Vaspin Is an Adipokine Ameliorating ER Stress in Obesity as a Ligand for Cell-Surface GRP78/MTJ-1 Complex

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It is unknown whether adipokines derived from adipose tissues modulate endoplasmic reticulum (ER) stress induced in obesity. Here, we show that visceral adipose tissue-derived serine protease inhibitor (vaspin) binds to cell-surface 78-kDa glucose-regulated protein (GRP78), which is recruited from ER to plasma membrane under ER stress. Vaspin transgenic mice were protected from diet-induced obesity, glucose intolerance, and hepatic steatosis, while vaspin-deficient mice developed glucose intolerance associated with upregulation of ER stress markers. With tandem affinity tag purification using HepG2 cells, we identified GRP78 as an interacting molecule. The complex formation of vaspin, GRP78, and murine oxygen-related protein 150 deacti-vation of the proteases, which are inhibited by recombinant human vaspin protein (rhVaspin) administration into DIO mice (7,8). Since vaspin belongs to serpin clade A (Serpina12) and interestingly, GRP78 heterozygosity has been shown to result in a compensatory increase in other ER chaperones, which enhanced overall ER capacity and consequently metabolic benefits (5).

We previously reported the cloning of visceral adipose tissue-derived serine protease inhibitor (vaspin), belonging to serpin clade A (Serpina12), and suggested that it may be a compensatory adipokine to improve insulin sensitivity with anti-inflammatory properties demonstrated by recombinant human vaspin protein (rhVaspin) administration into DIO mice (7,8). Since vaspin belongs to serpin, the identification of the proteases, which are inhibited by vaspin, may lead to the development of novel strategies in the treatment of obesity, diabetes, and insulin resistance; however, as of now molecular target(s) of vaspin and its mode of action are totally unknown (9). In later human studies, elevated serum vaspin concentrations in obesity (10), type 2 diabetes (11), and polycystic ovary syndrome (12) were reported, and it correlates with insulin resistance (homeostasis model assessment of insulin resistance) and C-reactive protein levels (13). Here, we show that vaspin exerts its compensatory actions on metabolic dysfunction associated with obesity by using genetic manipulation of the vaspin gene, i.e., in vaspin transgenic (Tg) and knockout mice. Further, we demonstrate that vaspin is a ligand for cell-surface-associated GRP78/MTJ-1 complex in the liver and provide a new functional insight into and molecular basis for the direct cross-talk between an adipokine and ER stress responses in obesity.
mouse vaspin (Serpina12) cDNA was inserted into the EcoRI site by blunt end ligation after filling-in reaction. BamHI and XhoI fragment was subjected to blunt end ligation into Smal site of pBluescript SKII+ (Stratagene), in which mouse aP2 promoter was inserted into EcoRV-FnuI site by blunt end ligation (14). The whole construct with HefIII and NotI, and transgene were generated. Microinjected C57BL/6Jcdl one-cell stage zygotes were oviduct transferred and permitted to develop to term. Three Tg founders were obtained, and Southern blot analysis was performed using Apol fragment of the transgene.

Genotyping of Tg mice was performed by PCR using primers 5ʹ-AAGGCTGTCGGCGCAATAG-3ʹ and 5ʹ-AGAGCTGCTGCTGTACAG-GCCATAGGTCG-3ʹ.

We also constructed a targeting vector for vaspin (Serpina12) using pBl-Hx-Neo-DT carrying neomycin-resistance gene (Neo) to target exon 2 (Supplementary Fig. 7A) (15). The 5ʹ and 3ʹ homology arms were amplified from mouse genomic DNA using TaKaRa Ex Taq. The following primer pairs were used to amplify the homology arms: 5ʹ-GGATCTGGAGAGGAACGAGG-3ʹ and 5ʹ-CCACCCATGGACATCACTCG-3ʹ (for the 5ʹ homology arm) and 5ʹ-CTGACTTATCTTTGAACATCGG-3ʹ and 5ʹ-CCACCATGTGCGAGGATG-3ʹ (for the 3ʹ homology arm). Homologous recombination of 129P2/OlaId embryonic stem cell clones was screened by Southern blot using BamHidigested genomic DNA with a 3ʹ probe. Recombination was further confirmed by Mulu-digested DNA with a 5ʹ probe and Snal-Cdigt-digested DNA with a Neo-probe (Supplementary Fig. 7A). Male chimeric mice were mated with C57BL/6Jcrl mice to generate F1 mice. F1 mice were heterozygous and the F2 generation was generated by crossing two F1 mice. F2 mice were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were treated with specific antibodies (1:200–1,000 dilution): GRP78 (C-20), PGC-1 (H-300), Na+/K+-ATPase and subjected to Western blot analysis.

