Expression of HBP2 in Human Spermatogonial Stem Cell-like Cells from Nonobstructive Azoospermia Patients and Its Role in G1/S Transition & Downregulation in Colon Cancer

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ABSTRACT

The HMG box containing protein (HBP) has a high mobility group domain and involved in the regulation of proliferation and differentiation of tissues. We screened *HBP2* in glioblastoma using Suppression Subtractive Hybridization (SSH) and isolated human spermatogonial stem cell-like cells (hSSC-like cells) derived from patients of nonobstructive azoospermia (NOA). Expression of HBP2 was analyzed by RT-PCR in undifferentiated stem cells (human Embryonic Stem Cells, hSSC-like cells 2P) and spontaneous differentiated stem cells (hSSC-like cells 4P). It was overexpressed in hESC and hSSC-like cells 2P but not in hSSC-like cells 4P. Also, the expression level of HBP2 was downregulated in colon tumor tissues compared to normal tissues. Specifically in synchronized WI-38 cells, HBP2 was highly upregulated until the G1 phase of the cell cycle and gradually decreased during the S phase.

Our results suggest that HBP2 was downregulated during the spontaneous differentiation of hSSC-like cells. HBP2 was differently expressed in colon tissues and was related to G1-progression in WI-38 cells. It may play a role in the maintenance of an undifferentiated hSSC-like cell state and transits from G1 to S in WI-38 cells.

This research was important that it identified a biomarker for an undifferentiated state of hSSC-like cells and characterized its involvement to arrest during cell cycle in colon cancer.

(Key words : HBP2, HMG-domain, hSSC-like cells, NOA, Cell cycle)

INTRODUCTION

HMG (high mobility group) box-containing proteins (HBP) have a high mobility group domain (Lesage *et al.*, 1994). HBP1, like members of the TCF/LEF and SOX families, is a sequence-specific HMG box transcription factor with significant roles in the regulation of proliferation and differentiation. Expression of HBP1 leads to cell cycle arrest even in the presence of optimal proliferation signals *in vitro* (Tevosian *et al.*, 1997). *In vivo*, HBP1 has been shown to regulate proliferation within differentiated adult tissues; after partial hepatectomy in transgenic mice carrying HBP1 under a hepatocyte specific promoter. As little as a two-fold increase in the level of HBP1 protein is sufficient to delay progression of hepatocytes through G1 to the peak of S phase by 10~12 hours. HBP1 can function as a direct transcriptional repressor of cell cycle targets such as the promoters for *n-Myc* and the CDK inhibitor p21 (Smith *et al.*, 2004). The mammalian *HBP2* gene has been isolated by functional complementation of cells unable to undergo DNA replication in fission yeast. HBP2, a protein with the HMG domain, belongs to the Sox (Sry-related HMG box) family of transcription factors (Sanchez-Diaz *et al.*, 2001). SRY has been identified in the sex determining regions of the human and mouse Y chromosomes (Denny *et al.*, 1992). SOX proteins regulate gene expression by binding to DNA via a conserved DNA-binding domain, the HMG box. Phylogenetic studies have determined that SOX family members segregate into ten groups (named A~J) on the basis of sequence similarities within the HMG box with many groups containing multiple members in the same organism, each having related gene functions. For instance, human and mouse genomes each encode 20 *Sox* genes (Wilson and Dearden, 2008).

Previously, we used the SSH (Suppression subtrac-

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tive hybridization) process to identify genes that are differently expressed in glioblastoma (Yu *et al.*, 2005). Our teams have also isolated human spematogonial stem cell-like cells derived from patients of NOA (Nonobstructive azoospermia) (Lee *et al.*, 2006).

In the current study, we showed that HBP2 was expressed in human Embryonic Stem Cells (CHA-hESC6) lines as well as hSSC-like cells 2P, but not in hSSC-like cells 4P. This gene is differently expressed in colon tissues and plays a role in the G1/S transition in WI-38 cells. This research was important since it identified a likely biomarker for determining the differentiation state of hSSC-like cells and because it showed that HBP2 is upregulated in undifferentiated cells.

MATERIALS AND METHODS

Cell Cultures

The WI-38 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). WI-38 normal lung fibroblast cells were routinely maintained in Eagle's minimal essential medium (EMEM), amino acids, non-essential amino acids supplemented with 10% fetal bovine serum (FBS), 0.1 M sodium pyruvate, streptomycin (100 μg/ml) and penicillin (100 U/ml). WI-38 cells were cultured at 37℃ in a humidified 5.0% CO_2 incubator. Human Embryonic Stem Cells (CHA-hESC6), undifferentiated human spermatogonial stem cell-like cells (hSSC-like cells 2P) and spontaneous differentiated human spermatogonial stem cell-like cells (hSSC-like cells 4P) were kindly provided by Dr. Lee at the Fertility Center of CHA General Hospital, CHA Research Institute (Lee *et al.*, 2006).

