

B-box and SPRY Domain Containing Protein (BSPRY) is Associated with the Maintenance of Mouse Embryonic Stem Cell Pluripotency and Early Embryonic Development

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Abstract. Mouse embryonic stem (ES) cells consist of heterogeneous populations with differing abilities to proliferate and differentiate. We previously demonstrated that the expression level of platelet endothelial cell adhesion molecule 1 (PECAM1)/CD31 was positively correlated with the undifferentiated state of mouse ES cells. In order to screen for a novel gene(s) involved in ES cell pluripotency, we performed an oligo microarray analysis and identified that B-box and SPRY domain containing protein (BSPRY) was expressed at high levels in PECAM1-positive cells. Two splice isoforms of BSPRY, BSPRY-1 and BSPRY-2, were expressed in undifferentiated ES cells and in blastocysts. Knockdown of BSPRY-1/2 in ES cells significantly reduced the number of undifferentiated colonies and caused increased expression of primitive ectoderm marker gene *Fgf5*. The overexpression of BSPRY-2 reciprocally increased the number of undifferentiated ES cells in the presence of LIF. Similarly, injection of BSPRY-1/2 siRNAs into 2-cell embryos caused developmental retardation and degeneration of embryos, and a significant decrease in the number of cells, especially in the inner cell mass (ICM), was observed at the blastocyst stage. Furthermore, microinjection of a BSPRY-1 expression vector into pronuclear stage embryos resulted in an increase in the hatching blastocysts rate after 120 h of culture. These results suggest that BSPRY-1 and BSPRY-2 are associated with both ES cell pluripotency and early embryonic development.

Key words: BSPRY, Early embryonic development, Embryonic stem cells

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Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts and maintain the pluripotency to differentiate into three germ layers. Recent studies have demonstrated that mouse ES cells consist of heterogeneous populations with differing abilities to proliferate and differentiate [1–3]. We previously reported that there was wide variation in the expression of platelet endothelial cell adhesion molecule 1 (PECAM1) [1]. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis revealed that marker genes for undifferentiated ES cells were highly expressed in PECAM1-positive cells. PECAM1-positive ES cells injected into 8-cell host embryos could contribute to the epiblast in a chimeric embryo, but PECAM1-negative ES cells failed to differentiate into epiblast cells. These results suggested that the PECAM1 expression level was positively correlated with the undifferentiated state of ES cells.

In order to search for a novel gene(s) involved in ES cell pluripotency, we analyzed gene expression profiles in PECAM1-positive and PECAM1-negative populations using an oligo microarray and isolated several genes that were expressed at high levels in PECAM1-positive

cells. We focused upon one of these genes, which encodes mouse B-box and SPRY domain containing protein (BSPRY) protein, because *in situ* hybridization analysis revealed that *Bspry* was also expressed at the ICM of blastocysts [4]. These results suggest that BSPRY may be involved in not only ES cell pluripotency but also early embryonic development.

BSPRY was originally identified as a binding partner of transient receptor potential vanilloid 5 (TRPV5) [5], part of the epithelial Ca²⁺ channel family. In kidney epithelial cells, the overexpression of BSPRY inhibited TRPV5-mediated Ca²⁺ influx, indicating that BSPRY is a negative regulator of Ca²⁺ transport [5]. In mice, there are two alternative splice isoforms, termed BSPRY isoform 1 (BSPRY-1) and isoform 2 (BSPRY-2). These have an almost identical amino acid sequences, containing B-box and SPRY domains, but exhibit differences at the C-terminal end.

The biological role of BSPRY-1 and BSPRY-2 in ES cells and early development remains unclear. In this study, we examined the function of BSPRY-1 and BSPRY-2 in ES cells and early embryonic development by knockdown and overexpression analysis. We found that BSPRY-1 and BSPRY-2 were associated with both the maintenance of ES cell pluripotency and early embryonic development. Our analysis also revealed that the two isoforms of BSPRY exhibited differing activity levels in ES cells and early embryos.

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Table 1. Primer sequences for qRT-PCR and cDNA cloning

Gene	Sense primer	Antisense primer
qRT-PCR		
<i>mBspry-1</i>	CACAATGGGTATCATGAGCCTCTG	CCTAGAAGCATGGCCTGACCTG
<i>mBspry-2</i>	CACAATGGGTATCATGAGCCTCTG	TTGTCCTTCGTATGCTGCAAGG
<i>uBspry*</i>	GAGAGGCTGCAGTTACAGAGTGC	TCTGAATCAGCTGGAGGTCATC
<i>mPou5f1(Oct3/4)</i>	GGCGTTCTCTTTGGAAAGGTGTTTC	CTCGAACCACATCCTTCTCT
<i>mNanog</i>	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
<i>mRex-1</i>	TCTTAATGACAGAGGCACTG	TCCCATCCCCTCAATAGCA
<i>mSocs3</i>	GCTTACTACATCTATTCTGGG	CTGATCCAGGAAGTCCCAGAA
<i>mGapdh</i>	ATCCCAGAGCTGAACGGGAAGCTACTGGC	GCATCGAAGGTGGAAGAGTGGGAGTTGCTG
<i>uGapdh*</i>	GAGCTGAACGGGAAGCTCAC	ATACCAGGAAATGAGCTTGAC
cDNA cloning		
<i>mBSPRY-1</i>	AAGAATTCACCACCATGTCCGCGGATGTCTCCGGG	AACTCGAGCTACCTAGAAGCATGGCCT
<i>mBSPRY-2</i>	AAGAATTCACCACCATGTCCGCGGATGTCTCCGGG	AACTCGAGCTACCACACTCCCACGACGAC

* Primer set recognizes common sequences from the mouse, human and dog.

