Characterization of Basigin Isoforms and the Inhibitory Function of Basigin-3 in Human Hepatocellular Carcinoma
Proliferation and Invasion

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Basigin, which has four isoforms, plays an important role in invasion of hepatocellular carcinoma (HCC). Detailed transcriptional regulation and functions of the basigin isoforms have not been reported except in the case of the predominant isoform basigin-2, which acts as inducer of matrix metalloproteinases (MMPs). Here we determined that basigin-2, basigin-3, and basigin-4 were the most abundant transcript variants in human cell lines. GeneRacer PCR and luciferase reporter assays showed that basigin-3 and basigin-4 were initiated from an alternative promoter. Basigin-3 and basigin-4 were widely expressed in various normal human tissues at the mRNA level and were upregulated in HCC tissues compared to normal tissues. Western blotting and confocal imaging showed that glycosylated basigin-3 and basigin-4 were expressed and localized to the plasma membrane. However, in cultured cell lines, only native basigin-3, and not basigin-4, was detected at protein level. Overexpression of basigin-3 inhibited HCC cell proliferation, MMP induction, and cell invasion in vitro and in vivo. Bimolecular fluorescence complementation assays and nuclear magnetic resonance (NMR) analysis indicated that basigin-3 interacted with basigin-2 to form hetero-oligomers. In conclusion, we systematically investigated the alternative splicing of basigin and found that basigin-3 could inhibit HCC proliferation and invasion, probably through interaction with basigin-2 as an endogenous inhibitor via hetero-oligomerization.

The stromal components of tumors interact with cancer cells to promote tumor growth and metastasis (34). One of the most important groups of mediators of the communication between tumors and their microenvironment is matrix metalloproteinases (MMPs), enzymes that are produced largely by various stromal cells rather than tumor cells. MMPs help cancer cells spread by breaking down the extracellular matrix (ECM) and other barriers (5). In many tumors, MMP expression is regulated mainly by tumor-stroma interactions via extracellular MMP inducer (EMMPRIN), also called CD147, HAb18G/CD147, or basigin (BSG) (12, 14, 37). Basigin is a highly glycosylated type I transmembrane protein belonging to the immunoglobulin (Ig) superfamily (2). The molecule is composed of an intracellular portion and an extracellular portion that contains two immunoglobulin-like domains and a single transmembrane region (26). Crystal structure resolution indicates that the extracellular portion of basigin comprises an N-terminal IgC2 domain and a membrane-proximal IgI domain (44). The IgC2 domain is required for counterreceptor activity (35), which is involved in MMP induction and oligomerization (43). The IgI domain is required for association with caveolin-1, which leads to decreased self-association on the cell surface (36). Several functions of basigin have been described in both physiological and pathological processes. For example, basigin is involved in energy metabolism (17), reproduction (10), and development (6). In numerous malignant solid tumors, basigin is overexpressed and promotes tumor progression, invasion, and metastasis by stimulating MMP secretion, which is the best-characterized function for this gene (11). In addition, it is noteworthy that increasing evidence suggests that N glycosylation and homo-oligomerization are essential for basigin to be fully functional (43, 44). Due to the pivotal role of basigin in cancer, this molecule has been termed a cancer-associated biomarker (23) and serves as a target for cancer therapy (40).

Alternative splicing (AS) and alternative promoters (AP) are important molecular mechanisms that generate a large number of mRNA variants and protein isoforms from the surprisingly low number of human genes, which greatly expands protein diversity (21, 27). AS, in correlation with AP,
may regulate tissue- or cell-specific transcription patterns and translation efficiency and produce protein isoforms with additional or deleted protein parts or domains, including different N or C termini, which could lead to changes in protein functions (3, 33). AS and AP are prevalent in numerous type I transmembrane adhesion molecules, such as HCAM (CD44) (28), NCAM (CD56) (38), and CEACAM1 (CD66a) (42), producing isoforms with a variety of domains and corresponding functions. Interestingly, there is increasing evidence that alterations in the splicing patterns of genes may be involved in the regulation of gene functions by generating endogenous inhibitor or activator isoforms (22, 30).

Currently, four basigin AS transcript variants (Homo sapiens basigin transcript variants 1, 2, 3, and 4) encoding different isoforms (basigin isoforms 1, 2, 3, and 4) are found in the NCBI Entrez Gene database. Of these four variants, the basigin-2 transcript (accession number NM_198589) is the most predominant splice variant, encoding the well-known basigin/CD147/EMMPRIN (1). The basigin-1 transcript (NM_001728) encodes a long, retina-specific isoform that is distinguished by an additional Ig-like domain (three Ig-like domains in total) in the extracellular portion (8). The other two, less widely found variants, basigin-3 (NM_198590) and basigin-4 (NM_198591), were first identified in human endometrial stromal cells and cervical carcinoma cell lines (1). Basigin-3 is a short isoform comprising only one Ig-like domain (Ig-I domain) in its extracellular portion (32); it interacts with the internalized basigin receptor-ligand complex (1). However, the detailed characteristics of basigin isoforms, such as transcriptional regulation, expression profiles, and functions that distinguish them from the well-known basigin-2, are not known.

To further understand the role of alternative splicing in the regulation of basigin expression and the biological functions of the isoforms, multiple approaches were employed in this study. We first determined the expression of basigin transcript variants in human cell lines. Then, we analyzed basigin variant exon arrangements, splicing patterns, and transcriptional regulation. The expression profiles of basigin-3 and basigin-4 were determined in 20 normal human tissues, hepatocellular carcinoma (HCC) cell lines, and adjacent normal tissues (ANTs) were collected after written informed consent from patients at the Xijing Hospital of the Fourth Military Medical Uni-

MATERIALS AND METHODS

The sequences of all primers and probes used in this work, as well detailed sources of samples in Human Total RNA Master Panel II, will be provided upon request.