Poly(A)⁺ RNA analysis. For quantitative real-time PCR analysis, cDNA synthesized from 2 μg of total RNA was analyzed in a sequence detector (model 7900; PE Applied Biosystems) with specific primers and SYBR Green PCR Master (PerkinElmer Life Sciences). The relative abundance of mRNAs was standardized with 36B4 mRNA as the invariant control. The mRNA expression was determined by StbBR green. Gene-specific primers are indicated in Supplementary Table 1.

Western blot analysis. Mice were starved overnight, anesthetized with pentobarbital and injected with 5 units of regular human insulin into the inferior vena cava, and liver was removed 5 min after the injection. Liver tissues were fractionated by discontinuous iodixanol gradients following protocol S36 of the manufacturer’s application sheets (OptiPrep; Invitrogen). The protein lysates of total and subcellular fractions of liver tissues as well as H-4-II-E-C3 were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were treated with specific antibodies (1:200–1,000 dilution): GRP78 (C-20), PGC-1 (H-300), Na/K-ATPase α1 (N-15), and vaspin (I-14) (Santa Cruz); vaspin (Strategic Biosolutions); phospho-Akt (Ser473), Akt, phospho-AMP-activated protein kinase (AMPK)α2 (401H), Sirt1 (C14H4), phospho-eIF2α (Ser51), and GAPDH (14C10) (Cell Signaling); calnexin (ab22505) and IRE1α (phospho-IRE1α) (Abcam); and Golgi 58 (58K-9) and DNAJ1 (1A2) at 4 °C. Antibody detection was followed by treatment with secondary antibodies conjugated with horseradish peroxidase and, finally, visualization with ECL Plus (GE Healthcare) and a luminescent image analyzer (LAS-3000mini; Fujifilm). Immunoprecipitation was performed using Universal Magnetic Co-IP kit (Active Motif). Cell-surface proteins of H-4-II-E-C3 were biotinylated and isolated using Pierce Cell Surface Protein Isolation kit.

Liquid chromatography–tandem mass spectrometry. Recombinant full-length mouse vaspin (vaspin-Ad) and vaspin tagged with calmodulin and streptavadin-binding peptides (pCTAP-vaspin-Ad) (InterPlay Mammalian TAP system; Stratagene) were prepared by Adenovirus Expression Kit (Takara) as previously described (8). Adenovirus vector-expressing vaspin tagged with calmodulin and streptavadin-binding peptides (pCTAP-vaspin-Ad) was introduced to HepG2. Soluble proteins were purified by CBP- and SBP-binding resin (InterPlay Mammalian TAP system; Stratagene) and subjected to SDS-PAGE and Coomassie blue staining. Visible bands were excised and in-gel digested with trypsin and analyzed with liquid chromatography–tandem mass spectrometry.

Pulldown assay and 125I-vaspin binding analysis. Coding sequences without signal peptide of vaspin21-413 and GRP78510-604 were ligated to KpnI/XhoI and Sall/NotI sites of pTNT vector (Promega), respectively. Coding sequence without signal peptide of vaspin21-413 was ligated to BamHII-XhoI site, GRP78 to Sall-NotI and α-antitrypsin (α1AT)570-675, and chimeric vaspin21-413α1AT570-675 and α1AT570-675vaspin21-413 to EcoRI-NotI of pET-42a vector. Pulldown assay was performed with MagneGST Pull-Down System (Promega). rhVaspin was iodinated with specific activity of 7.8 μCi/μg (PerkinElmer) and used for solid-phase assay. Cell-binding assay of cultured H-4-II-E-C3 was performed using 125I-vaspin and subjected to Scatchard analysis. Liver membrane fractions were isolated, and the binding activity of 125I-vaspin was also investigated (9).

α2-macroglobulin (α2M) (Abnova), anti-GRP78 (N-20), and anti-GRP78 (C-20) (Santa Cruz) were used to amplify the homology arms: 5ʹ-AGAGGCTGTCGGCGCAATAG-3ʹ and 3ʹ-AGAGGCTGTCGGCGCAATAG-3ʹ.

