elderly male (73-year-old) were obtained from the Body Donor Center (Nanjing Medical University, China), and fetal testes were obtained from accidentally aborted six-month-old fetuses (Clinical Reproductive Center, Nanjing Medical University, China). Macroscopic and histological examinations were used to confirm that these samples kept their structural integrity. Testis tissue samples from 12 azoospermic patients aged 21-46 years were acquired via biopsy. One of them was diagnosed as having Sertoli cell-only syndrome (SCOS), the other 11 were diagnosed as having spermatogenic arrest at different stages.

### 2.2 Construction of human testis cDNA microarray

Protocols for human testis cDNA microarray construction, human embryo and adult testis cDNA probe preparation, hybridization, and signal analysis have been described in detail recently [3].

### 2.3 Sequencing and analysis of interest clones

The differentially expressed cDNA plasmids were extracted and purified in mini-preps (QIAprep Spin Miniprep Kit; Qiagen, Hilden, Germany). The full length of the insert was sequenced in an autosequencer (ABI model 377, Perkin-Elmer, Norwalk, USA). The generated sequences were subjected to BLAST analysis (http:// www.ncbi.nlm.nih.gov) to determine the sequence homology with other species and the gene locus on the human chromosome. Meanwhile, the nucleotides and the deduced protein were also analyzed by Gene Runner (http://www.generunner.com), SMART (http://www. smart.embl-heidelberg.de/) and Multiple Sequence Alignment by ClustalW (http://www.ebi.ac.uk/clustalw/). After sequence identification and analysis, a novel testisspecific gene, named BGR-like gene, was found. Its complete sequence has been received by GenBank

### (accession number AY009107).

# 2.4 Expression of BGR-like gene in human embryo, adult and elderly testes and Leydig cells

To determine the expression of BGR-like gene in various developmental stages testes, BGR-like gene-specific RT-PCR was carried out on cDNA from fetal, adult and elderly testes and human Leydig cells. For the human Leydig cell preparation, a fraction of a testis (47-yearold) was used to isolate Leydig cells according to techniques previously described [16]. Briefly, a piece of tissue was finely minced and was treated twice with 0.04 % collagenase (type I, Sigma Chemical Co., USA; 130 U·mg<sup>-1</sup>) and 0.01 % trypsin inhibitor in a 1:1 mixture of Ham's F12 medium: Dulbecco's Modified Eagle Medium (DMEM: F12, Gibco BRL, Gathersburg, MD, USA), under constant agitation at 34 °C for 40 min. After this digestion procedure, cells obtained from the supernatants were purified by the use of a discontinuous Percoll density gradient and the interface between 34 % and 60 % was collected. Cells were maintained in the culture medium mentioned above for 24 hours and then replaced with the same medium containing 10 % horse serum for 6 days.

The total RNA was extracted from the various ..... (μρρτοχία ατοίχ 3.5 μg·μL<sup>-1</sup>) using TRIzol Reagent (Gibco). Reverse transcription was performed in 15 µL reaction mixture. First, 1 µL of total RNA, 1 µL random primer (0.2 µg·mL<sup>-1</sup> Sangon Co., Shanghai, China) and 7 µL diethyl pyrocarbonate (DEPC) water were mixed and incubated at 70 °C for 5 min; then 3 µL M-MLV RT 5 × buffer, 0.75  $\mu$ L dNTP (20 mmol·L<sup>-1</sup>), 0.25  $\mu$ L Rnasin (50 U· $\mu$ L<sup>-1</sup>), 1  $\mu$ L M-MLV Reverse Transcriptase (10 U·µL<sup>-1</sup>), Promega Co., Madison, WI, USA), 1 µL DEPC water were added and incubated at 37 °C for 1 hour and then 95 °C for 5 min. The various cDNAs were PCR amplified with the BGR-like-specific primers: P1: 5'-ACCTGGAATGACTGGAACC-3' (nt135-nt153); P2: 5'-TCTGTGGAATCCGGCTAG-3' (nt520-nt537), which spanned an intron. The desired fragment was 403 bp. The reagents in 20 µL PCR reaction volume were as follows:  $10 \times PCR$  buffer 2 µL, 25 mmol·L<sup>-1</sup> Mg<sup>2+</sup> 1.5  $\mu$ L, 20 mmol·L<sup>-1</sup> dNTPs 0.15  $\mu$ L, Tag DNA polymerase (5 U· $\mu$ L<sup>-1</sup>) 0.15  $\mu$ L, 12.2  $\mu$ L distilled water, 1  $\mu$ L of each primer (5 pmol· $\mu$ L<sup>-1</sup>), cDNA sample 2  $\mu$ L. PCR was carried out using PE9600 (Perkin-Elmer, Wellesley, MA, USA) under these conditions: one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, followed by one cycle at 72 °C for 10 min.  $\beta$ -actin was used as the positive control of the cDNA templates. The primer sequences for human  $\beta$ -actin cDNA were: P1: 5'- CGGTTGGCCTTG-GGGTTCAGGGGGG-3'; P2: 5'-ATCGTGGGGGGGGG-CCCCAGGCACCA-3'. The desired fragment was 247 bp. The products of PCR were analyzed by 1.5 % agarose gel electrophoresis.