**Cell lines and human tissue samples.** The FHCC-98 and SMMC-7721 human HCC cell lines have previously been described (41). Other tumor or normal cell lines, including A549, 786-O, PC-3, SW480, MD-MBA-231, OZQ (normal human hepatic cells), HEK-293, Fb (human skin fibroblasts), and NIH 3T3, were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured using standard protocols. Human Total RNA Master Panel II was purchased from Clontech (Mountain View, CA). A total of 30 paired pathologically confirmed HCC tissues and adjacent normal tissues (ANTs) were collected after written informed consent from patients at the Xijing Hospital of the Fourth Military Medical Uni-

**DNA extraction and RNA isolation.** Total DNA was harvested from cultured cells or tissues using the RNasea mikit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the PrimerScript reverse transcription-PCT (RT-PCR) kit (TaKaRa, Shiga, Japan). PCR products were performed using Ex Taq HS (TaKaRa) with the following program: 94°C for 2 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and a final step of 10 min at 72°C. PCR products were analyzed using agarose gel electrophoresis and cloned into the pMD18-T (TaKaRa) vector for sequencing.

The GeneRacer kit (Invitrogen, Carlsbad, CA) was used to perform 5′ rapid amplification of cDNA ends (5′-RACE) and 3′-RACE. Poly(A)+ RNA was first purified from total RNA using the Oligoex mRNA minikit (Qiagen, Hilden, Germany) to generate RACE-ready cDNA. 5′-RACE and 3′-RACE nested PCRs were performed with GeneRacer primers and gene-specific primers. Amplified products were cloned and sequenced using the TOPO TA cloning kit (Invitrogen).

**Bioinformatics analysis of promoter region and dual-luciferase reporter assay.** The 5′ flanking sequence of basigin-3 and basigin-4 were analyzed using the computer program PROSCAN (Dan Prestridge, University of Minnesota; http://www-bimas.cit.nih.gov/molbio/proscan/) with default parameters. Based on the predicted results for the promoter region, several primers and forward strands. In a series of 5′-terminal deletion mutants was designed and subcloned into the pGL3-basic vector (Promega, Madison, WI). The normal hepatic cell line OZQ was transfected with 0.3 pmol of pGL3 plasmid containing the insert to be measured and 0.03 pmol of the pRL-TK vector (Promega) using Lipofectamine 2000 (Invitrogen). The pRL-TK was used as an internal control and pGL3-basic as negative control (NC). An hTERT/pcDNA3 vector containing the basigin-2 core promoter (bp −217 to +37 of the basigin-2 transcription initiation site [TSS]) was used as a positive control (19). At 24 h after transfection, cells were lysed, and the luciferase activities were measured as previously described (18).

**Generation and purification of recombinant basigin-2 and basigin-3.** The RNA fragments encoding extracellular portions of basigin-2 and basigin-3 were subcloned into the pET22a vector (Novagen, Madison, WI). The proteins were expressed in the Escherichia coli BL21 (DE3) Golden strain.

**Antibodies.** HAB18 is a monoclonal antibody (MAb) against the Ig-C2 domain (specific to basigin-2) (20). Recombinant protein B23ID-D4P (Igl domain, consisting of 64 residues from T121 to C185 of basigin-2) and synthetic polypeptide B23ID-D4P-N85 (85 residues from N-terminal to residue 170 of basigin-2) were used as antigens for the preparation of rabbit polyclonal antibodies B23ID and B4N11, respectively, against the Ig domain (specific to both basigin-2 and basigin-3) and basigin-4. Preparation and purification of antibodies were performed using the EZ sulfhydryl mKHL antibody preparation and purification kit (Pierce, Rockford, IL). Antibodies against green fluorescent protein (GFP) (sc-9996), MMP-2 (sc-10736), MMP-9 (sc-10737), and a-tubulin (sc-80666) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Proliferating cell nuclear antigen (PCNA) antibody (clone pc10) was from Chemicon (Billerica, MA). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) or Cy3 were from Pierce (Rockford, IL).

**Vector construction and stable transfection.** The coding regions of basigin-2, basigin-3, and basigin-4 were inserted into pEGFP-N1 (Clontech). Stable transfectants were screened with G418 (Calbiochem, San Diego, CA) after transfection.

**Deglycosylation and Western blotting.** Cultured cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz) on ice, and total protein was deglycosylated using peptide N-glycosidase F (PNGase F) (NEB, Beverly, MA) following the supplied protocol. Western blotting was performed according to standard protocols using Immunoblot-H polyclonal antibody (PVD) membranes (Millipore, Bedford, MA). For immunoblotting, membranes were incubated with the primary antibody (0.5 μg/ml) for 2 h, followed by 1 h of incubation with HRP-conjugated secondary antibody (0.2 μg/ml). Finally, the blots were washed and the signals were visualized using the ECL Plus kit (Amersham, Buckinghamshire, United Kingdom).
RNA interference. Small interfering RNAs (siRNAs) si34 (5′-CCA ACA UGA AGC AGU CGG ATT-3) and si219 (5′-CAC CGU GGU CGC CGG GAA Att-3) were designed by Dharmacon (Lafayette, CO) to specifically downregulate both basigin-3 and basigin-4 but not basigin-2. Cells were transfected with siRNA using Lipofectamine 2000. siGAPDH (5′-GUA UCA CAG CCU CAA GUA-3′) and siNOR (5′-UUC UCC GAA CGU GUC AGT TT-3′) were used as positive and negative controls, respectively, under similar conditions.

Confocal imaging. Cells plated in chamber slides were fixed in 4% paraformaldehyde for 20 min and permeabilized in phosphate-buffered saline (PBS) supplemented with 0.5% Triton X-100. After blocking, cells were incubated with 1 μg/ml HAB18 for 2 h and then incubated with Cy3-conjugated anti-mouse IgG for 1 h at room temperature. Slides were counterstained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and examined using the Nikon AI confocal laser Microscope system (Tokyo, Japan).

Cell proliferation assay. Cells were plated in sextuplicate in 96-well plates (2 × 103 per well) in 100 μl complete medium and allowed to attach overnight. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (20 μl at 5 mg/ml, Sigma, St. Louis, MO) was added every 24 h and incubated for 4 h. The supernatant was discarded, the precipitate was dissolved in 200 μl dimethyl sulfoxide (DMSO), and plates were read with a microplate reader at 570 nm.

Gelatin zymography. Gelatin zymography was performed using 10% SDS-PAGE containing 1 mg/ml gelatin. Transfected HCC cells (10,000 per well) were cultured alone or cocultured with fibroblasts (0.5 × 105 per well) in 96-well plates. After attachment, the cells were washed and incubated in serum-free medium for 12 h. The supernatants were then collected and prepared in nonreducing loading buffer. After electrophoresis, SDS was removed using 2.5% Triton X-100 to renature gelatinases. Gels were then incubated at 37°C for 16 h in developing buffer (0.1 M Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM CaCl2, and 0.02% Brij-35) and stained with Coomassie blue R-250. MMP activity was visualized as clear bands against the blue-stained gelatin background.