Colon Tissues

Colon tissues specimens (four proximal colon tissues) from Korean patients were donated by Dr. Kim at Bundang CHA General Hospital, Seongnam, Korea as described (Park *et al.*, 2007).

Isolation of RNA

Total RNA was extracted from cell lines using TRI REAGENT (Molecular Research Center; Cincinnati, Ohio, USA). Cells grown in a monolayer were lysed directly in a culture dish using the following procedure: first, media were poured off and 1 ml TRI REAGENT was added to each 10 cm^2 of culture dish. The homogenate was stored for 5 min, at room temperature to permit the complete dissociation of nucleoprotein complexes. The homogenate was then supplemented with 0.2 ml chloroform per 1 ml TRI REAGENT and shaken vigorously for 15 sec. The resulting mixtures were stored at room temperature for 5 min and centrifuged at 13,000 rpm for 15 min at 4℃. Next, the aqueous phase was transferred to a fresh tube and mixed with 0.5 ml of isopropanol. The sample was stored at room temperature for 5 min and centrifuged at 13,000 rpm for 8 min at 4℃. The supernatant was removed and the RNA pellet was washed with 1 ml of 70% ethanol and subsequently centrifuged at 13,000 rpm for 5 min at 4℃. After the ethanol was removed, RNase-free water (generated by diethyl pyrocarbon (DEPC) treatment was used to resuspend the pellet. The concentration of each RNA sample was measured prior to the synthesis of high quality cDNA by determining the 260/280 nm ratio. Roughly 1 μg of total RNA was treated with 1 μl of DNase I (Roche; Germany) at 37℃ for 20 min, followed by incubation at 65℃ for 10 min, to remove genomic DNA contaminants.

Synthesis of cDNA and Analysis of RT-PCR

Reverse transcription-polymerase chain reactions (RT-PCR) were performed on RNA isolated from hESCs, hSSC-like cells, normal colon tissues and colon tumor tissues. Full-length cDNA was synthesized using the SuperScript™ First-strand Synthesis System (Invitrogen; Carlsbad, CA, USA). To normalized mRNA amounts, levels of the housekeeping gene 18S rRNA were determined. Specific primers were designed as 20 mers (Table 1). PCR were performed using the TaKaRa EX Taq™ kit using the following PCR condition, an initial incubation for 5 minutes at 94℃, followed by 35 cycles of: i) 30 seconds at 94℃ (denaturation), ii) 30 sec at 55℃ (annealing) and iii) 30 sec at 72℃ (extension), finally, a 5 min incubation at 72℃ was performed. The products of the PCR amplifications were analyzed on a 1.0% agarose gel.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using a SYBR Green I kit (Qiagen; Hilden, Germany). Specific primers were designed as 20 mers. Calibrated and normalized gene expressions levels were determined using the housekeeping gene (18S rRNA). The PCR conditions are as follows: first, a 10 min incubated at 9 4℃ was performed followed by 45 cycle of: i) 20 sec at 94℃, ii) 20 sec at 55℃ and iii) 25 sec at 72℃. All PCR were performed in a Roter-gene 6 (Corbett Research; Sydney, Australia). All plates were analyzed in triplicate and the average copy numbers were calculated as described (Jung *et al.*, 2008).

Serum Stimulation

WI-38 was seeded at a density of 1×10^6 cells/100 mm dishes. When the cells were 70% confluent, cells were grown in serum-free medium for 48 h and then

Table 1. Sequences of primer pairs

Primer names	Sequence (5′-3′)	TM.	Product size
HBP2 (RT-PCR)	F : 5′-ACACCCCAGGCTTGATAACC-3′ R : 5′-TTCCACAGCGTTTTACAGGC-3′	55℃	578 bp
HBP2 (qRT-PCR)	F : 5′-GATCCCGCAGCATTAAACAA-3′ R : 5′-GCAAGTCTTCTTGGGTGTGG-3′	55℃	129 bp
Cyclin D (RT-PCR)	F : 5′-ATGTTCGTGGCCTCTAAGATGA-3′ R : 5′-CGTGTGAGGCGGTAGTAGGACAG-3′	55℃	374 bp
Cyclin E (RT-PCR)	F : 5′-TCTCAGAAAGATGTTGGGAAGC-3′ R : 5′-TGAATCTGACTTCCTCCCTTGA-3′	55℃	331 bp
18S rRNA	F : 5′-TACCTACCTGGTTGATCCTG-3′ R : 5′-GGGTTGGTTTTGATCTGATA-3′	55℃	255 bp

TM: temperature; bp: base pair; RT: reverse transcription; qRT: quantitative real time.

supplied with fresh 10% FBS-containing medium. After 10% FBS treatment, cells were harvested at different time points: 0, 0.5, 1, 2, 4, 6, 12, 24, 30 and 48 hours as described (Choi *et al*,. 2005; Choi *et al.*, 2006).