#### 2.5 Expression profiles of BGR and BGR-like gene

BLAST-nucleotide showed that BGR-like gene is highly homologous to the previously identified BGR gene including BC022027, AK0933433, AJ577571, AY358766 and AK057412. In order to determine the expression profile of BGR, primers which can amplify all six splicing variants were designed in common regions of the six sequences. The primers sequences were as following: P3 (located at 1133–1152 bp in BGR-like gene): 5'-CCGCATGGCTAAGACTCTCG-3'; P4 (located at 1563-1582 bp in BGR-like gene): 5'-CCACCAGCA-GTGATAAGGAT-3'. The PCR product was 449 bp in size. cDNAs of 16 different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocytes) from a commercial Human Multiple Tissue cDNA panel (MTCTM Panels I and II, Cat#K1420-1 and K1421-1, Clontech) were used in this study. PCR reaction condition used were one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by one cycle at 72 °C for 10 min.  $\beta$ -actin was used as a positive control.

In addition, the expression profile of the BGR-like gene was also worked out. The PCR mixture, condition and BGR-like-specific primers were the same as used above.

## 2.6 BGR-like expression in the normal spermatogenesis testes and testes of male infertile patients

Four normal fertile men and 12 azoospermic testes were used in this study. The testicular tissues were obtained via biopsy from 12 infertile men at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). The clinical diagnoses based on testicular biopsy were Sertoli cell-only syndrome (SCOS) in one patient and spermatogenic arrest at different stages in the other 11 patients. Total RNA (approximately  $3.5 \ \mu g \cdot \mu L^{-1}$ ) was extracted using Trizol reagent and then reverse transcribed to cDNA with avian myelobastosis virus reverse transcriptase. All the following procedures, including cDNA synthesis, BGR-like gene amplification and electrophoresis were performed as described above.  $\beta$ -actin was used as the positive control.

#### 3 Results

#### 3.1 cDNA microarray hybridization

The results of hybridization with embryo and adult testis probes indicated that the clone named BGR-like gene was highly expressed in adult testes. The signal intensities hybridized with embryo testis probe and with adult testis probe were 3.37 and 169.05, respectively. Obviously, the expression level in adult was about 50-fold higher than that in the embryo (Figure 1).

## 3.2 Sequence identification and analysis of BGR-like gene

The full BGR-like gene cDNA length is 2500 bp and has a 1437-bp open reading frame from nt 582–2018. The putative protein was 479 amino acids. The methionine at nt 582–584 was the initiation site because there was an upstream stop code TAG at nt 480–482 (Figure 2). A BLAST search in the human genome database localized the BGR-like gene to human chromosome 19 (NT\_011255.14|Hs19\_11412). One clone in chromosome 19 (length = 7 286 004 bp) contains the genomic sequence of BGR. The BGR-like gene is spliced by 16 exons and 15 introns (Figure 3), encompassing 57 435 bp genomic DNA (from 6075 670 bp to 6133 105 bp) in NT\_011255.14. A BLAST search of the contig map



Figure 1. cDNA hybridization images showing differential expression of the BGR-like gene in 6-month-old fetal testes and adult human testes. The arrows indicate the BGR-like gene cDNA and the hybridization intensities in fetal and adult testes are 3.37 and 169.05, respectively. Hybridization intensity is 50-fold higher in adult than that in fetal testis.

showed that all exons were located with chromosome 19p13.3, so the BGR-like gene was mapped to chromosome 19p13.3.

BLAST-nucleotide showed that the BGR-like gene was highly homologous to the previously identified BGR gene including BC022027, AK0933433, AJ577571, AY358766 and AK057412. They belonged the same UniGene cluster Hs.99344. Splicing comparison indicated that six sequences had eight identical exons in the C-terminal of the cDNAs. The difference between the BGR-like gene and the other five sequences is that the BGR-like gene had a shorter exon1, and lacked exon5 and exon 7 (Figure 3). Because of the changing of the nucleotide acids, the sequences of proteins differed. The BLAST-protein search revealed that the BGR-like gene protein was 187 amino acids shorter than NP112186 (BC022027, AK0933433 and AJ577571 encoded the same protein), 137 amino acids shorter than AAQ89126 (AY358766 encoded protein) and 13 amino acids longer than BAB71476 (AK057412 encoded protein) at its Nterminal end, indicating that the six genes were transcripts from the same DNA and had then undergone different splices.