RESULTS

Vaspin improves insulin sensitivity in vivo. Vaspin Tg C57BL/6Jcrl mice under the control of aP2 promoter were produced (Supplementary Fig. 1A), and the vaspin levels in serum and adipose tissues derived from three independent lines (line 1, line 10, and line 58) were comparable, although copy numbers of transgene were different in the three lines (Supplementary Fig. 1B–D). At 25 weeks of age, the body weight gain was ameliorated by ~8% (Fig. 1A) and fat pad and liver weight were significantly reduced (Fig. 1B) in Tg mice of line 1 compared with WT mice. Mice fed with HFHS chow demonstrated similar weight reduction in all three lines (Supplementary Fig. 1E), and the representative data of line 1 are shown. Although the pathological features of steatohepatitis such as infiltration of inflammatory cells were not observed in either vaspin Tg or WT mice, by Oil Red O staining, the lipid droplets in the liver were apparently reduced (Fig. 1C), and triglyceride content was also significantly decreased in HFHS chow Tg mice, suggesting that liver may be one of the major target organs of vaspin. In the sera, leptin and insulin levels were significantly reduced at 13 weeks of age in HFHS chow mice, but there were no differences at 25 weeks of age (Supplementary Fig. 2A–C). The results suggested that vaspin may not completely compensate for insulin resistance associated overall with obesity and aging. In addition, vaspin overexpression did not alter the serum concentration of adiponectin (Supplementary Fig. 2D). Gel filtration HPLC indicated a small weight reduction in the fraction of small dense LDL cholesterol (Supplementary Fig. 2E–G). Glucose tolerance and insulin tolerance tests demonstrated improvement of insulin sensitivity in Tg mice (Fig. 1D and E and Supplementary Fig. 3A and B). In the liver tissues, real-time PCR demonstrated the reduction of gluconeogenic genes G6pc and Pepck and lipogenesis genes Acc, Fasn, and Scd1 (Supplementary Fig. 4A–H). In proinflammatory cytokines, serum interleukin-6 levels were significantly reduced in

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vaspin Tg HFHS chow mice (Supplementary Fig. 4I–K). To survey the gene expression profile in adipose tissues, we further evaluated the expression of 22,626 genes by Mouse Gene 1.0 ST Array (Affymetrix). Among 22,626 genes, 350 genes and 26 immune response genes revealed significant variation across the samples. Hierarchical clustering analysis of these genes revealed that mesenteric gene expression profile in vaspin Tg mice fed with high-fat chow was on same branch as in mice fed with normal chow, suggesting that the expression pattern of immune response genes became close to that of control mice fed with normal chow (Supplementary Fig. 5A and B). Food intake and locomotor activities were unaltered (Supplementary Fig. 6A–C), and VO2 did not changed in vaspin Tg HFHS-diet mice with ANCOVA with body weight as a covariate (Supplementary Fig. 6D). The hypertrophy of adipocytes was ameliorated and the crown-like structures were also reduced in Tg HFHS chow mice (Supplementary Fig. 6E).
For further confirmation of the phenotype of Tg mice, the standard gene-targeting method was used to produce vaspin knockout mice (Supplementary Fig. 7A). Elevation of mRNA and protein expression of vaspin in the epididymal adipose tissues and serum was observed in vaspin+/+ HFHS chow mice; however, it was not observed in vaspin−/− mice (Supplementary Fig. 7B). Vaspin−/− HFHS-diet mice demonstrated increase in body weight, fat pad, and liver weight (Fig. 2A and B). The size of adipocytes and the number of crown-like structures increased in vaspin−/− mice (Fig. 2C and Supplementary Fig. 6F), and deterioration of insulin sensitivity was observed (Fig. 2D and E and Supplementary Fig. 3C and D). Serum total cholesterol and small dense LDL cholesterol levels were significantly increased in vaspin−/− mice (Supplementary Fig. 8A–C). Serum leptin levels increased at 25 weeks of age in vaspin−/− mice compared to vaspin+/+ HFHS mice (Supplementary Fig. 8D).
age and, in accordance with the results in vaspin Tg mice, serum concentration of adiponectin was not altered in vaspin−/− mice, suggesting that the vaspin-mediated improvement of insulin tolerance is not dependent on the adiponectin pathway (Supplementary Fig. 8D and E). Tri-glyceride contents prominently increased in vaspin−/− and vaspin+/- HFHS chow mice compared with vaspin+/+ mice (Supplementary Fig. 8F and G). The impact of gene manipulation of vaspin on the body and fat pad weight was rather less impressive compared with the changes in insulin sensitivities and triglyceride contents in the liver. We thus speculated that secreted vaspin from visceral adipose tissue may exert such effects directly acting on liver cells and further investigated to identify the vaspin-interacting molecules in the liver.