RESULTS

Previously, our team indentified DEGs (differently expressed genes) in glioblastoma by SSH (Yu *et al.*, 2005). We screened the expression levels of those genes in hSSC-like cells 2P and hSSC-like cells 4P. HBP2 was downregulated as the progression to differentiation. The expression level of HBP2 in undifferentiated cells (CHA-hESC6, hSSC-like cells 2P) remained highly compare to its level in differentiated cells (hSSC-like cells 4P). Therefore, HBP2 is downregulated as the differentiation of spermatogonia lineage cells (Fig. 1).

HBP2 was differently expressed in colon tissues. We performed RT-PCR in normal and corresponding tumor tissues from four colon cancer patients. HBP2 was upregulated in normal colon tissues compared to tumor tissues (Fig. 2).

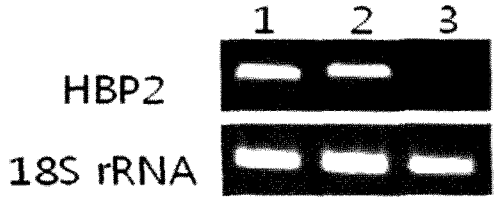

Fig. 1. Expression pattern of HBP2 in hESC and hSSC-like cells. Lane 1: hESC-CHA6 cell line, Lane 2: Undifferentiated hSSC-like cells 2P, Lane 3: Spontaneous differentiated hSSC-like cells 4P. 18S rRNA was used as the reference gene.

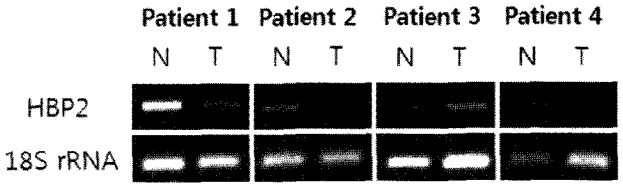

Fig. 2. Expression pattern of HBP2 in colon tissues. N: normal colon tissues, T: tumor colon tissues. 18S rRNA was used as the reference gene.

To analyzed the association of HBP2 in cell cycle regulation, we performed serum stimulation. Normal WI-38 lung fibroblast cells were cultured at a density of 70%, and then treated with serum-free medium for 48 hours. After 48 hours, 5% serum-containing medium was added and RNA samples were isolated at the indicated times (Choi *et al.*, 2005; Choi *et al.*, 2006). By using this method, we could arrest the WI-38 cells at the GO/G1 boundary in cell cycle (Russo *et al.*, 2001). The G1/S transition occurs between 4 hours and 6 hours in synchronized WI-38 cells. Expression of HBP2 was significantly increased until 4 hours and gradually decreased in WI-38 cells treated with 10% serum for 6 to 12 hours. Hence, HBP2 seems to be upregulated during the G1 phase and downregulated in the S phase (Fig. 3).

DISCUSSION

In the human adult male, a population of diploid stem-cell spermatogonia continuously undergo self-renewal and produce progenitor cells, thereby initiating the complex process of cellular differentiation that re-

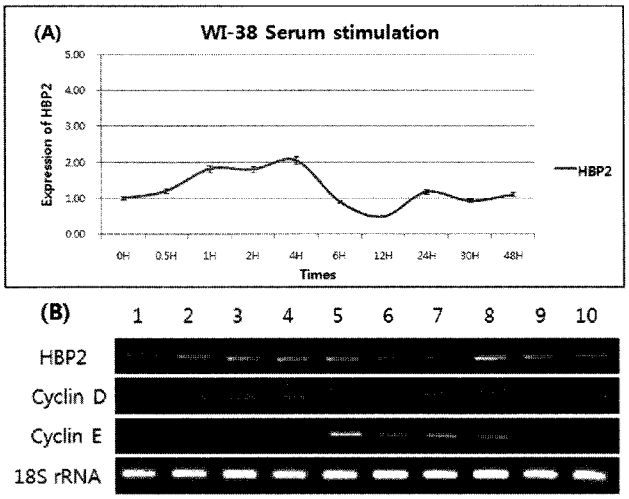

Fig. 3. Serum stimulation. HBP2 expression levels at the proposed G1/S transition in WI-38 cells. (A) qRT-PCR were analyzed using Roter-gene 6 (Corbett Research). Each gene level was calibrated using the housekeeping gene (18S rRNA). (B) Expression of HBP2 in serum-stimulated cells. Lane 1: 0 h, Lane 2: 0.5 h, Lane 3: 1 h, Lane 4: 2 h, Lane 5: 4 h, Lane 6: 6 h, Lane 7: 12 h, Lane 8: 24 h, Lane 9: 30 h, Lane 10: 48 h.