Additionally, the BGR-like gene protein had sequence homology with bubblegum of human, mouse, rat and *Drosophila* (Figure 4). The sequence positive of BGRlike gene with bubblegum of human, mouse and rat is approximately 69 % and with *Drosophila* bubblegum approximately 66 %. Analysis of the amino acid sequence of the BGR-like gene to the data bank of motifs using Gene Runner software (http://www.generunner.com) showed that there was a putative AMP-binding domain signature. In the regions of putative AMP-binding domain and bubblegum FACS signature motif, the BGRlike gene is highly conserved (Figure 4).

# 3.3 Expression of BGR-like gene transcript in human testes and Leydig cells

Our RT-PCR analyses revealed that the BGR-like gene transcript was expressed more highly in adult testes than in fetal as the result of hybridization, and was moderately expressed in elderly testes. BGR-like gene transcripts were also found in human Leydig cells (Figure 5).

# 3.4 Tissue distribution of the BGR-like gene and BGR transcripts

The expression of the BGR-like gene and BGR were

GGCTGGGATGGAACGTTCTGGAAGGAAGAAGAAAACACCTGAGTGACCAGATGTCCCAGC	60	TACCTATAGGCGAGTTGTATGGGTTGAGTGAGAGCTCGGGACCCCACACGATATCCAACC	1320
TCCAGGTGCCTTGCCAGATGGCCAGAACCACACCTCTTGAAGAGTGACAGTGCTGTGGAG	120	I P I G E L Y G L S E S S G P H T I S N	
CATGGTTTCTGCACACCTGGAATGACTGGAACCCCCAAAGACTCAAGAAGGAGCTAAAGAT	180	AGAATAACTACAGGCTTCTAAGCTGTGGCAAGATCTTGACTGGGTGTAAGAATATGCTGT	1380
CTTGAAGTAGACATGAATAAAACAGAAGTTACTCCCAGGCTGTGGACCACCTGTCGAGAT	240	Q N N Y R L L S C G K I L T G C K N M L	
GGAGAAGTCCTTCTGAGGCTATCCAAACACGGACCAGGCCATGAGACCCCGATGACCATC	300	TCCAGCAGAACAAGGATGGCATTGGGGAGATCTGCCTCTGGGGTAGGCACATCTTCATGG	1440
CCTGAATTTTTTCGAGAGTCAGTCAACCGATTTGGAACTTATCCAGCCCTCGCATCCAAG	360	FQQNKDGIGEICLWGRHIFM	
AATGGCAAAAAGTGGGAAATTCTGAATTTCAACCAGTACTATGAGGCTTGTCGGAAGGCT	420	GCTATCTGGAAAGTGAGACTGAAACTACAGAGGCCATCGATGATGAAGGCTGGCT	1500
GCAAAATCCTTGATCAAGCTGGGTTTGGAGCGTTTCCACGGAGTTGGTATCCTGGGGTTT	480	G Y L E S E T E T T E A I D D E G W L H	
AACTCTGCAGAGTGGTTTATCACTGCTGTTGGTGCCATCCCTAGCCGGATTCCACAGAGCA	540	CTGGGGATCTGGGCCAGCTGGACGGTCTGGGTTTCCTCTATGTCACCGGCCACATCAAAG	1560
GCCTAGAGCCCCTAAAAGCGATCATCCAGTACAGACTGCCAATGAAGAAGAACAACAACT	600	S G D L G Q L D G L G F L Y V T G H I K	
м к к n n n		AA <u>ATCCTTATCACTGCTGGTGG</u> TGAGAATGTGCCCCCCATTCCTGTTGAGACCTTGGTTA	1620
TGTACTCTTGGGATGATTTCATGGAACTTGGCAGAAGTATCCCTGACACCCAACTGGAGC	660	EILITAGGENVPPIPVETLV	
LYSWDDFMELGRSIPDTQLE		AGAAGAAGATCCCCATCATCAGTAACGCCATGTTAGTAGGAGATAAACTGAAGTTTCTGA	1680
AGGTCATCGAGAGCCAGAAGGCGAATCAATGCGCAGTGCTCATCTACACTTCAGGGACCA	720	K K K I P I I S N A M L V G D K L K F L	
Q V I E S Q K A N Q C A V L I Y T S G T		GCATGTTGCTGACGCTGAAGTGTGAGATGAATCAGATGAGCGGAGAACCTCTGGACAAGC	1740
CAGGCATACCCAAGGGAGTGATGCTCAGTCATGACAACATCACGTGGATTGCAGGAGCAG	780	SMLLTLKCEMNQMSGEPLDK	
T G I P K G V M L S H D N I T W I A G A		TGAACTTCGAGGCCATCAACTTCTGTCGGGGTCTGGGCAGCCAGGCATCCACCGTGACTG	1800
TGACAAAGGACTTTAAACTGACAGACAAGCATGAGACGGTGGTTAGCTACCTCCCACTCA	840	L N F E A I N F C R G L G S Q A S T V T	
V T K D F K L T D K H E T V V S Y L P L		AGATTGTGAAGCAGCAAGACCCCCTGGTCTACAAGGCCATCCAGCAAGGCATCAATGCTG	1860
GCCATATTGCAGCACAGATGATGGACATCTGGGTACCCATAAAGATTGGGGCGCTCACAT	900	E I V K Q Q D P L V Y K A I Q Q G I N A	
SHIAAQ MMDIW VPIKIGALT		TGAACCAGGAAGCCATGAACAATGCACAGAGGATTGAAAAGTGGGTCATCTTGGAGAAGG	1920
ACTTTGCTCAAGCAGATGCTCTCAAGGGCACCTTGGTAAGTACTCTAAAGGAGGTAAAAC	960	V N Q E A M N N A Q R I E K W V I L E K	
Y F A Q A D A L K G T L V S T L K E V K		ACTTTTCCATCTATGGTGGAGAGCTAGGTCCAATGATGAAACTTAAGAGACATTTTGTAG	1980
CTACTGTCTTCATTGGAGTGCCTCAAATTTGGGAGAAGATACATGAGATGGTGGAGAAAA	1020	DFSIYGGELGPMMKLKRHFV	
PTVFIGVPQIWEKIHEMVEK		CCCAGAAATACAAAAAACAAATTGATCACATGTACCACTGACTG	2040
ATAGTGCCAAGTCCATGGGCTTGAAGAAGAAGGCATTCGTGTGGGCAAGAAACATTGGCT	1080	А Q К Y К К Q I D Н М Y Н	
N S A K S M G L K K K A F V W A R N I G		CTCAGCTGTTCTGATGCCTTCAGCAGGAAGACCTCATTGCAATAAGTGAAATGCTGCTCT	2100
TCAAGGTCAACTCAAAAAAGATGTTGGGGAAATATAATACTCCCCGTGAGCTACCCGCATGG	1140	AGGTAGAAGCTCTCCCTGCTGTTTTTAAGAAGCCACATTCCTCATTGGTCAGTTTCTTGA	2160
FKVNSKKMLGKYNTPVSYRM		TTGTTCGTCTGTTGGAGAGGTGCTCCCTAGAAGAACCTGCCATACGTTTCAAAGCAATAA	2220
CTAAGACTCTCG TGTTCAGCAAAGTCAAGACATCCCTTGGCTTGG	1200	AATCACTGTATATCTTTCTAAGGACCTTCAAGTCATGACTCCAGGGAAGCCTATTGGGAA	2280
A K T L V F S K V K T S L G L D H C H S		GTTTACTAAAAACTGCCTGATTTACAAGAAAGACCTGAACTTGTGGGCTCCCATTTGATT	2340
TTATCAGTGGGACTGCGCCCCTCAACCAAGAGACTGCCGAGTTCTTTCT	1260	TTTTTCTCCTCAGGGGACTCAGACATTAGAAAGAAAAAGCCTCACAGATTTGAAGAACTG	2400
FISGTAPLNQETAEFFLSLD		GACCCCCAAATTAATCACCTGCCTGGAAGCAACTGGGAAACCCTTCCAATAAGTCCTGAT	2460
		AATAAAGCACTTCAGGGAAAAAAAAAAAAAAAAAAAAAA	2500