Materials and Methods

Animals

All animals were treated according to the Animal Care Committee of the National Institute of Agrobiological Sciences.

Cell culture

Mouse ES cell line TT2 [6] cells were cultured on STO cells in Knockout Dulbecco's Modified Eagle's (KO-DME) media (Invitrogen, Carlsbad, CA, USA) supplemented with 17.5% KnockOut Serum Replacement (Invitrogen), 10^{-4} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 1 mM nonessential amino acids (Invitrogen) and 20 ng/ml leukemia inhibitory factor (LIF), produced as conditioned media from Cos-7 cells expressing human LIF. ES cell differentiation was induced by LIF withdrawal.

qRT-PCR

Total RNAs from mouse adult tissues, ES cells and blastocysts were isolated using an RNeasy Micro Kit (QIAGEN, Hilden, Germany). Single-strand cDNA were synthesized from total RNA by M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and purified using a QIAquick PCR Purification Kit (QIAGEN). qRT-PCR was performed using LightCycler 480 SYBR Green I (Roche, Indianapolis, IN, USA) on a LightCycler 480 (Roche). The primer sequences used for this study are shown in Table 1. The reaction conditions for the PCRs consisted of 95 C for 5 min, followed by 45 cycles of 95 C for 10 sec, primer annealing at 64 C for 5 sec and elongation at 72 C for 10 sec. A fluorescent signal was read after each cycle following elongation at 72 C.

Synthesis of BSPRY-1/2 specific small inhibitory RNAs (siRNAs)

Single-stranded RNAs were transcribed using an *in vitro* Transcription T7 Kit (Takara Bio, Otsu, Shiga, Japan) from *Bspry-1/2* PCR fragments corresponding to nucleotides 190-493 of the mouse *Bspry-1/2* coding region (GenBank accession No. AK035588) and containing opposing T7 promoter sequences. Double-stranded RNAs

(dsRNAs) were generated from two complementary stands by heating to 75 C for 5 min and then slow cooling to room temperature. After treatment with DNase I (Takara Bio) and purification with an RNeasy MinElute Cleanup Kit (QIAGEN), dsRNAs were digested by a Dicer enzyme (Takara Bio) to produce 21-23 bp siRNAs. The siRNAs were purified using Microcon-100 (Millipore, Billerica, MA, USA) and EZ-pure spin columns (Millipore) to remove residual large dsRNAs and proteins [7]. Then siRNAs were labeled with carboxyfluorescein (FAM) using a Silencer siRNA Labeling Kit (Ambion, Austin, TX, USA). FAM-labeled BSPRY-1/2 siRNAs were precipitated, washed with 70% ethanol, dried and resuspended in PBS. The quality of FAM-labeled BSPRY siRNAs were then assessed using agarose gel electrophoresis. The fluorescein (FITC)-labeled negative control siRNAs were purchased from QIAGEN.

Transfection of siRNAs in ES cells and enrichment of fluorescence-positive cells

ES cells were seeded into a 24-well plate (BD Falcon, Franklin Lakes, NJ, USA) at a density of 2.5×10^5 cells/well. The next day, 250 ng FAM-labeled BSPRY-1/2 or FITC-labeled control siRNAs were transfected using X-treme GENE siRNA Transfection Reagent (Roche). After 24 h of transfection, the fluorescence-positive cells were isolated by fluorescence-activated cell sorter (FACS) to enrich the siRNA-introduced cells and were immediately lysed in Buffer RLT (QIAGEN) for qRT-PCR of *Bspry-1* and *Bspry-2* genes to assess the effect of siRNAs. Sorted cells were plated on a 60 mm gelatin-coated culture dish at a density of 1×10^4 cells/dish for Western blot analysis or 1×10^3 cells/dish for an alkaline phosphatase (AP) assay and qRT-PCR for marker genes. The seeded cells were cultured in KO-DME media supplemented with 14% fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 10^{-4} M 2-mercaptoethanol, 1 mM nonessential amino acids and LIF for 2 days for Western blot analysis and for 4 days for the AP assay and qRT-PCR for marker genes.

Alkaline phosphatase (AP) assay

AP staining was carried out with an Alkaline Phosphatase Detection Kit (Sigma-Aldrich), according to the manufacturer's instructions.

Briefly, ES cells were fixed in 4% paraformaldehyde in PBS for 30 sec at room temperature and washed two times with PBS. ES cells were then stained with freshly prepared AP solution at room temperature for 15 min. Tight round ES colonies that were strongly stained for AP were judged as undifferentiated colonies, and colonies surrounded by flattened and AP-negative cells were judged as differentiated (Fig. 2F).

Plasmid construction

BSPRY-1 and BSPRY-2 genes were cloned by RT-PCR using a TT2 cell cDNA. The primer sequences are shown in Table 1. PCR products were subcloned into the *EcoRI* and *XhoI* site of pcDNA3.1 (Invitrogen). Constructs were sequenced before transfection.

Plasmid transfection into ES cells

Lipofectamine Plus (Invitrogen) was used to co-transfect pcDNA3.1, pcDNA3.1-BSPRY-1, pcDNA3.1-BSPRY-2, pCAG-wtSTAT3 and pCAG-dnSTAT3 [8] vectors with pEGFP-N vector (Invitrogen) into ES cells. For the BSPRY-1 and BSPRY-2 overexpression experiment, GFP-positive cells were enriched by FACS and then subjected to qRT-PCR for *Bspsy-1* and *Bspsy-2* genes or were cultured in the presence of LIF for 4 days for performance of the AP assay and qRT-PCR for marker genes. ES cells transfected with wtSTAT3 and dnSTAT3 vectors were subjected to FACS and qRT-PCR at 48 h after transfection.