sults in mature spermatozoa (Brinster and Zimmermann, 1994). A cure for NOA through reproductive medicine is still lacking (Chan and Schlegel, 2000). Some patients with symptoms of NOA successfully restored their reproduction ability by ICSI (Intracytoplasmic sperm injection) or ROSI (Round spermatid injection) in cases where sperm was derived from testicular tissues with focal spermatogenesis (Devroey *et al.*, 1995). In general, however, artificial insemination is the most commonly recommended therapy for patients exhibiting MA (maturation arrest) at a specific stage of spermatogenesis or with SCOS (Sertoli cell-only syndrome) by isolating hSSC-like cells from patients NOA (Lee *et al.*, 2006). We could not maintain hSSC-like cells over long-term culture. Specifically, the colonies disappeared before the 10th consecutive passage (Nagano *et al.*, 1998). Therefore, we need to increased life-span in hSSC-like cells during spontaneous differentiation.

HBP2 had been identified by our team as a gene of interest using SSH of glioblastoma (Yu *et al.*, 2005). HBP2 has a conserved HMG domain (Lesage *et al.*, 1994). HMG box protein is one which represses the expression of a set of genes activated by E2F, contributing to the cells entrance into the G0 phase (Shih *et al.*, 1998). In addition, it was bound and regulated by RB and other pocket proteins (p107, p130). HBP1 was involved in G1 regulation in an animal model of proliferation (Lavender *et al.*, 1997). HBP1 could regulate cell cycle progression in differentiated tissues (Shih *et al.*, 2001). The inhibition of proliferation suggests that HBP1 might have a tumor suppressor function in the breast (Kim *et al.*, 2006). The HBP1-mediated suppression of Wnt signaling was also consistent with growth suppression in colon cancer cells, genes such as cyclin D1 are constitutively activated through mutation in the Wnt pathway (Sampson *et al.*, 2001).

Compared to the diverse studies on HBP1, research on HBP2 is quite scarce. To date only yeast studies have been performed. HBP2 activated transcription at G1/S transition fission yeast *Sch. pombe* (Sanchez-Diaz *et al.*, 2001). In the current study, we analyzed the upregulated expression level of HBP2 in undifferentiated stem cells and normal colon tissues. The expression level of HBP2 was downregulated during spontaneous differentiated hSSC-like cells (Fig. 1). Expression pattern of HBP2 in hESCs showed function of undifferentiated potential, because hSSC-like cells were adult stem cells. HBP2 was upregulated in normal colon tissues compared to colon tumors (Fig. 2). HBP2 was played an important role cell proliferation in undifferented stem cells but it has no connection with proliferation of colon cancer. HBP2 differently processed proliferation in stem cell and colon cancer. In addition, HBP2 seems to progress G1/S in WI-38 cells. Treatment with serum-free medium for 48 h synchronized the WI-38 cells at G0 and treated serum-containing medium each dish. The cells re-cycled cell cycle and transited G0/G1 stage. HBP2 expression levels gradually increased until 4 h (2-fold), and decreased after 8 h of serum stimulation in WI-38 cells (Fig. 3). We confirmed the G1/S transition period (after 4 h) by analyzing the expression pattern of cyclin D and E (Fig. 3) (Akli and Keyomarsi, 2003). Therefore, HBP2 maintains the undifferentiated state in stem cells and it seems to regulate G1 progression in WI-38 cells like *Sch. pombe*.

In summary, HBP2 has an HMG domain which participates in the regulation of proliferation and differentiation. HBP2 maintains the undifferentiated state and is downregulated in colon cancer tissues. We purpose that overexoressed-HBP2 in hSSC-like cells 4P will increase life-span and don't progress tumorigenesis. In further studies, we will expand life-span in hSSC-like cells 4P. We will confirm HBP2 that it plays roles of tumor suppression and cell cycle arrest in other tumor tissue like HBP1. In addition, we will attempt to use HBP2 as a biomarker for an undifferentiated state of hSSC-like cells.

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