In vitro invasion assay. MilliCell chambers (12-mm diameter with 8-μm pores) (Millipore) were precoated with Matrigel (BD, Bedford, MA) on the upper side. A total of 1 × 105 serum-starved HCC cells alone or together with 0.5 × 105 fibroblasts were added to the upper compartment in medium supplemented with 0.1% serum, and the chambers were placed into 24-well plates with medium containing 10% serum. After 24 h at 37°C, invaded cells on the lower membrane surface were fixed and stained with 0.1% crystal violet. Invasive activity was quantified by counting nine high-power fields (HPFs) (400×) per chamber. Mean values were obtained from at least three individual chambers for each experimental point per assay.

Orthotopic HCC model. In vivo fluorescence imaging, and animal studies. Male BALB/c nu/nu mice at 4 to 6 weeks of age were provided by the Laboratory Animal Research Center of FMMU, and the animal study was reviewed and approved by the FMMU Animal Care and Use Committee. Stably transfected FHCC-98 cells (2 × 106) were resuspended in 100 μl Matrigel and injected subcutaneously into the right flanks of nude mice. When the tumor reached a size of ~1 cm3, the mice were sacrificed. The tumors were resected, cut into 1-mm3 sections under aseptic conditions, and then implanted under the liver capsules of the corresponding lobes of nude mice. The health status and body weights of mice were observed every other day.

The animals were imaged weekly for 35 days using a Xenogen IVIS kinetic imaging system (Caliper, Hopkinton, MA). For in vivo fluorescence imaging, mice were anesthetized with isoflurane and then allowed the determination of the TIS. Due to the exon arrangement and transcription initiation site (TIS) of basigin-2 have been reported previously (7, 24), whereas those of basigin-3 and basigin-4 are unknown. Thus, we performed GeneRacer PCR on mRNA extracted from two HCC cell lines, FHCC-98 and SMMC-7721. GeneRacer PCR ensured that only capped full-length transcripts were amplified and allowed the determination of the TIS. Due to the exon sharing of basigin variants, we designed exon junction gene-specific primers, 1-4R, 1-5R, and 7-8F, to distinguish between them. 5′-RACE was performed using the GeneRacer 5′ primer and 1-4R or 1-5R, and 3′-RACE was performed with 7-8F and the GeneRacer 3′ primer. In both cell lines, only one band was amplified using 1-4R, 1-5R, and 7-8F (Fig. 1B). The second round of seminested PCR, these products were cloned into pcDNA3.1-Venus1 or pcDNA3.1-Venus2 (generous gifts from S. W. Michnick, University of Montreal), respectively (29). Equal amounts of Venus1 and Venus2 were cotransfected into HEK293 cells, and the fluorescence was determined at 24 h after transfection.

NMR spectroscopy. Uniformly 13C-labeled, 15N/C-labeled, and 1H/15N/labeled basigin-2 proteins were prepared for nuclear magnetic resonance (NMR) analysis. NMR samples were dissolved at 1 mM in NMR buffer (50 mM Tris [pH 7.0], 50 mM NaCl, 0.01% NaN3, 0.006% 2,2-dimethyl-2-silapentane-4-sulfonic acid sodium [DSS] in 90% H2O–10% D2O with and complete EDTA-free protease inhibitor cocktail [Roche, Mannheim, Germany]). All NMR experiments were performed at 298 K on a Bruker Avance 600-MHz (with cryoprobe) or 800-MHz (with cryoprobe) NMR spectrometer. Backbone chemical shift assignments were based on a two-dimensional (2D) 1H-13C heteronuclear single-quantum coherence (HSQC) spectrum and 3D HNCA, HNCAC, HN(CA)CO, HB(CA)C, HB(CA)NC, HN(CAC)O, and HN(CA)CO experimental data (31). NMR spectra were processed using the NMRPipe software (4) and analyzed using NMRView software (13). The chemical shift in the 1H dimension was directly referenced to DSS, whereas the chemical shifts in the 13C and 15N dimensions were indirectly referenced to DSS.

Statistical analysis. All statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS, Chicago, IL). The differences between luciferase activity and mRNA expression were assessed using Student’s t test. Each in vitro quantitative test was independently replicated, and the data were calculated as means ± standard errors of the means (SEM). Two-way repeated-measures analysis of variance (ANOVA) and Bonferroni tests were used to measure the proliferation curve in vitro and in vivo. One-way ANOVA was used to compare the MMP production, transfilter cell number, fluorescence signals, tumor volume, and grayscale value of immunohistochemistry staining in different groups. All the statistical tests were two sided, and a P value of <0.05 was considered significant.

Nucleotide sequence accession numbers. The full-length cDNA sequences of basigin-3 and basigin-4 have been annotated and deposited in the GenBank database under accession numbers GU557064 and GU557065, respectively.

RESULTS

Basigin-2, basigin-3, and basigin-4 are the most abundant transcript variants in human cell lines. According to the Entrez Gene database, the basigin gene is composed of 10 exons spanning approximately 15,000 nucleotides (nt) and is transcribed into 4 mRNA variants that encode protein isoforms. The expression of basigin variants was determined using RTPCR with variant-specific primer pairs. Using primers corresponding to sequences within exons 1, 2, and 7, we amplified three distinct bands in 7 types of human tumor cell lines and 3 types of normal cell lines (Fig. 1A). These bands were cloned and sequenced, and they corresponded to basigin-2 (primer 2F-7R) and basigin-3 and basigin-4 (primer 1F-7R), respectively. We also observed that the expression levels of basigin-2, -3, and -4 in tumor cell lines were higher than those in normal cell lines. However, basigin-1 (primer 3F-7R) was not detected in these cell lines.