Production of anti-BSPRY-1/2 monoclonal antibodies

In order to prepare mouse monoclonal antibodies against BSPRY-1/2, a BSPRY-2 (GenBank accession No. NP_619594.1, amino acids 1-486) full-length recombinant protein was injected into BALB/c mice (Charles River Laboratories Japan, Yokohama, Japan). Hybridoma cells were generated by fusion of P3U1 myeloma cells with immunized spleen cells. Antibodies were purified from hybridoma cell culture using a HiTrap Protein G HP Column (GE Healthcare, Little Chalfont, UK), according to the manufacturer's protocol.

Western blot analysis

Cultured cells were lysed in RIPA buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, 1% NP40, 2 mM MgCl₂, 2 mM CaCl₂ and 0.1% NaN₃ and then sonicated using a Bioruptor (Cosmo Bio, Tokyo, Japan). Protein samples (10 µg/lane) were loaded onto a 10% polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). The membranes were blocked with 5% Blocking Agent (GE Healthcare) in PBS-Tween and immunoblotted with anti-BSPRY-1/2 (1:1000) or anti- α -Tubulin (Invitrogen, 1:1000) diluted with Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Osaka, Japan) followed by horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare) at a dilution of 1:1000 in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo). Immunoreaction signals were visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare) and exposed to Amersham ECL Hyperfilm (GE Healthcare).

Collection of embryos, microinjection and culture in vitro

CD-1 female mice (Charles River Laboratories Japan) were

superovulated with an intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (PMSG) followed 48 h later by 5 IU human chorionic gonadotrophin (hCG). These treated females were then mated with CD-1 male mice (Charles River Laboratories Japan). For microinjection with siRNAs, fertilized 2-cell embryos were recovered from mated female mice 48 h after hCG injection and incubated in drops of mKSOM consisting of 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH₂PO₄, 0.2 mM MgSO₄, 1.7 mM CaCl₂, 25 mM NaHCO₃, 0.2 mM Na⁺ pyruvate, 1 mM glucose, 0.2 mM glutamine, 10 mM Na⁺ lactate, 0.01 mM tetrasodium EDTA and 1 g/l BSA under 5% CO₂ in air. Approximately 3 µl of 20 µM FAM-labeled BSPRY-1/2 siRNAs or FITC-labeled control siRNAs, were microinjected into one blastomere of a 2-cell stage embryo with 0.5 mg/ml dextran-tetramethylrhodamine (dTMR, 10,000MW, Molecular Probes, Eugene, OR, USA), which was used as fluorescent tracer dye. To confirm cell fate, embryos were observed at 24 h and 96 h using a fluorescence microscope.

For overexpression analysis of *in vitro* embryonic development, pronuclear stage embryos were obtained from superovulated CD-1 female mice 21 h after hCG injection. pcDNA3.1-BSPRY-1, pcDNA3.1-BSPRY-2, or pEGFP-N (control) vectors were microinjected into the pronuclei of embryos [9], which were then cultured in drops of mKSOM. Developmental rates to the blastocyst stage were assessed at 96 and 120 h after injection.

Statistical analysis

All data are presented as means \pm SEM. *In vitro* development data were analyzed using chi-square tests. Other data were analyzed using ANOVA followed by the Student's *t* test. P values of <0.05 were considered statistically significant.

Results

Expression of *Bspsy* isoforms in adult tissues and various cell lines

The mouse *Bspsy* gene encodes two isoforms, *Bspsy-1* (GenBank accession number AK035588) and *Bspsy-2* (GenBank accession number NM_138653), with differing C-terminal ends due to alternative splicing (Fig. 1A). BSPRY-1 and BSPRY-2 proteins are composed of 473 (52.7 kDa) and 486 amino acids (54.7 kDa), respectively. Amongst the amino acid sequences of BSPRY-1 and BSPRY-2, 465 amino acids are conserved, including the B-Box and SPRY domains (Fig. 1A).

To examine the expression levels of both isoforms in mouse adult tissues, ES cells and blastocysts, qRT-PCR was performed using *Bspsy-1*- and *Bspsy-2*-specific primers. Both *Bspsy-1* and *Bspsy-2* were strongly expressed in the testis and were also detected in the lung, kidney, spleen, intestine, uterus, ovary, ES cells and blastocysts. Neither isoform was detected in the brain, liver, muscle and heart. The expression level of *Bspsy-2* was 10–70% lower than that of *Bspsy-1* in each tissue, ES cells and blastocysts (Fig. 1B).

We examined *Bspsy* expression in several cell lines by qRT-PCR using a primer set recognizing mouse, human and dog sequences. Strong expressions of BSPRY mRNA were detected in MDCK and ES cells but not in Cos-7, HeLa, Balb3T3 and NIH3T3 cell lines (Fig. 1C). As shown in Fig. 1D, the result of Western blot analysis