**Vaspin interacts with GRP78.** Serpin clade A members consist of classical serine proteases inhibitors, such as anti-trypsin (SERPINA1) and antichymotrypsin (SERPINA3), hormone-binding proteins (SERPINA6, corticosteroid-binding globulin; SERPINA7, thyroid-binding globulin), and angiotensigen (SERPINA8). The diverse function of serpin clade A members led us to further screen and search for the molecules interacting with vaspin. We failed to demonstrate that vaspin inhibits the activities of known serine proteases. Vaspin failed to bind to cortisol and thyroxine, and vaspin did not alter the protease cascades in renin angiotensin and complement systems. We further screened the interacting molecules by tandem affinity tag purification. pCTAP-vaspin-Ad was applied to HepG2 cells, purified by calmodulin and streptavidin resin, subjected to SDS-PAGE and in-gel digestion with trypsin, and analyzed by LC-MS/MS, and we identified GRP78 as an interacting molecule in HepG2 cells. Since GRP78 was not isolated in the nontransfected cell lysates, GRP78 specifically bound to vaspin but not to calmodulin and streptavidin resin (Fig. 3A). Immunoprecipitation study indicated the presence of vaspin-GRP78 complex in HepG2 cells supplement with rhVaspin in the culture media, and it was not found in HepG2 cells, in which vaspin was not expressed (Fig. 3B). Pull-down assay demonstrated the physical binding between in vitro–translated GRP78 and glutathione S-transferase (GST)-vaspin recombinant proteins (Fig. 3C) as well as between GST-GRP78 and in vitro–translated vaspin proteins (Fig. 3D). By pull-down assay, GST-vaspin−/−/−/413 failed to bind to GRP78, but GST-α1AT−/−/−/250−251/vaspin−/−/−/13 bound to GRP78, suggesting that GRP78 binds to the NH2-terminal half of vaspin, avoiding the reactive site loop at COOH-terminal, which is a conserved domain of serpin (Supplementary Fig. 9A). The main of GRP78 are important for the vaspin-induced phosphorylation of Akt. In pull-down and solid-phase binding assays, α2M did not efficiently compete with the binding between vaspin and GRP78 (Supplementary Fig. 12A and B); however, vaspin and α2M were colocalized in the sinusoidal front of liver cells in vaspin Tg mice (Supplementary Fig. 12C). Since α2M is abundant in serum (−100 mg/dL) and serum vaspin levels have been reported to be −1−10 ng/mL, the binding affinity of vaspin is quite high compared with α2M and excess amount of α2M is required to compete with vaspin.

Phosphorylation of Akt was enhanced in Tg and suppressed in vaspin−/− mice treated with HFHS diet (Fig. 4A). Similarly, phosphorylation of AMPK was markedly enhanced in Tg mice and suppressed in vaspin−/− mice without alterations in the expression of Sirt1 or peroxisome proliferator–activated receptor γ coactivator 1α (Fig. 4A and Supplementary Fig. 13). The additional of rhVaspin in the culture also increased the phosphorylation of Akt and AMPK in H-4-II-E-C3 in a dose-dependent manner (Fig. 4B). The application of anti-GRP78 (N-20) and anti-GRP78 (C-20) completely abrogated the vaspin-induced phosphorylation of Akt (Fig. 4C). The activation of AMPK was also efficiently repressed by anti-GRP78 (N-20) and anti-GRP78 (C-20) (Fig. 4C). Although the vaspin-binding site on GRP78 is still unknown, the data suggested that ATP domain at NH2-terminal and highly helicated COOH-terminal lid domain of GRP78 are important for the vaspin-induced intracellular signaling. Since GRP78 was recruited from ER to plasma membrane fractions under ER stress, we also investigated the ER stress–related markers in vaspin Tg and vaspin−/− HFHS mice. Among ER stress–related factors, upregulation of ATF6, pell2α, and pIRE1α by HFHS treatments was ameliorated in vaspin Tg mice; in contrast, they were upregulated in vaspin−/− HFHS mice compared with vaspin+/+ mice (Supplementary Fig. 13).