Figure 2. Nucleic acid and deduced amino acid sequences of the cDNA for the BGR-like gene. Underlining and bold show polymerase chain reaction (PCR) primers for the determination of expression profile. Grey highlighted areas indicate the putative adenosine monophosphate (AMP)-binding domain. Boxes show fluorescence-activated cell sorter (FACS) signature motif.

analyzed by RT-PCR in 16 different human organs. Detection of  $\beta$ -actin mRNA was used as an internal control. The  $\beta$ -actin-specific fragment could be amplified in all organs tested. BGR mRNA was predominantly expressed in testis and pancreas; lower in heart, lung, liver, kidney, small intestine and colon; and almost imperceptibly in human brain, placenta, muscle, prostate, thymus, spleen, ovary and leukocyte. But a BGR-like gene-specific fragment could be detected only in the testis. In the remaining organs including pancreas, kidney, colon, spleen, white blood cells, prostate, ovary, thymus, heart, small intestine, lung, placenta, liver, brain and muscle, the BGR-



Figure 3. Transcript and splicing comparison of the BGR-like gene with homologous genes. Seven homologues originate from one gene and consist of 16 exons. Identical exons are shown with black rectangles. The difference is shown with empty rectangles. The BGR-like gene lacks exon 5 and exon 7. The length of exon 1 and exon 16 varies among homologues. Arrows show the initiation of the open reading frame. •, the primers used in reverse transcription (RT)-PCR. The genome represents the corresponding DNA sequence of the BGR gene in chromosomes.

like gene transcript could not be found (Figure 6).

## 3.5 Abnormal expression of the BGR-like gene in the testes of patients with male infertility

A total of four normal fertile men and 12 patients with azoospermia were included in the present study. RT-PCR studies indicated that BGR-like gene mRNA existed in the four testes of normal fertile men and was not expressed in some of the patients' testes with abnormal spermatogenesis including spermatogenesis arrest, Sertoli cell-only syndrome and spermatogenesis disturbance. Expression of  $\beta$ -actin was detected in the testes of all the patients (Figure 7).