The exon arrangement and transcription initiation site (TIS) of basigin-2 have been reported previously (7, 24), whereas those of basigin-3 and basigin-4 are unknown. Thus, we performed GeneRacer PCR on mRNA extracted from two HCC cell lines, FHCC-98 and SMMC-7721. GeneRacer PCR ensured that only capped full-length transcripts were amplified and allowed the determination of the TIS. Due to the exon sharing of basigin variants, we designed exon junction gene-specific primers, 1-4R, 1-5R, and 7-8F, to distinguish between them. 5′-RACE was performed using the GeneRacer 5′ primer and 1-4R or 1-5R, and 3′-RACE was performed with 7-8F and the GeneRacer 3′ primer. In both cell lines, only one band was amplified using 1-4R, 1-5R, and 7-8F (Fig. 1B). The second round of seminested PCR, these products were cloned
and sequenced. The sequences showed that basigin-3 and basigin-4 were initiated from the same TIS and shared similar 3' ends with poly(A) tails in both HCC cell lines. This TIS, which has not previously been described, was located at the adenine residue 15 nt upstream of the previously reported TIS in RefSeq NM_198590 and NM_198591 and 1,227 nt upstream of the TIS of basigin-2 (NM_198589) (Fig. 1C).

Basigin-3 and basigin-4 are transcribed from an alternative promoter in the 5' flanking region of exon 1. Basigin-3/4 and basigin-2 are transcribed from different TISs, indicating the presence of an alternative promoter in the 5' flanking region of exon 1. We analyzed the sequence region 1,500 nt upstream and 100 nt downstream from the TIS (+1) using the PROSCAN program. The results showed a promoter predicted in the region from −730 to −480, which contained relatively dense distributions of potential binding sites for various transcription factors, such as Sp1, EARLY-SEQ1, AP-2, T-Ag, and PuF. We then analyzed the transcriptional activities of the 5' flanking region using luciferase reporter assays. A series of deletions from the 5' direction was tested (Fig. 1C and D). The results showed that the vector AP-1075/pGL3, composed of 1,075 bp upstream and 33 bp downstream from the TIS of exon 1, displayed strong promoter activity, which was approximately 11-fold higher than the activity produced by the promoterless vector pGL3-basic (P < 0.05), and a 5' deletion vector, AP-656/pGL3 (nt −656 to +33) showed a high activity similar to that of AP-1075/pGL3 (P < 0.05). However, AP-473/pGL3 (nt −473 to +33) and other 5' deletion vectors showed a dramatic loss (approximately 70%) of luciferase activity (P < 0.05). We then constructed an internal deletion vector, AP-1075-del/pGL3, that excluded the region from −656 to −473, which showed activity similar to that of AP-473/pGL3.

These results indicated that the region from −656 to −473 upstream of exon 1 had promoter activity. At the same time, the vector containing the promoter for basigin-2 (BSG2P/pGL3) displayed strong promoter activity, approximately 5-fold higher than that of AP-1075/pGL3.

By aligning the cDNA sequences with the genomic DNA sequence (NT_011125) using the program Vector NTI Advance 11 (Invitrogen) and analyzing the results of promoter activity assays, we confirmed that basigin variants were transcribed from two alternative promoters. Basigin-1 and basigin-2 are transcribed from the previously described promoter upstream of exon 2 (24). Basigin-2 is translated into two Ig-like domain isoforms, producing the well-known basigin protein. Due to exon 3 retention, basigin-1 includes an additional Ig-like domain (8). Both basigin-3 and basigin-4 are transcribed from an alternative promoter upstream of exon 1. Basigin-3 is translated from the start codon at exon 5, resulting in the lack of an N-terminal IgC2 domain (1). Exon 4 skipping results in basigin-4 having a longer open reading frame (ORF), with a unique N terminus (11 amino acid residues) encoded by exon 1. All four basigin isoforms differed only in the N terminus of the extracellular portion and shared a conserved core sequence encoded by exons 5 through 10, including the IgI domain, transmembrane region, and intracellular portion (Fig. 1E).

Differential expression of basigin mRNA variants in human normal and tumor tissues. To determine whether basigin mRNA variants were expressed in human tissues, we designed specific TaqMan primer and probe pairs to amplify splice variants using real-time RT-PCR of Human Total RNA Master Panel II (Fig. 2A). The results showed that basigin-2 was highly expressed in the heart, kidney, skeletal muscle, and testis. Basigin-3 was highly expressed in the bone marrow, fetal liver, lung, testis, and thymus, and the expression profile of basigin-4 was similar to that of basigin-3. The average expression level of basigin-2 in normal human tissue was higher than those of basigin-3 (~1,300 fold) and basigin-4 (~6,600 fold). Therefore, the basigin mRNA variants were expressed in a wide range of normal tissues, suggesting that the alternative promoter usage and the splicing of basigin are regulated. We also detected the expression of basigin variants in HCC tissues (Fig. 2B). The expression levels of basigin-2 and basigin-3 in HCC tissues were significantly higher than those in ANTs (P = 0.0039 and P = 0.0014, respectively), which demonstrated that basigin variants were upregulated in HCC.

Basigin-3 and basigin-4 mRNAs are translated to protein isoforms with glycosylation and subcellular localizations. To determine whether all basigin mRNA variants could encode protein isoforms, their coding regions were cloned into a GFP-tagged mammalian expression vector and transfected into HCC cells. After screening with G418 and fluorescence detection, stable FHCC-98 and SMMC-7721 HCC cell lines that overexpressed basigin isoforms tagged with GFP at their C termini were obtained. Because basigin-2 can be found in the high-glycosylation (HG) or low-glycosylation (LG) state, the glycosylation of basigin isoforms makes it difficult to identify them by molecular weight. To overcome this difficulty, we

![Diagram](image-url)
removed the carbohydrate residues with PNGase F before detecting isoforms using Western blotting with antibodies specific to the different basigin epitopes (Fig. 1E and Fig. 3A and B). Using MAb HAb18 (which binds to the IgC2 domain), endogenous basigin-2 (~30 kDa for the deglycosylated form) and overexpressed GFP-fused basigin-2 (~60 kDa for the deglycosylated form; the molecular mass of GFP is 29 kDa) were detected in HCC cells (Fig. 3C). Using the polyclonal antibody (PAb) B23ID (which binds the IgI domain), GFP-fused basigin-3 (~50 kDa for the deglycosylated form) and GFP-fused basigin-4 (~55 kDa for the deglycosylated form) were detected in addition to endogenous basigin-2 and GFP-fused basigin-2. We also found an additional band at 20 kDa in all transfected cell lines that was detected using the PAb B23ID. Using the anti-GFP MAb, the deglycosylated GFP-fused basigin isoforms were also detected, similar to with the B23ID PAb. The glycosylated forms were present as two bands with a higher molecular weight, which demonstrated that all basigin isoforms fused with GFP could be glycosylated in HG and LG states. However, the B4N11 PAb could bind only to GFP-fused basigin-4. These results demonstrated that basigin-3 and basigin-4 mRNAs encoded the protein isoforms in their glycosylated forms.