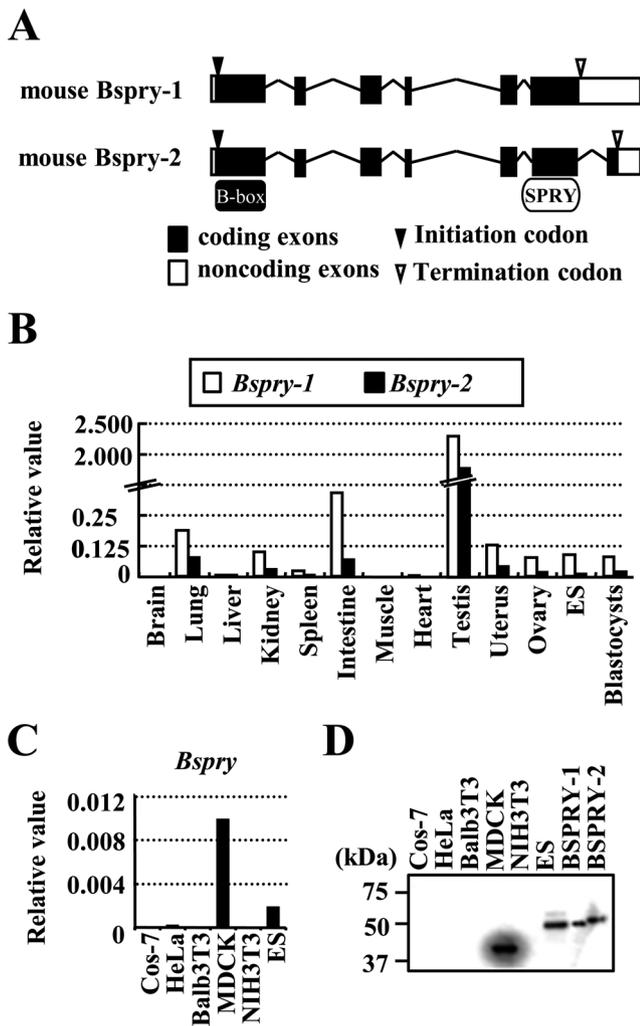


Fig. 1. Expression profile of BSPRY isoforms. **A:** Diagram showing *Bspry* isoforms and their differing 3' mRNA ends, along with accompanying amino acid sequences. The filled boxes show the coding exons and open boxes show noncoding exons. Solid and open arrowheads indicate the positions of the initiation and termination codons, respectively. The locations of the B-box and SPRY domains are indicated at the bottom of the diagram. **B:** Expression patterns of *Bspry-1* and *Bspry-2* in adult mouse tissues, ES cells and blastocysts. Values were normalized to *Gapdh* expression. Expression of *BSPRY-1/2* mRNA (**C**) and protein (**D**) in various cell lines. *Bspry-1/2* and *Gapdh* mRNA were detected with primer sets recognizing common sequences in the mouse, human and dog. BSPRY-1 and BSPRY-2 in (**D**) applied lysates harvested from Cos-7 cells transfected with BSPRY-1 and BSPRY-2 expression vectors, respectively.

was agreement with the results from qRT-PCR. A band observed at approximately 40 kDa in renal MDCK cell line corresponded to the predicted size for dog BSPRY (GenBank accession number XP_538804), which has a calculated molecular mass of 39.2 kDa. The band detected in ES cells was identical in size to that in Cos-7 cells transfected with mouse *Bspry-1* cDNA, indicating that BSPRY-1 protein is predominantly expressed in ES cells, similar to its mRNA expression.

BSPRY-1/2 knockdown results in ES cell differentiation

To examine whether BSPRY-1/2 is involved in ES cell pluripotency, we knocked down BSPRY-1/2 by RNA interference (RNAi). ES cells were transfected with FAM-labeled BSPRY-1/2 siRNAs, and fluorescent-positive cells (Fig. 2A) were subjected to analysis. As shown in Fig. 2B and 2C, BSPRY-1 and BSPRY-2 mRNA and protein was efficiently repressed by BSPRY-1/2 siRNAs. The number of total colonies was not altered in BSPRY-1/2 siRNAs (Fig. 2D), but the relative rates of undifferentiated ES colonies compared with the control group were significantly reduced (Fig. 2E). qRT-PCR analysis showed that the expression level of the primitive ectoderm marker *Fgf5* was slightly increase in BSPRY-1/2 siRNA-transfected ES cells, but there were no effects upon the expression of pluripotent marker genes such as *Pou5f1* (*Oct3/4*), *Rex-1* and *Nanog*, and other differentiated marker genes such as the primitive endoderm marker *Gata4* and the mesoderm marker *Brachyury* (*T*) (Fig. 2G).

BSPRY-1/2 suppression cause developmental retardation of early embryos *in vitro*

Next, we examined the effect of BSPRY-1/2 knockdown in early embryonic development, because BSPRY-1/2 was also expressed in blastocysts. To investigate the affects of siRNAs on cell differentiation and cell fate during early development, siRNAs were injected into one blastomere of a 2-cell stage embryo. As shown in Table 2, there were no significant differences in developmental rates between the control and BSPRY-1/2 siRNAs injection groups for up to 96 h after injection. However, at 120 h after injection, the percentage of hatching stage embryos was significantly reduced in the group injected with BSPRY-1/2 siRNAs compared with the control group (39.3% vs. 89.7%, $P < 0.05$), and this led to a consequent increase in the rate of blastocyst stage embryos compared with the control group (21.3% vs. 0%, $P < 0.05$). The rate of degenerated embryos was also increased in the group injected with BSPRY-1/2 siRNAs, as compared with the control group (39.3% vs. 10.3%, $P < 0.05$). Additionally, a significant reduction in the mean number of ICM cells, but not trophoblast (TE) cells, was observed at the blastocyst stage at 96 h after injection (Table 3). Marked differences in morphology were not observed between BSPRY-1/2 and control siRNA-injected blastocysts, and there was no difference in the number of cells and populations between fluorescent-positive and fluorescent-negative cells in the ICM and TE (Fig. 3C, D).