#### 4 Discussion

To study the mechanism of germ cell differentiation, it is useful to isolate and characterize the genes specifically expressed in testes. For this purpose, we have isolated specific cDNAs developmentally expressed in spermatogenesis [3]. In this paper, we describe one of these, named the BGR-like gene, which is highly expressed in human adult testes. The new findings include its cDNA and deduced amino acid sequences, its characterization

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analysis, the specific tissue distribution, age-dependent expression in human testes and an abnormal expression of this gene in patients with male infertility. These results strongly suggest that the BGR-like gene is involved in spermatogenesis.

The importance of the BGR-like gene in spermatogenesis was first identified by a close correlation beteen the expression level of the BGR-like gene and testicular development. Hybridization of cDNA microarray has indicated about 50-fold higher expression of the BGR-like gene in adult testes than in embryo testes, indicating its development-dependent expression. Further examination on development-dependent expression using RT-PCR on cDNA isolated from various developmental stages of human testes was performed. The BGR-like gene transcript was expressed almost imperceptibly in fetal testes, highly in adult testes, and moderately in elderly testes (Figure 5). Developmentally, spermatogenesis does not occur in male embryos. In adults, spermatogenic cells undergo successive mitotic, meiotic and post-meiotic phases, and then form mature sperm. In elderly males, altered sex hormone concentrations result from functional disturbances and a gradual reduction in Leydig cells [17], spermatogenesis weakens, and the quantity and

BGR-like	
HBG	MPRNSGAGYGCPHGDPSMLDSRETPQESRQDMIVRTTQEKLKTSSLTDRQPLSKESLNHA 60
MBG	MPRGSEAGYCCLSRDSNMPDSRDDQQQGASLGTSQDNSQTSSLIDGQTLSKESPSHG 57
RBG	MPRSSEAGYCCLSRDSNMPDSRDDQQQGASMGTSPDNSQTSSLIDGRTLSKESPSHG 57
DBG	MSTIDALYNRPGPNRLRQADA 21
BGR-like	
HBG	LELSVPEKVNNAQWDAPEEALWTTRADGRVRLRIDPSCPQLPYTVHRMFYEALDKYGDLI 120
MBG	LELSAPEKARAASLDGAEEALWTTRADGRVRLRLEPFCTQRPYTVHQMFYEALDKYGNLS 117
RBG	LELSAPEKARAASLDASEEALWTTRADGRVRLRLEPFCTQLPYTVHQMFYEALDKYGNLS 117
DBG	YRTTNRQDAVKIRMAKDGIGAEEPISVPGLLKRTVNNYGDYP 63
BGR-like	
HBG	ALGFKR-QDKWEHISYSQYYLLARRAAKGFLKLGLKQAHSVAILGFNSPEWFFSAVGTVF 175
MBG	$\label{eq:linear} ALGFKR-KDKWERISYYQYYLIARKVAKGFLKLGLERAHSVAILGFNSPEWFFSAVGTVF~176000000000000000000000000000000000000$
RBG	$\label{eq:linear} ALGFKR-KDKWERISYYQYYLIARKVAKGFLKLGLERAHSVAILGFNSPEWFFSAVGTVF~176000000000000000000000000000000000000$
DBG	ALRTKNGKNGYHTVTYKQYEQKVHQVAKAFIKLGLEEHHSVGVLAFNCAEWFYSAMGAIH 123
BGR-like	
BG	AGGIVTGIYTTSSPEACQYIAYDCCANVIMVDTQKQLEKILKIWKQLPHLKAVVIYKE 237
MBG	AGGIVTGIYTTSSPEACQYISHDCRANVIVVDTQKQLEKILKIWKDLPHLKAVVIYQE 234
RBG	AGGIVTGIYTTSSPEACQYIAHDCRANVIVVDTQKQLEKILKIWKDLPHLKAVVIYQE 234
DBG	ARGIIAGIYTTNSADAVQHVLESSHAQIVVVDDAKQMDKIHAIRDKLPKLKAAIQIQEPY 183
BGR-like	MKKNNNLYSWDDFMELGRSIPDTQLEQVIESQKANQCAVLIYTSGTTGIPKGVMLSH 57
HBG	- PPPNKMANVYTMEEFMELGNEVPEEALDAIIDTQQPNQCCVLVYTSGTTGNPKGVMLSQ 296000000000000000000000000000000000000
MBG	-PPPKKMANVYTMEELIELGQEVPEEALDAIIDTQQPNQCCVLVYTSGTTGNPKGVMLSQ 293
RBG	-PPPKKMANVYTMEELIELGQEVPEEALDAIIDTQQPNQCCVLVYTSGTTGNPKGVMLSQ 293
DBG	SPYLKKEDGYYRWSEIESMNVSDVEDQYKTRLENVAINECCCLVYTSGTVGMPKGVMLSH 243
DCD 111	
BGK-11Ke	DN11W1AGAV1KDFKL1D-KHE1VVSYLPLSH1AAQMMD1WVP1K1GAL1YFAQADAL 114
NDG	DNTTWTARYGSQAGDTRFAEVQQEVEVSYLFLSHTAAQTYDLWTGTQWGAQVCFAEFDAL 356
MBG	UNTIWIARYGSQAGDIQPAEVQQEVIVSYLPLSHIAAQIYDLWIGIQWGAQVCFADPDAL 353
KBU	
DRC	DNTIFDVRGIVKAMDRVVVGAESIVSYLPLSHVAAQTVDIYTCAFVAGCIWFADKDAL 301
	****