GFP fused to the C termini of basigin isoforms can be used to determine subcellular localization using fluorescence microscopy. Overexpressed basigin-3 and basigin-4 proteins were directly observed using GFP excitation (green), and the endogenous basigin-2 was detected through staining with MAb HAb18 and Cy3-labeled secondary antibody (red). Confocal imaging showed that GFP-fused basigin-3 and basigin-4 were localized mainly at the plasma membrane with minor staining in the cytoplasm in two stable HCC cell lines, similar to the

FIG. 2. Expression profile of basigin mRNA variants in human normal and tumor tissues. (A) Basigin variants were differentially expressed in normal human tissues. Basigin mRNA variants were specifically amplified using real-time RT-PCR from total RNA extracted from 20 normal human tissues. Data are presented as means ± SEM. The expression of basigin variants was calculated as the normalized ratio of the basigin copy number to the β-actin copy number in 100 ng total RNA. (B) Overexpression of basigin variants in HCC tissues. The expression levels of basigin variants were calculated as the log10 value of the normalized ratio.
subcellular localization of basigin-2 (Fig. 3D). We also observed that GFP-fused basigin-3 and basigin-4 localized with basigin-2 at the cell membrane. These results demonstrated that basigin-3 and basigin-4 localized to the plasma membrane, possibly due to a signal peptide, and the localization indicated interaction between the basigin isoforms.

**Endogenous basigin-3 is expressed under standard cell culture conditions.** The novel 20-kDa band could be detected only using the B23ID PAb and not using either the HAb18 or the B4N11D antibody, indicating that it contained an IgI domain but not the IgC2 domain or the N terminus of basigin-4. We hypothesized that this band might be endogenous basigin-3, which has previously been identified using matrix-assisted laser desorption ionization-tandem mass spectrometry (MALDI MS-MS) sequencing (1). To confirm this hypothesis, we performed RNA interference in FHCC-98 and SMMC-7721 HCC cells expressing the basigin protein isoforms.
cells with two siRNAs specific to exon 1 of basigin. Both siRNAs, si34 and si219, significantly knocked down basigin-3 and basigin-4 mRNA expression (34.8% and 35.6% of the value for the blank control, respectively), whereas the mRNA expression of basigin-2 was unchanged. Western blot analysis showed that the expression level of deglycosylated basigin-2 was unchanged but that the 20-kDa protein was significantly knocked down using both siRNAs, suggesting that the 20-kDa protein is endogenous basigin-3 (Fig. 3E). We next detected the expression of endogenous basigin-3 in cultured cells using B23ID. Western blot analysis showed that the basigin-3 protein was widely expressed at a low level under standard cell culture conditions at less than approximately 5% of the basigin-2 protein level (Fig. 3F). We also observed that the protein level of basigin-3 in normal cell lines was lower than that in cancer cell lines. Additionally, endogenous basigin-4 protein was not detected, probably due to the low mRNA transcriptional level.

**Basigin-3 inhibits HCC cell proliferation and invasion in vitro.** Of the four basigin isoforms, only the function of basigin-2 has been well defined. Studies on basigin-1 and basigin-4 are limited by their retina-specific expression and unknown expression patterns, respectively. Therefore, we focused on the function of basigin-3, especially in HCC. Considering the homology between basigin-3 and basigin-2, we simultaneously investigated the roles of basigin-3 and basigin-2 in HCC proliferation and invasion. To examine the role of basigin isoforms in the proliferation of HCC cells, we performed MTT cell proliferation assays with stably transfected FHCC-98 and SMMC-7721 cells. In four stably transfected FHCC-98 cells, the cells that overexpressed basigin-2–GFP grew at the highest rate, and the proliferation rate of cells that overexpressed basigin-3–GFP was the lowest (Fig. 4A). There were significant differences between the proliferation of cells with basigin-2–GFP or basigin-3–GFP and the control cells at days 4, 5, and 6 (P < 0.01 as assessed using two-way repeated-measures ANOVA and Bonferroni tests). The results with SMMC-7721 cells were similar to those with FHCC-98 cells. These results suggest that the overexpression of basigin-2 can promote HCC cell proliferation; in contrast, basigin-3 inhibits HCC cell proliferation.

Gelatin zymography studies revealed the ability of tumor cells to induce fibroblasts to secrete MMP-2 and MMP-9. Our previous work showed that HCC cells cultured alone could secrete only low levels of MMPs (41). Thus, we compared the MMP secretion abilities of FHCC-98 cells transfected with different basigin isoforms or siRNAs cocultured with human fibroblasts. As shown in Fig. 4B, cocultured FHCC-98 cells and fibroblasts (2:1) exhibited strong MMP secretion. The grayscale analysis showed that MMP secretion was significantly decreased in cells transfected with basigin-3–GFP compared with negative control (NC) cells (Fig. 4B) (P < 0.01). The cells transfected with basigin-2–GFP had considerably increased MMP secretion compared with NC cells (Fig. 4B) (P < 0.01). When basigin-3–GFP expression was silenced, the secretion of MMPs was slightly increased. These results suggest that up-regulation of basigin-3 expression can reduce MMP secretion. In contrast, the overexpression of basigin-2 significantly up-regulated MMP expression. In addition, we cocultured HCC cells with mouse fibroblast NIH 3T3 cells. The results were similar to those for the coculture with human fibroblasts (data not shown). These results indicate that human basigin can induce mouse fibroblast MMP secretion, which established a foundation for studying the in vivo function of basigin in the orthotopic transplantation tumor model.

Using a transwell invasion assay, the number of cells that migrated through the filter was significantly decreased in the FHCC-98 cells transfected with basigin-3–GFP compared with NC cells (Fig. 4C) (P < 0.01). In contrast, transfection with basigin-2–GFP greatly increased the number of cells that migrated through the filter compared with NC cells (Fig. 4C) (P < 0.01). Gelatin zymography and invasion assays suggested that basigin-2 significantly increased the invasiveness of FHCC-98 cells, while basigin-3 played an opposite role.