Two isoforms of BSPRY exhibit differing activity levels in ES cells and early embryos

Because BSPRY-1/2 siRNAs were designed based on a region common to the two isoforms, it is not clear which isoform has a more important role in the maintenance of ES cell pluripotency and early embryogenesis. To address this question, we performed overexpression experiments of each isoform in ES cells and early development. BSPRY-1 or BSPRY-2 expression vector was co-transfected with GFP vector into ES cells, and the next day, GFP-positive cells were sorted to separate them from the non-transfected cells. qRT-PCR analysis confirmed that expression of *Bspry-1* and *Bspry-2* mRNA was specifically increased in BSPRY-1 and BSPRY-2 expression vector-transfected ES cells, respectively (Fig. 4A). After 4 days of culture in the presence of LIF, the number of undifferentiated

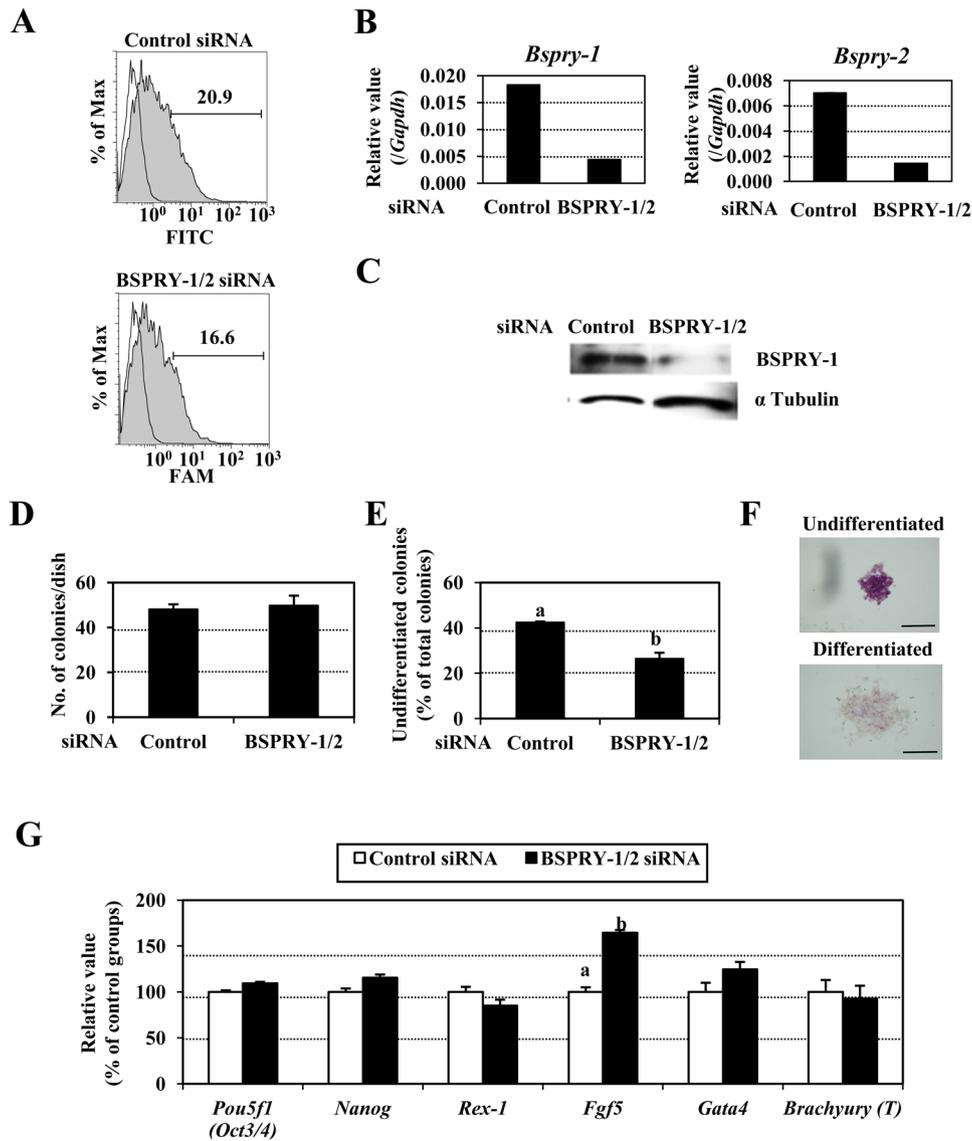


Fig. 2. The effect of BSPRY-1/2 knockdown upon ES cell pluripotency. **A:** FACS profiles of ES cells transfected with FITC-labeled negative control siRNA and FAM-labeled BSPRY-1/2 siRNAs at 24 h after transfection. Gray histograms and open histograms represent the FACS profiles of transfected and non-transfected ES cells, respectively. **B:** siRNA-mediated knockdown of *Bspry-1* and *Bspry-2* at 24 h after transfection. **C:** Detection of BSPRY-1 protein expression level in ES cells at 72 h after transfection with BSPRY-1/2 siRNAs. **D:** Comparison of the number of total colonies and (**E**) the percentage of undifferentiated colonies in ES cells at 5 days after transfection with BSPRY-1/2 or control siRNAs. Colonies were fixed, stained and judged as undifferentiated AP-positive colonies (**F**, upper panel) or differentiated AP-negative colonies (**F**, lower panel). **G:** qRT-PCR analysis for the pluripotent and differentiation marker genes in ES cells at 5 days after transfection with BSPRY-1/2 or control siRNAs. Data are expressed as means \pm SEM for triplicate wells and as relative expression values compared with the control group. Values with different letters are significantly different ($P < 0.05$). Scale bars = 100 μ m.

colonies was significantly increased in BSPRY-2-overexpressing cells (Fig. 4C). Although there was no significant difference, the number of undifferentiated colonies in the BSPRY-1-overexpressing cells group was slightly higher than that of the control group (Fig. 4C). Neither isoform had an apparent effect upon the number of total colonies and the expression levels of the pluripotent marker

and differentiation marker genes (Fig. 4B, D).