Figure 4 (Continued).

BGR-like	KGTLV	STLKEV	/KPT\	/FIG\	/PQIW	EKIH	EMVEKN	ISAKS	IGLK	KAF	VWAR	NIG	FKVNS	SKKM	174
HBG	KGSLV	NTLREV	EPTS	SHMGV	PRVW	EKIME	ERIQEV	AAQSO	GFIRF	RKMLI	LWAM	SVT	LEQN-	-LTC	415
MBG	KGTLV	NTLREV	EPTS	SHMGV	PRVW	EKIME	ERIQEV	AAQSO	GFIRF	KML	LWAM	SVT	LEQN-	-LTC	412
RBG	KGTLV	NTLREV	EPTS	SHMGV	PRVW	EKTMF	ERTQEV	AAQSO	FIRE	RKML]	WAM	SVT	LEQN-	-LTC	412
DBG	KGTLV	KSLQDA	RPTF	RFMGV	PRVY	EKFQI	ERMVAV	ASSS	GSLKK	MLA	SWAK	GIT	LKHY-	-MVS	360
	** **	*	**	*4	**	** *	k	*			**				
PCD-1:1-		TDVCVI	MART	PI VEC	WWW	ei ei i	Meuce	TCOT	UDI M	DET A	CEEL	CI D	TDICI	21 VC	094
UDC	DCCDI	VDETTE	DI ADA		WVDO	ALCE/	WCOW	EVCM	ALTING					SL10	475
MBC	PGODL	AFFIIF			IN V RQ		INCQUIN	FIGA		IE I QI		JUN	TRU I	IG1G	470
NDG	PONDL	APP 150			ARVRQ.	ALGE	INCQUIN	IEVCA		ETQ	DEEL	JLN	TRUT	luiu	472
RBG	PSNDL	CCEDV	TAV		NVVVO	ALGEI	INCQUIT	ACAA	APMIA	AE I QI	XPPL	JLN	IKLIA	MEC	472
DDG	<i>f</i> 0 <i>V</i> 22	GGFKIN	TAK	sL1M2	SKVKQ.	ALGFI	JKVLIL	JASAAr	APM5r	'E I N	MIFL	SLD.	LKIVI	AFG	420
			*	*	*	**		4	este	**	**	*		*	
BGR-like	LSESS	GPHTIS	SNQNN	VRLI	.SCGK	ILTG	CKNMLF	QQNKI	GIGE	EICL	WGRH	IFM	GYLES	SETE	294
HBG	LSETS	GPHFMS	SPYN	YRLY	/SSGK	LVPGG	CRVKLV	NQDAE	EGIGE	EICL	WGRT	IFM	GYLNM	1EDK	535
MBG	LSEST	GPHFMS	SPYN	YRLY	(SSGR	VVPGG	CRVKLV	NQDAI	)GIGE	EICL	WGRT	IFM	GYLNM	1EDK	532
RBG	LSEST	GPHFMS	SPYN	VYRLY	(SSGR	VVPGG	CRVKLV	NQDAI	)G I GF	EICL	WGRT	IFM	GYLN	1EDK	532
DBG	MSETA	GCHTIC	CLPDS	SVGLN	NT I GK	TLPG	CESKFI	NKDAN	IGHGE	ELCI	RGRH	VFM	GYIDN	KEK	480
	**	* *		*	*	**	k		* *1	* *	**	**	**		
BGR-like	TTEAI	DDEGWI	.HSGI	LGQI	LDGLG	FLYV	FGHIKE	EILIT	AGGEN	WPP	IPVE	TLV	KKKII	PIIS	354
HBG	TCEAI	DEEGWL	.HTGI	AGRI	DADG	FLYI	FGRLKE	ELIITA	AGGEN	WPP	VPIE	EAV	KMELI	PIIS	595
MBG	TCEAI	DSEGWI	.HTGI	MGRI	.DADG	FLYI	FGRLKF	ELIIT/	AGGEN	WPP	VPIE	EAV	KMELI	PHS	592
RBG	THEAI	DSEGWL	.HTGE	MGRI	.DDDG	FLYI1	FGRLKE	ELIITA	AGGEN	WPP	VPIE	EAV	KMELF	PIIS	592
DBG	TEESL	DDDCWL	HSGE	LGF V	/DDKG	YVSL1	IGRSKE	EIIITA	AGGEN	NIPP	VHIE	NTI	KKELI	DAIS	540
	* *	* **	* **	* *	* *	×	* **	****	****	* **	*	:	*	**	
BGR-like	NAMLV	GDKLKF	LSML	LTLK.	CEMNO	QMSGE	PLDKL	NFEAI	NFCR	GLGS	SQAST	TTV	SIVKQ	-QD	413
HBG	NAMLI	GDQRKF	LSML	LTLK	CTLD	PDTSD	QTDNL	TEQAM	EFCQ	RVGS	SRATI	VSI	SIIEK	-KD	654
MBG	SAMLI	GDQRKF	LSML	LTLK	CTLD	PETSE	PTDSL	TEQAV	EFCQ	RVGS	SKAST	VSI	SIVGQ	-RD	651
RBG	SAMLI	GDQRKF	LSML	LTLK	CTLNI	PETSE	PTDNL	TEQAV	EFCQ	RVGS	SKAST	VSI	SIVGQ	-KD	651
DBG	NAFLV	GEQRKY	LTVL	ITLK	TEVD	(DSGE	PLDEL	SHESS	VWVK	SLGV	/EHK1	VSI	DILAA	GPC	600
	* * :	* *	* *	***			* *			*	2	*	*		