**Basigin-3 inhibits HCC proliferation and invasion in nude mice.** To further define the inhibitory function of basigin-3, we used an orthotopic HCC model in nude mice. As shown in Fig. 5A, the growth of a tumor can be observed by detecting fluorescence at the transplantation site. All the mice were analyzed using an ROI of the same size, and the fluorescence intensity within the ROI was analyzed. The basigin-3–GFP group had the smallest fluorescence area and the weakest fluorescence intensity. The strongest fluorescence intensity and the largest fluorescence area were observed in basigin-2–GFP mice. No signal was detected in the NC mice (Fig. 5A). The growth curve showed that the proliferation of basigin-3–GFP tumors was the slowest and that the proliferation of the basigin-2–GFP tumors was the fastest. There were significant differences between the fluorescence signals in basigin-2–GFP mice or basigin-3–GFP mice and the GFP control mice at days 21, 28, and 35 (Fig. 5B) (P < 0.01 as assessed using two-way repeated-measures ANOVA and Bonferroni tests). All the mice were sacrificed after the last imaging, the tumors were removed, and the tumor volumes were measured. The basigin-3–GFP mice had the smallest tumors, which were all single tumors with no liver or distant-organ metastasis. In contrast, basigin-2–GFP mice had the largest tumors and two to three local liver metastases, while four of six mice had mesentery metastasis and two of six mice had stomach metastasis. All mice had ascites, rectal prolapse, and cachexia and no distant organ metastasis (Fig. 5A). One-way ANOVA showed that there was no significant difference between the tumor volumes of the GFP group and the NC group (P > 0.05), whereas the tumor volumes of the basigin-3–GFP group were dramatically lower than those of the NC group (P < 0.01), and the basigin-2–GFP group tumor volumes were significantly higher than those of the NC group (Fig. 5C) (P < 0.01). These results suggested that basigin-3 inhibited HCC cell proliferation and metastasis in vivo, while basigin-2 promoted the proliferation and metastasis of HCC.

H&E staining showed that all four tissues examined were tumors. We further examined the expression of GFP, PCNA, MMP-2, and MMP-9 using immunohistochemistry (Fig. 5D). GFP was expressed in all tumors except in the NC group, proving that the detected tumor tissues were derived from stably transfected HCC cells with the GFP tag. Proliferating cell nuclear antigen (PCNA) localizes to the nucleus and reflects cell proliferation status. In our work, we found that PCNA was expressed in the HCC cell nuclei. The PCNA labeling index was calculated as the percentage of positively stained nuclei. There was no significant difference between the GFP and NC mice (P > 0.05), whereas the PCNA labeling
index of the basigin-3–GFP mice was significantly lower than that of the NC group (P < 0.01). Furthermore, the basigin-2–GFP group PCNA labeling index was dramatically higher than that of the NC group (Fig. 5E) (P < 0.05). To investigate the effect of the basigin isoforms on HCC microenvironments, we analyzed MMP-2 and MMP-9 expression. Using image optical density analysis with Image-Pro Plus, we determined that the staining intensity of MMP-2 and MMP-9 in the basigin-3–GFP group was significantly lower than that in the NC group (P < 0.01), while that in the basigin-2–GFP group was higher than that in the NC group (P < 0.05). There was no significant difference between the GFP and NC groups (Fig. 5F) (P > 0.05). These results suggested that basigin-3 significantly inhibited the in vivo proliferation of HCC cells and MMP secretion by the surrounding stromal cells, thereby inhibiting ECM degradation and tumor invasion.

FIG. 4. Roles of basigin-3 and basigin-2 in HCC cell proliferation and invasion in vitro. (A) In vitro proliferative ability was measured for 6 days using an MTT assay in HCC cells transfected with basigin isoforms and a blank control. Data are presented as optical density (OD) at 570 nm (mean ± SEM from three replicate experiments). (B) MMP secretion was analyzed using gelatin zymography in HCC cells after transfection with basigin isoforms and siRNAs. Left, gelatin zymography; right, grayscale analysis of at least three independent experiments. The data are shown as the percentage compared with the MMP levels of NC cells. * P < 0.01 compared to the control level, as determined using one-way ANOVA. (C) In vitro tumor cell invasion assay of FHCC-98 cells transfected with basigin isoforms. Left, representative images showing the cell density on the filter. Right (graph), the number of cells migrating through the filter was counted and plotted as the mean number of migrating cells per optic field in three independent experiments. * P < 0.01 compared to the control level, as determined using one-way ANOVA.
FIG. 5. Basigin-3 inhibits HCC proliferation and invasion and the expression of GFP, MMP-2, and MMP-9 in a nude mouse model. (A) *In vivo* fluorescence images of the orthotopic HCC model in nude mice. The colored region represents the GFP fluorescence signal of HCC cells in nude mice. Right, signal intensity scale. Increasing red color indicates increasing signal strength, while weaker signal strengths are represented by 2600 LIAO ET AL. MOL. CELL. BIOL.
Basigin-3 interacts with basigin-2 to form hetero-oligomers.

Due to the important role of oligomerization in basigin function, we explored the interaction between basigin-3 and basigin-2 using bimolecular fluorescence complementation (BiFC) assays and NMR. The BiFC technique is based on the formation of a fluorescent complex from two separate nonfluorescent fragments, which are brought together by the association of two interacting partner proteins fused to the fragments, and it is utilized for direct visualization of protein-protein interactions under physiological conditions (15). The extracellular portions of basigin-3 and basigin-2 were separately fused to the C termini of two complementary fluorescent fragments, Venus1 (residues 1 to 158) and Venus2 (residues 159 to 238), respectively. When Venus1–basigin-3 and Venus2–basigin-2 were coexpressed in HEK-293 cells, Venus fluorescence was visible. In addition, when Venus1–basigin-3 and Venus2–basigin-3 or Venus1–basigin-2 and Venus2–basigin-2 were coexpressed, Venus fluorescence was also detected. As a control, no fluorescence was observed when Venus1–basigin-3 or Venus1–basigin-2 was coexpressed with Venus2 (Fig. 6A). These results indicated that basigin-3 interacted with basigin-2 in living cells and that basigin-3 and basigin-2 could form homodimers.

To further determine the binding interface of basigin-3 and basigin-2, we performed backbone NMR resonance assignments for basigin-2. Nearly all backbone NH chemical shift assignments were obtained, except for residues G153, S156, R157, F158, F159, V160, and S161. These residues comprise a short α-helix and part of a β-strand in the crystal structure. The missing NH signals were probably due to the intermediate time scale of conformational exchange, which caused broadening of the NMR signals beyond detection.