Next, we investigated the effect of BSPRY-1 and BSPRY-2 overexpression upon early embryonic development. At 96 h after injection, the hatching blastocyst rate of the BSPRY-1 overexpression group was significantly higher than that of the control group (16.8% vs. 6.1%, $P < 0.05$, Table 4). At 120 h after injection, the percentages

Table 2. Effect of BSPRY-1/2 knockdown upon early embryonic development *in vitro*

siRNA	No. of analyzed embryos	96 h		120 h		
		Blastocyst (%)	Hatched (%)	Blastocyst (%)	Hatched (%)	Degenerated (%)
Control	58	30 (53.6)	23 (39.7)	0 (0) ^a	52 (89.7) ^a	6 (10.3) ^a
BSPRY-1/2	61	38 (62.3)	13 (21.3)	13 (21.3) ^b	24 (39.3) ^b	24 (39.3) ^b

Values with different superscripts within the same column are significantly different ($P < 0.05$). Data were from three replicates.

Table 3. Comparison of the numbers of cells in the ICM, TE and whole blastocysts injected with BSPRY-1/2 and control siRNAs at 96 h after injection

siRNA	No. of analyzed embryos	ICM	TE	Total
Control	15	26.9 ± 2.4 ^a	95.8 ± 5.3	122.7 ± 6.3
BSPRY-1/2	9	19.3 ± 1.3 ^b	85.8 ± 7.3	105.1 ± 8.1

Values with different superscripts within the same column are significantly different ($P < 0.05$).

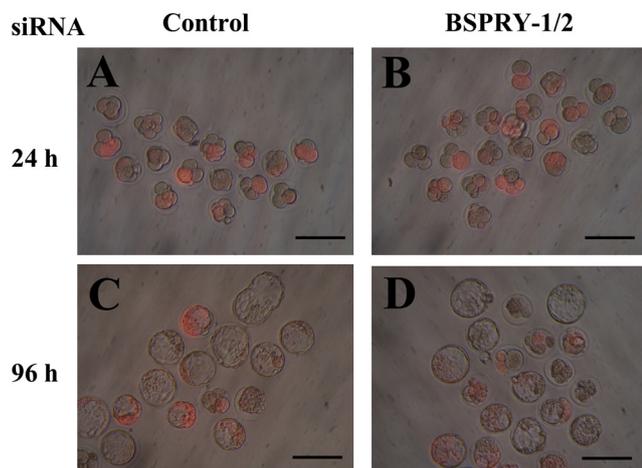


Fig. 3. Fluorescent images of embryos injected with Control or BSPRY-1/2 siRNAs. siRNAs were injected into a blastomere of the 2-cell stage embryos with dextran-tetramethylrhodamine, and the embryos were observed at 24 h (A, B) and 96 h (C, D) after injection.

of the blastocyst stage in the control and BSPRY-1 groups were 29.2% and 11.1% ($P < 0.05$), and the hatching rates were 36.3% and 65.6% ($P < 0.05$), respectively. However, BSPRY-2 overexpression had no significant effect upon embryonic development (Table 4).

Changes in *Bspry-1* and *Bspry-2* expression during ES cell differentiation

In order to investigate the relationship between BSPRY and ES cell differentiation, expression levels of *Bspry-1*, *Bspry-2* and pluripotent marker genes were measured sequentially over 3 days after LIF withdrawal. A decrease in *Pou5f1* (*Oct-3/4*) was observed on day 3 following LIF withdrawal. *Nanog*, *Rex-1* and *Socs3*, known to be downstream targets of LIF/STAT3 signaling, began to decrease on day 2 after LIF withdrawal. On the other hand, the expressions of *Bspry-1* and *Bspry-2* quickly increased on the day after LIF withdrawal and maintained a 1.5-fold value for following

3 days (Fig. 4A). Because there are a number of signaling pathways downstream of the LIF receptor and gp130 besides STAT3, such as the phosphatidylinositol 3-kinase (PI3K) and Ras/Erk pathways, we introduced dominant-negative STAT3 (dnSTAT3, Y705F) specifically to block the LIF/STAT3 pathway [8, 10]. As expected, reduction in the expression levels of *Nanog*, *Rex-1* and *Socs3* were observed at 48 h after transfection, but *Bspry-1* and *Bspry-2* expressions remained unchanged (Fig. 4B).

Discussion

We previously identified BSPRY as a candidate molecule involved in ES cell pluripotency and early embryonic development [4]. BSPRY has been reported as a negative regulator of Ca^{2+} transport in MDCK cells and the mouse kidney [5], but its functions in ES cells and early embryogenesis remain unclear. In this study, we reported for the first time the functional role of BSPRY-1 and BSPRY-2 in the maintenance of ES cell pluripotency and early embryonic development. Our data also suggested the possibility that the two BSPRY isoforms have differing activity levels in ES cells and early embryos.

Schenker *et al.* observed high expression levels of two *Bspry* transcription products in the mouse testis by Northern blot analysis [11]. In agreement with these earlier results, our qRT-PCR data indicated that *Bspry-1* and *Bspry-2* were highly expressed in the mouse testis. Van de Graaf *et al.* further reported that *Bspry* was expressed in the kidney, small intestine, lung and uterus [5], and we additionally confirmed that *Bspry* was expressed in the ovary, ES cells and blastocysts (Fig. 1B) and that *Bspry-1* was the predominant isoform in these tissues.