Figure 4 (Continued).

BGR-like	PLVY	KAIQQ	GIN/	AVNQEAM	NNAQRI	EKWV	/ILE	KDFSIY	GGELGPM	MKLF	RHF	[VAQ]	KYKKQ	473
HBG	EAVY	QAIEE	GIRF	RVNMNAA	ARPYHI	QKWA	ILE	RDFSIS	GGELGPI	MKLK	RLT	VLE	KYKGI	714
MBG	EAVY	QAIHE	GIQF	RVNANAA	ARPYHI	QKWA	ILQ	RDFSIS	GGELGPI	MKLK	RLT	VLRI	KYKDI	711
RBG	EAVY	QAIHE	GIQF	RVNANAA	ARPYHI	QKWA	ILE	RDFSIS	GGELGPI	MKLK	RLT	VLE	KYKDI	711
DBG	PKVWI	KSIED	AIKF	RANKQSI	SNAQKV	QKFI	ILP	HDFS1P	TGELGPI	LKVK	RNV	VSKI	IYADE	660
	*	*	*	*		*	**	****	****	* *	*	*	*	
DCD-11ko														
DOR-11Ke	1 DI M	Y I I	- 41	79										
HBG	IDIM	YQEQK	- 47 M 72	79 24										
HBG MBG	IDIM IDSF IDSF	Y QEQK Y QEQK	- 47 M 72 Q 72	79 24 21										
HBG MBG RBG	IDIM IDSF IDSF IDSF	Y NEQK Y QEQK Y QEQK	- 47 M 72 Q 72 Q 72	79 24 21 21										
HBG MBG RBG DBG	IDIM IDSF IDSF IDSF IDSF	Y II Y QEQK Y QEQK Y QEQK Y A	- 41 M 72 Q 72 Q 72 - 66	79 24 21 21 66										

Figure 4. Amino acid sequence comparison of the BGR-like gene protein with bubblegum of human, mouse, rat and *Drosophila* by using software ClustalW. HBG, human bubblegum; MBG, mouse bubblegum; RBG, rat bubblegum; DBG, *Drosophila* bubblegum; \*, identities in amino acid sequence. Grey highlighted areas indicate the putative AMP-binding domain. Boxes show FACS signature motif.

quality of sperm decreases. Thus, we suggest that in human testes, the BGR-like gene is developmentally regulated and may play a role in testicular development and/ or spermatogenesis.

Consistent with its potential role in spermatogenesis, the BGR-like gene was expressed specifically in human testes. Sequence analysis showed that the BGR-like gene was a new mRNA splice variant of other BGR transcripts, four of which came from testes. We designed two primers



Marker Fetal Adult Old Leydig Blank

Figure 5. BGR-like gene expression in human fetal testes, adult testes, aged testes and human Leydig cells.  $\beta$ -actin was used as a control. BGR-like gene transcript was expressed more highly in adult testes than in fetal, and moderately expressed in elderly testes. The BGR-like gene specific fragment was also found in human Leydig cells.

located in the common region of the six sequences and determined their tissue distribution by RT-PCR. The common BGR fragment was not only detected in the testes but also found in the pancreas, heart, lung, liver, kidney, small intestine and colon. The results indicated that the expression of BGR mRNA was widely distributed in human tissues. We further examined the expression profile of the BGR-like gene mRNA in 16 human organs and found that the BGR-like gene was highly and exclusively expressed in testes. Based on these results, we believe that the BGR-like gene. The high tissue specificity of the BGR-like gene expression pattern provided compelling evidence for a crucial role of the BGR-like gene in spermatogenesis.