Protein-protein interactions can change the chemical environment, resulting in NH chemical shift changes for the residues at the interface. Therefore, we identified the interaction interface of basigin-3 and basigin-2 by comparing the NH chemical shift differences between free basigin-2 and basigin-2 together with basigin-3. After the addition of basigin-3, we observed chemical shift changes along with peak broadening for some NH signals from basigin-2, indicating direct binding between basigin-3 and basigin-2 (Fig. 6B). An overlay of the 2D 1H-15N HSQC spectra of basigin-2 with different concentrations of basigin-3 revealed that a number of NH cross-peaks shifted gradually as the basigin-3 concentration increased. Residues with a combined NH chemical shift difference ($\Delta_{\text{comb}}$) between free basigin-2 and basigin-2–basigin-3 (1:3) of greater than 0.05 ppm included S154, W82, and G83. Residues with a $\Delta_{\text{comb}}$ value of less than 0.05 ppm but more than 0.03 ppm included M123, M176, L101, E84, H115, A122, V125, and S130 (Fig. 6B). In addition, residues H170, E155, E168, W139, H102, S162, M151, E172, K148, and S163 displayed significant intermediate exchange line broadening with basigin-3 titration, in which the NH peak intensities of these residues were significantly lower than those of the residues in free basigin-2 (Fig. 6C). From mapping these residues affected by basigin-3 binding onto the crystal structure of basigin-2, it was apparent that most of the residues could be mapped to an area of the membrane-proximal IgI domain of basigin-2, which was located around the residues with a missing NH signal (Fig. 6D). It is likely that the residues with the missing NH signal were involved in the interaction, probably comprising the interaction surface. Taking the results together, we conclude that basigin-3 interacts with basigin-2 at the interface of the IgI domain and forms hetero-oligomer complexes. Because basigin-3 consists mainly of IgI domains, this result suggests that basigin-3 can also form homodimers.

**DISCUSSION**

In our study, we demonstrated that in addition to the predominant splice variant basigin-2, basigin-3 and basigin-4 were the most abundant variants expressed, and they were upregulated in HCC tissues compared to normal tissues. Basigin-3 and basigin-4 mRNAs were initiated from an alternative promoter, and they encoded protein isoforms in their glycosylated forms, which were localized to the plasma membrane. In cultured cell lines, native basigin-3 interacted with basigin-2 to form hetero-oligomers and inhibited HCC cell proliferation, MMP induction, and cell invasion. These findings suggest that basigin-3 is one of the most important isoforms of the basigin family, and it may be involved in regulating the functional activity of basigin-2 via hetero-oligomerization.

First, we identified the transcription and the expression of basigin variants. Because of the retina-specific expression of basigin-1, only basigin-2, -3, and -4 were detected in cultured cells. We were most interested in studying the function of basigin in tumors; therefore, we did not pursue studies on basigin-1. Our results also indicated that the expression levels of basigin-2, -3, and -4 in tumor cell lines were higher than those in normal cell lines. After the detection of the three basigin isoforms, we found that the first exon of basigin-3 and basigin-4 was different from that of basigin-2. GeneRacer PCR showed that basigin-3 and basigin-4 were initiated from the same TIS and shared similar 3′ ends with poly(A) tails in both HCC cell lines. This is the first report of an alternative promoter in the basigin gene. Using 5′ flanking region luciferase reporter assays, we determined that the critical alternative promoter was located between positions −656 and −473 up-

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increasing blue color. After the last imaging, the mice were sacrificed, and the livers were excised. The tumor size was measured in the resected liver. (B) Effect of basigin-3 on HCC proliferation detected using in vivo imaging. The ROI fluorescence intensity was recorded as photons/sec/cm²/sr. (C) Quantitative analysis of tumor volume in the four groups. *P < 0.01 compared to the negative-control group, as determined using one-way ANOVA. (D) H&E staining and immunohistochemistry show the role of basigin-3 in HCC proliferation and invasion. The expression of MMP-2 and expression of MMP-9 were detected mainly in the stroma, whereas the expression of PCNA was detected mainly in the HCC nuclei. (E) Basigin-3 inhibits the expression of PCNA in HCC. The PCNA labeling index was calculated as the percentage of positively stained nuclei. *P < 0.05 compared to the negative-control group, as determined using one-way ANOVA. (F) Basigin-3 inhibits the expression of MMPs in HCC. The expression of MMPs was calculated as optical density relative to the NC group. *P < 0.05 compared to the negative-control group, as determined using one-way ANOVA.
stream of exon 1. In our study, we showed that the BSG gene had a complex transcriptional expression pattern that encoded four mRNA variants through the use of alternative splicing and the existence of an internal alternative promoter. The N terminus can be alternatively spliced to produce four isoforms that share a conserved IgI domain, transmembrane region, and intracellular portion.

Furthermore, the expression of the three basigin isoforms was detected in human normal and tumor tissues. Our results showed that the basigin mRNA variants were expressed in a wide range of normal tissues in the fetal liver, heart, kidney, skeletal muscle, testis, and thymus. The tissue distribution may reflect the biological function of the genes. The heart and skeletal muscle are the main energy metabolism organs. Strenuous exercise or hypoxia activates anaerobic glycolysis, resulting in the massive accumulation of lactic acid. The interaction of basigin with monocarboxylate transporter (MCT) allows for the transport of lactic acid out of the cell, which may explain the high expression of basigin in the heart and skeletal muscle (13). In addition, basigin is involved in sperm maturation (45), fertilization, implantation (42), and thymic development (43), which explains the expression of basigin in the testis and thymus. The average expression level of basigin-2 in normal human tissue was higher than those of basigin-3 and basigin-4. This may be due to the fact that basigin-2 promoter activity was much stronger than that of the upstream alternative promoter (shown in Fig. 1D). However, detailed mechanisms of splicing regulation of basigin are still unclear. We also found that the expression of basigin variants in HCC tissues was significantly higher than that in ANTs, which demonstrated that basigin variants were upregulated in HCC.