Overexpression of BSPRY-2 in ES cells significantly induced an increase in the number of undifferentiated ES colonies in the presence of LIF (Fig. 4C). Conversely, in early embryos, the overexpression of BSPRY-1 resulted in a significant increase in hatching blastocyst rate, whilst BSPRY-2 had no effect (Table 4). Initially, we constructed C-terminal V5-tagged BSPRY-1, but it lost activity during early embryonic development (data not shown). These results suggest that the C-terminal end of BSPRY is most likely to be functionally important. Birkenfeld *et al.* reported that rat BSPRY contains a 14-3-3 binding motif, and it translocated from the cytoplasm to the

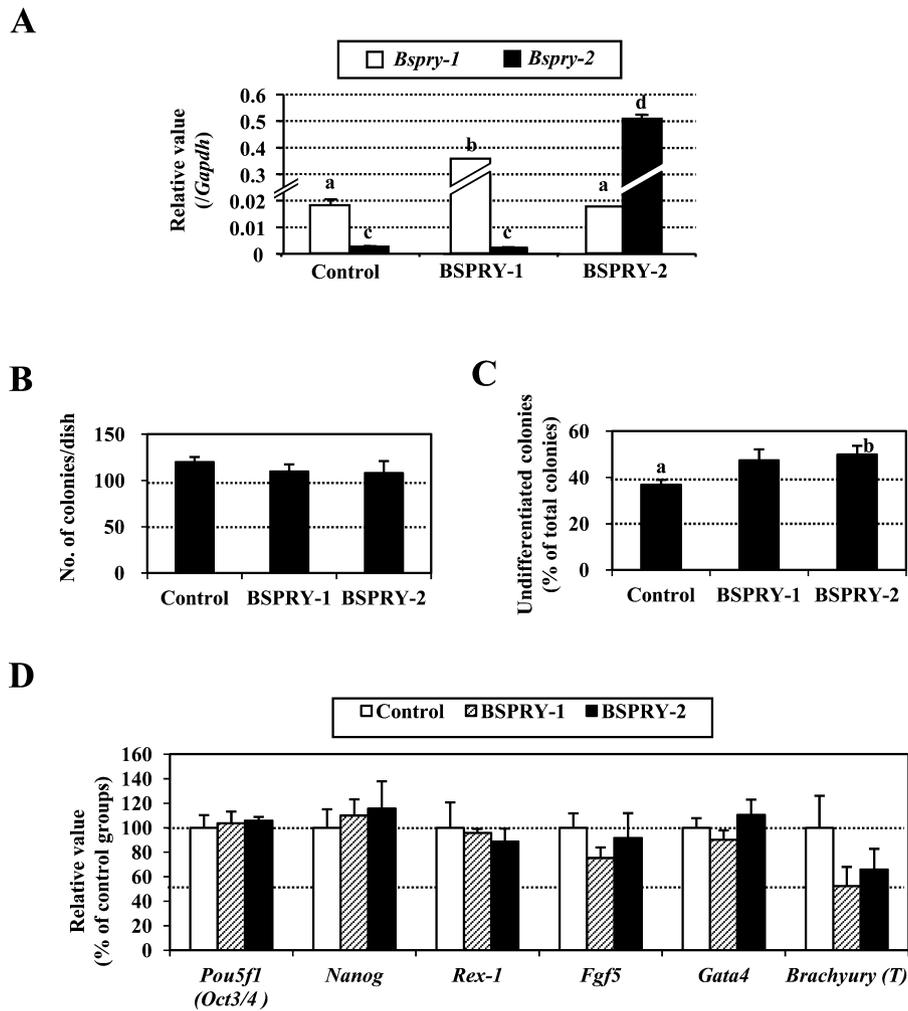


Fig. 4. The effect of BSPRY-1 or BSPRY-2 overexpression upon ES cell pluripotency. BSPRY-1 and BSPRY-2 expression vectors were co-transfected into ES cells with GFP expression vector, and GFP-positive cells were enriched by FACS at 24 h after transfection and cultured for 4 days in the presence of LIF. A: qRT-PCR analysis confirmed increases of *Bspry-1* and *Bspry-2* expression levels in ES cells transfected with the BSPRY-1 and BSPRY-2 expression vectors, respectively. Values were normalized to *Gapdh* expression. B–D: Comparison of the number of total colonies and (C) the percentage of undifferentiated colonies in BSPRY-1- or BSPRY-2-overexpressing ES cells at 5 days after transfection. The percentage of undifferentiated colonies was increased in BSPRY-2 over-expressing ES cells. D: qRT-PCR analysis for the pluripotent and differentiation marker genes in BSPRY-1- or BSPRY-2-overexpressing ES cells at 5 days after transfection. Data are shown as means ± SEM for triplicate wells. Values with different letters are significantly different (P<0.05).