To further confirm that vaspin is an authentic ligand for cell-surface GRP78 complex, we performed the knockdown experiments in vaspin Tg mice and H-4-II-E-C3 cells. The expression of GRP78 was reduced by ~40% by the treatment of in vivo–injected siRNA-GRP78 (Fig. 5A). Glucose tolerance tests revealed that siRNA-GRP78 treatment deteriorated glucose tolerance in both vaspin Tg and WT mice, and the insulin-sensitizing action of overexpressed vaspin disappeared in vaspin Tg mice (Fig. 5B and C). Binding assay using 125I-vaspin in isolated membrane fractions of the liver and H-4-II-E-C3 cells revealed...
high-affinity binding with $K_d$ values $22.2 \times 10^{-9}$ and $0.57 \times 10^{-9}$ mol/L, respectively. The treatments of siRNA-GRP78 and shRNA-GRP78 lentivirus did not alter the binding affinity ($24.0 \times 10^{-9}$ and $0.53 \times 10^{-9}$ mol/L in liver and H-4-II-E-C3 cells, respectively); however, they reduced $B_{\text{max}}$ both in vivo and in vitro (Fig. 5D and E).

**DISCUSSION**

GRP78 is an ER chaperone that binds to hydrophobic patches of nascent polypeptides in ER and serves as a switch for the induction of UPR. GRP78 directly binds to IRE1α, ATF6, and PERK under normal conditions. Under ER stress, the available pool of GRP78 is occupied by

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**FIG. 3.** Identification of GRP78 and vaspin complex by tandem affinity tag purification. A: pCTAP-vaspin-Ad was applied to HepG2, and purified products are analyzed by SDS-PAGE. In-gel digestion with trypsin and analysis by LC-MS/MS revealed that band 1 is GRP78 and band 2 is vaspin. B: HepG2 was treated with vaspin recombinant protein. Vaspin-GRP78 complex was immunoprecipitated by vaspin antibody and blotted with GRP78 antibodies. C: Pull-down assay using GST-vaspin fusion protein and in vitro–translated GRP78. D: Pull-down assay using GST-GRP78 fusion protein and in vitro–translated vaspin. (A high-quality color representation of this figure is available in the online issue.)
preventing the aggregation of unfolded proteins, and GRP78 releases IRE1α, ATF6, and PERK, which are transducers of the UPR signals (1). Beyond the ER retention, GRP78 is also associated with various anchor membrane proteins on the cell surface and functions as a receptor for various ligands and a signaling hub for cell survival or death (18–21). In many tumor cells, α2M binds to cell-surface GRP78/MTJ-1, activates phosphatidylinositol 3-kinase/Akt signal to suppress apoptotic pathways, concomitantly upregulates NF-κB, and induces UPR so that cell proliferation occurs (22–25). In addition to α2M, teratocarcinoma-derived growth factor-I (Cripto) and GRP78 form a complex at the cell surface and enhance the cell growth by inhibiting transforming growth factor-β signaling events (26). Truncated cadherin and GRP78 form a complex at the cell surface and enhance the cell growth by inhibiting transforming growth factor-β signaling events (26). 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inhibits the insulin- and ER stress–induced hepatic steatosis in db/db mice (6). Although GRP78 heterozygocity attenuates the diet-induced obesity and insulin resistance, Ye et al. (5) discuss the hypothesis that compensatory upregulation of GRP94 and protein disulfide isomerase, as well as other adaptive UPRs, is responsible for the therapeutic effects. Since the combination of GRP78 with ligands and associated anchor proteins exerts diverse biological consequences...
such as cell survival and apoptosis, vaspin itself, anti-GRP78 antibodies, or GRP78-interacting small molecules mimicking beneficial action of vaspin need to be screened for the treatment of metabolic syndrome. In autoimmune diseases, anti-citrullinated protein antibodies, specific autoantibodies in rheumatoid arthritis, bind to GRP78 and enhance NF-κB activity and tumor necrosis factor-α production (28). Thus, anti-GRP78 antibodies and GRP78-interacting small molecules should be functionally tested as to whether they have antiapoptotic/proapoptotic potentials or anti-inflammatory/proinflammatory properties. α2M binds to cell-surface GRP78/MTJ-1, induces NF-κB activity, and activates phosphatidylinositol 3-kinase/Akt signal, which results in antiapoptosis, cell proliferation, and survival of tumor cells (21). Thus, the signals downstream of vaspin-GRP78/MTJ-1 pathways may induce NF-κB activity and production of proinflammatory cytokines in the setting of obesity and metabolic syndrome. In the current study, however, vaspin ameliorated ER stress and exerted anti-inflammatory action in liver via vaspin-GRP78/MTJ-1 pathway (Fig. 6). Similar findings have been reported, i.e., that vaspin inhibits NF-κB activation and prevents tumor necrosis factor-α–induced expression of intracellular adhesion molecule-1 in vascular smooth muscle cells (29).