Recently, basigin-3 was listed in the GenBank database as a nonsense-mediated mRNA decay (NMD) candidate gene that would be degraded by NMD. Alternative splicing strongly af-

FIG. 6. Interaction between basigin-3 and basigin-2. (A) The interaction between basigin-3 and basigin-2 was detected using BiFC. HEK-293 cells coexpressing the BiFC constructs indicated were cultured for 24 h, and fluorescence was observed under a fluorescence microscope. (B) Overlay of the 2D $^1$H-$^1$C HSQC spectra of basigin-2 with different concentrations of basigin-3. The blue peaks were from free basigin-2, red peaks from basigin-2–basigin-3 (1:2), and green peaks from basigin-2–basigin-3 (1:3). Residues with combined NH chemical shift differences of $\geq$0.04 ppm and large NH peak intensity changes (less than 25% of free protein) are shown with the one-letter amino acid code and the residue number. (C) Upper panel, plots of combined NH chemical shift differences versus residue number. The combined chemical shift differences were calculated using the empirical equation $\Delta_{\text{comb}} = [\Delta_{\text{HN}}^2 + (\Delta_{\text{N}}/6.5)^2]^{1/2}$, where $\Delta_{\text{HN}}$ and $\Delta_{\text{N}}$ represent the chemical shift difference of $^1$H and $^1$N, respectively. Lower panel, plots of NH signal intensity ratio of free basigin-2 to basigin-2–basigin-3 (1:3) versus residue number. Residues without assignment are indicated by short red bars. (D) Mapping of the binding interface between basigin-3 and basigin-2. The perturbed residues were mapped on the structure of basigin-2 (residues with intensity ratios of $\geq$3.0 are shown in magenta, $\geq$2.0 but $<$$3.0$ in violet, and $\geq$1.8 but $<$$2.0$ in pink). Residues with $\Delta_{\text{comb}}$ of $\geq$0.05 ppm are shown in cyan and $<$$0.04$ but $<$$0.05$ ppm in aquamarine, and residues without assignment are shown in slate.

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fects gene expression by generating protein isoform diversity. However, up to one-third of human AS events create a premature termination codon that triggers NMD followed by mRNA degradation (25). NMD can be inhibited by specific protein biosynthesis inhibitors, such as cycloheximide (CHX); therefore, we treated HCC cells with CHX and detected the expression of basigin mRNA variants as previously described (45). Our results showed that the expression levels of basigin-2 and basigin-3 did not increase significantly, which indicated that basigin-3 could be translated into a protein rather than being degraded by NMD (data not shown). In addition, previous work using MALDI MS/MS protein sequencing has also demonstrated that basigin-3 is translated from its second open reading frame (1).

Our study also aimed to elucidate the expression pattern of the basigin protein isoforms. Antibodies are the most important tool in protein research, but we could not specifically assess the endogenous expression of basigin protein isoforms with any existing BSG antibodies because they could not distinguish between the isoforms. Thus, we raised the following antibodies that could distinguish between the isoforms: HAb18, which was specific to basigin-2; B23ID, which was specific to basigin-2 and basigin-3; and B4N11P, which was specific to basigin-4. Using these affinity-purified polyclonal antibodies and specific siRNAs, we showed that basigin-3 and basigin-4 mRNAs encoded translated proteins. Basigin-2 is subject to post translational glycosylation modifications. Our results revealed that basigin-3 and basigin-4 protein isoforms were also glycosylated.

Because of the tissue specificity of basigin-1 and the low expression of basigin-4, we only investigated the biological functions of basigin-2 and basigin-3. Previous studies have shown that basigin-2 plays an important role in tumor invasion and metastasis, mainly via regulating fibroblasts and tumor cells to secrete MMPs that disrupt the tumor microenvironment (31), but little was known about the function of basigin-3. In our study, we examined the role of basigin-3 in HCC invasion and metastasis in vitro and in vivo. Our data suggested that basigin-3 inhibited HCC cell proliferation, migration, and invasion by decreasing MMP secretion, contrary to the results obtained for basigin-2. There is increasing evidence that alterations in the splicing patterns of genes contribute to the regulation of gene function by generating protein isoform diversity. For example, a bZIP-type transcription factor, OsABI5, undergoes alternative splicing. OsABI5 variants may have overlapping and distinct functions to fine-tune gene expression together with OsVP1 (46).

Homologomerization is a prerequisite for a number of proteins to be fully functional. Basigin-2 stimulates MMP expression in stromal cells through the formation of basigin-2 homo-oligomers. Because basigin-3 contains only the IgI domain, it may not stimulate MMP secretion in stromal cells. Thus, we hypothesized that basigin-3 forms a hetero-oligomer with basigin-2 through its IgI domain to act as an endogenous inhibitor that regulates basigin-2 function.

Therefore, we investigated whether basigin-2 and basigin-3 interacted with each other. There are many ways to study protein-protein interactions, the most common of which include coimmunoprecipitation (co-IP), fluorescence resonance energy transfer (FRET), BiFC, Biacore, and others. Unlike many in vitro protein interaction assays, BiFC does not require protein complexes to be formed by a large proportion of the proteins or at stoichiometric proportions. Instead, BiFC can detect interactions among protein subpopulations with weak interactions and low expression due to the stable complementation of the fluorescent reporter protein (16). The BiFC results showed that basigin-3 interacted with basigin-2 in living cells and that basigin-3 and basigin-2 could homodimerize. Previous studies have reported that basigin-2 forms homodimers, which is a prerequisite for basigin-2 function. The homodimerization of basigin-3 indicated that the IgI domain conferred basigin polymerization and that the absence of IgC2 would not affect this process. However, no previous research has shown polymerization between basigin-3 and basigin-2.

NMR is one of the most accurate methods of investigating
protein-protein interactions; it directly determines the protein interface from the protein space structure. Thus, we investigated the binding interface of basigin-3 and basigin-2 using NMR. From the NMR results, we concluded that basigin-3 interacted with basigin-2 at the interface of the IgI domain and formed hetero-oligomer complexes, which is consistent with the hypothesis of the functional mechanism of basigin-3. As shown in Fig. 7, we hypothesize that basigin-3 may competitively bind to basigin-2, forming hetero-oligomers through IgI domain interaction. This hetero-oligomerization results in the inhibition of MMP induction, which requires basigin-2 homo-oligomerization.

In conclusion, our study is the first to examine the detailed transcriptional regulation, expression profile, and function of the basigin isoforms in HCC. Our results show that basigin-3 is one of the most important isoforms of the basigin family, and it inhibits HCC cell proliferation, MMP induction, and cell invasion, probably via hetero-oligomerization with basigin-2. These are features of basigin that were previously unrecognized, and this knowledge significantly broadens the importance of the BSG gene.

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