Table 4. Effect of BSPRY-1 or BSPRY-2 overexpression upon early embryonic development *in vitro*

	No. of analyzed embryos	96 h		120 h		
		Blastocyst (%)	Hatched (%)	Blastocyst (%)	Hatched (%)	Degenerated (%)
Control	95	48 (50.2)	6 (6.1) ^a	28 (29.2) ^a	35 (36.3) ^a	31 (34.1)
BSPRY-1	105	63 (60.0)	19 (16.8) ^b	11 (11.1) ^b	71 (65.6) ^b	22 (25.2)
BSPRY-2	109	50 (44.9)	18 (15.4)	24 (22.0)	54 (47.5)	30 (34.3)

Values with different superscripts within the same column are significantly different (P<0.05). Data were from four replicates.

submembranous and perinuclear regions when co-transfected with 14-3-3 ζ [12]. In addition, Schenker *et al.* reported that the SPRY domain in a proximal portion of the C-terminal region is suggested to be a protein-binding module [11]. Thus, it is possible that BSPRY-1 and BSPRY-2 also interact with specific binding partners via these domains, and the differing C-terminal ends of the two BSPRY isoforms may affect the strength of interaction with partner proteins. Identification of the binding partners of BSPRY-1 and BSPRY-2 would help to distinguish the precise role of the two isoforms in ES cells and early embryos.

BSPRY-1/2 knockdown in embryos caused retardation of development to the hatching blastocyst stage and led to a reduction in the number of ICM cells (Tables 2, 3). To demonstrate the direct effect of BSPRY-1/2 knockdown, we injected BSPRY-1/2 siRNAs into one blastomere of a 2-cell stage embryo and traced the cell fates derived from each blastomere. However, at least in embryos that proceeded to the blastocyst stage, we were unsuccessful in detecting any difference in cell fate and number between BSPRY-1/2 siRNA-injected and non-injected blastomere derivatives (Fig. 3D). Recently, it was reported that BSPRY was involved in Ca²⁺ influx as an inhibitory factor of TRPV5 in kidney epithelial cells. Su *et al.* confirmed that TRPV5 was highly expressed in the mouse blastocyst by microarray analysis [13], and we also verified its strong expression in blastocysts, whereas the level was undetectable in ES cells (data not shown). Ca²⁺ is an essential signal for many cellular processes in embryogenesis, e.g., fertilization [14], cavitation [15, 16], blastocoel formation [15] and blastocyst outgrowth [17–19]. Taken together, BSPRY-1 may be involved in cell differentiation and division in the early embryo via regulation of Ca²⁺ influx with TPRV5, and it may be one reason why the activity level of the two isoforms of BSPRY is different in early embryo and ES cells.

Dominant-negative STAT3 did not affect the expressions of *Bspry-1* and *Bspry-2*, at least within 48 h, suggesting that *Bspry-1* and *Bspry-2* were not the direct downstream targets of the LIF/STAT3 pathway. On the other hand, *Bspry-1* and *Bspry-2* expressions were temporally increased for 3 days following LIF removal and then decreased at 4 days (Fig. 5A). *Lefty* [20], *Id1* and *Id2* [21] are also rapidly upregulated after LIF removal [22]; however, these factors work to determine the directions of cell differentiation rather than to prevent cell differentiation. Conversely, overexpression of BSPRY-1 and BSPRY-2 increases the undifferentiated colonies in ES cells, and so we hypothesized that the expressions of BSPRY-1 and BSPRY-2 are partially suppressed by LIF signaling pathways besides STAT3 or other important pathways for maintenance of ES cell pluripotency and that reductions in these signals would cause upregulation of the expressions of BSPRY-1 and BSPRY-2 to complement the reduced signals. Further studies are needed to define the functional role and action mechanism of BSPRY-1 and BSPRY-2 in ES cells and early embryonic development.

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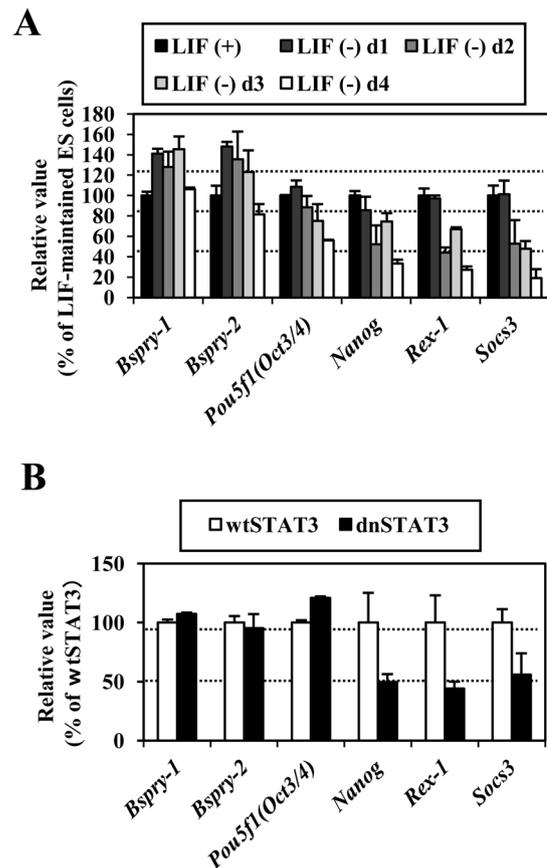


Fig. 5. Expression patterns of *Bspry-1* and *Bspry-2* in differentiating ES cells. A: Changes in the expression levels of *Bspry-1*, *Bspry-2* and marker genes (*Soccs3*, *Rex-1*, *Nanog* and *Pou5fl* [*Oct-3/4*]) in ES cells cultured with or without LIF. Results are expressed as relative values compared with ES cells in the presence of LIF. B: Changes in expression levels of *Bspry-1*, *Bspry-2* and marker genes in ES cells overexpressing wtSTAT3 and dnSTAT3. wtSTAT3 and dnSTAT3 expression vectors were co-transfected into ES cells with GFP expression vector, and GFP-positive cells were enriched at 48 h after transfection and subjected to qRT-PCR. The results are expressed as relative values compared with wtSTAT3-transfected ES cells.

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