There are some limitations to the current investigation. Since the treatment with siRNA-GRP78 deteriorated glucose tolerance in both vaspin Tg and WT mice in the current study, the GRP78-induced beneficial effects in obesity were not completely dependent on the vaspin-mediated pathway; thus, targeting the GRP78 may be beneficial rather than using vaspin itself for the development of new therapeutic modalities. Another limitation is that direct biological actions of vaspin on adipose tissues were not clearly demonstrated in the study. GRP78 is also expressed in the adipocytes in vivo, and vaspin is expressed in both intracellular and extracellular compartments. Vaspin may exert various biological effects on adipocytes such as reduction of adipocyte size and anti-inflammatory action by interacting with cell-surface GRP78 or ER-associated GRP78. Precise mechanisms of the direct action of vaspin on adipose tissues remain to be investigated in future studies. The last limitation is that where vaspin binds to the region of GRP78 is still uncharacterized. α2MG binds to the region at amino acids 98–115 (30) and competed with the binding of vaspin on GRP78. In addition, both anti-GRP78 N-20 and C-20 antibodies interfered with the binding of GRP78, vaspin binding, and the subsequent signaling events. It is still not clear whether α2MG and anti-GRP78 antibodies directly compete with vaspin or interfere with the vaspin binding by the alteration of conformation or function of GRP78 molecules.

Poor prognosis of the majority of tumors correlates with the expression of cell-surface GRP78 and presence of autoantibodies, which mediates prosurvival signals and responses to cellular stress. Thus, blocking anti-GRP78 antibodies, small molecule inhibitors, anti–cancer agent conjugated with vehicle targeted to GRP78, or GRP78 promoter–driven expression of suicide genes may be expected in the treatment of cancers (18). Vaspin indeed has a beneficial role in obesity in the metabolic dysfunctions, and the only concern to be addressed is whether vaspin promotes the tumor growth by acting on the cell-surface GRP78 on tumor cells. However, the lack of apparent carcinogenesis processes in vaspin Tg mice in our experiments negates the possibilities of development of neoplasm. Taken together, targeting GRP78 by vaspin or vaspin-mimicking antibodies or small molecules is beneficial for therapeutics for obesity and its related insulin resistance by ameliorating ER stress in the liver.

ACKNOWLEDGMENTS
This research was supported by Grant-in-Aid for Young Scientists (B) (24790926) to A.N., Grant-in-Aid for Scientific Research (B) (23390241), Challenging Exploratory Research (23659470), Grant-in-Aid for Scientific Research on Innovative Areas (“Molecular Basis and Disorders of Control of Appetite and Fat Accumulation”) (23126516) to J.W., and Grant-in-Aid for Scientific Research (A) (21249053) to H.M. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

No potential conflicts of interest relevant to this article were reported.

A.N. and J.W. designed and performed most of the experiments, conceived the study, and wrote the manuscript. I.I. designed and performed most of the experiments. S.T. performed Tg and knockout animal experiments. K.Hig. performed Tg and knockout animal experiments and prepared rhVaspin. K.M., M.K., K.I., T.T., A.K., K.Hid., and J.E. performed Tg and knockout animal experiments. C.S.H. and D.O. performed immunohistochemistry. Y.M. and R.M. performed quantitative RT-PCR. S.K. and Y.I. derived and cultured mouse embryonic stem cells and performed blast cyst injection. H.M. conceived the study and wrote the article. J.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Yoko Saito and Dr. Noriko Yamamoto at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences for excellent technical assistance.
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