Although there is a close relationship between the BGR-like gene and spermatogenesis, the exact function of the BGR-like gene protein remains elusive. Homologous comparison of BGR-like gene transcript revealed that BGR-like gene protein also had sequence homology with bubblegum of human, mouse, rat and *Drosophila* which were proved to have ACS activity and belong to the ACS super-family. The sequence positive of the BGR-like gene with bubblegum of human, mouse and rat is 69 % and with *Drosophila* bubblegum 66 %. Interestingly, the BGR-like gene also contains the two conserved functional motifs that are common to these and related



Figure 6. Electrophoresis showing expression profiles of the BGR and BGR-like gene. BGR mRNA was predominantly expressed in testes and pancreas; weakly in the heart, lung, liver, kidney, small intestine and colon; and almost imperceptibly in the human brain, placenta, muscle, prostate, thymus, spleen, ovary and leukocyte. The BGR-like gene was specifically expressed in testes. All organs expressed  $\beta$ -actin. M, marker; He, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; Sk, skeletal muscle; Ki, kidney; Pa, pancreas; Sp, spleen; Th, thymus; Pr, prostate; Te, testis; Ov, ovary; Si, small intestine; Co, colon; Le, blood leukocyte; Bl, blank.



Figure 7. BGR-like gene expression in testes of normal fertile men and azoospermia patients. (A): BGR-like gene expression in four normal fertile men' testes. (B): RT-PCR studies examined BGR-like gene expression in testes of 12 patients with Sertoli cell-only syndrome (1), spermatogenesis arrest (2–6) and spermatogenesis disturbance (7–12). BGR-like gene specific fragment was not detected in patients 1, 2, 5, 8, 9, 10 and 12. As a control, the lower panel displayed the expression level of  $\beta$ -actin in corresponding patients.

enzymes (Figure 4). One of the regions highly conserved in the BGR-like gene is the putative domain for the ATPdependent covalent binding of AMP. The AMP-binding domain is required for fatty acid uptake, because mutations within this motif abolish uptake activity [18]. The other conserved domain is believed to represent the FACS signature motif. The motif is essential for catalytic activity and functions in part to promote fatty acid chain length specificity and may compose part of the fatty acid binding site within the enzyme [19]. The conserved primary structure suggests, at least to some extent, that its function may also be conserved and important. As for the bubblegum family, human and mouse bubblegum are reported to be very long-chain acyl-CoA synthetase (VLCS) or long-chain acyl-CoAsynthetase (LACS) and play a central role in very long-chain fatty acid (VLCFA) or long-chain fatty acid (LCFA) metabolism. Their mRNA is expressed in the brain, adrenal gland and testes, which are affected by X-ALD [13, 14]. In adult mice, interstitial Leydig cells were found to highly express bubblegum mRNA and protein. The rat bubblegum ortholog is a gonadotropin regulated LACS (GR-LACS) [15]. GR-LACS mRNA is expressed abundantly in Leydig cells of the adult testis and relatively low in the ovary and brain [15]. In vivo, treatment with a densensitizing dose of human chorionic gonadotropin caused transcriptional down-regulation of GR-LACS expression in Leydig cells. The similar amino acid sequences, the presence in the BGR-like gene protein of a putative AMP-binding domain and FACS signature motif and testis expression prompted the hypothesis that the BGR-like gene may also be expressed in Leydig cells. To test this hypothesis, we investigated the expression of the BGR-like gene in human Leydig cells. The result showed that the BGR-like gene indeed expressed in Leydig cells which are known to be essential cells for spermatogenesis. In Leydig cells, androgen biosynthesis is active, depends highly upon the availability of steroid precursors. This requirement may be contributed by the acyl-CoA synthetase activity of the BGR-like gene and related enzymes. In testes, cholesterol is generated from endogenous precursors rather than derived from circulating lipoproteins, which is the case in other steroidogenic tissues [20]. For this reason, the BGR-like gene may be important in the maintenance of adequate cholesterol pools required for steroid biosynthesis in the testes. The hypothesis that the BGR-like gene encodes an ACS must be tested by determining enzymatic activities of the protein.

The present study also revealed abnormal expression of the BGR-like gene in infertile men. Among the twelve patients we studied, seven of them had no expression of the BGR-like gene in the testes while all four fertile adults expressed the BGR-like gene. Our results showed that a lack of expression of the BGR-like gene is correlated with azoospermia: loss of the BGR-like gene might affect steroidogenesis and can interfere with spermatogenesis. The function of the BGR-like gene and its role in male infertility can be examined further using point- or null mutation of the BGR-like gene.

In conclusion, the present study has provided evidence implicating the possible involvement of the BGRlike gene in spermatogenesis. Its development-dependent expression, tissue distribution and protein characteristics point to a possible role for the BGR-like gene in spermatogenesis. Further research is required to determine the exact role and regulation mechanism of the BGRlike gene protein in spermatogenesis and its relationship with clinical male infertility.

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