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Original Article

Dkk3/REIC, an N-glycosylated Protein, Is a Physiological Endoplasmic Reticulum Stress Inducer in the Mouse Adrenal Gland

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Dickkopf 3 (Dkk3) is a secreted protein belonging to the Dkk family and encoded by the orthologous gene of *REIC*. *Dkk3/REIC* is expressed by mouse and human adrenal glands, but the understanding of its roles in this organ is still limited. To determine the functions of Dkk3 in the mouse adrenal gland, we first identified that the mouse Dkk3 protein is N-glycosylated in the adrenal gland as well as in the brain. We performed proteome analysis on adrenal glands from *Dkk3*-null mice, in which exons 5 and 6 of the *Dkk3* gene are deleted. Two-dimensional polyacrylamide gel electrophoresis of adrenal proteins from wild-type and *Dkk3*-null mice revealed 5 protein spots whose intensities were altered between the 2 genotypes. Mass spectrometry analysis of these spots identified binding immunoglobulin protein (BiP), an endoplasmic reticulum (ER) chaperone. To determine whether mouse Dkk3 is involved in the unfolded protein response (UPR), we carried out a reporter assay using ER-stress responsive elements. Forced expression of *Dkk3* resulted in the induction of distinct levels of reporter expression, showing the UPR initiated by the ER membrane proteins of activating transcription factor 6 (ATF6) and inositol-requring enzyme 1 (IRE1). Thus, it is possible that Dkk3 is a physiological ER stressor in the mouse adrenal gland.

Key words: *Dkk3* knockout mouse, adrenal gland, glucose-regulated protein 78, proteome, endoplasmic reticulum stress

Dickkopf3 (*Dkk3*) encodes a secreted protein that belongs to the Dkk family, which comprises 5 members in mammals, Dkk1 to Dkk4 and Dkk-like 1 (Dkkl1) [1,2]. The founding member, Dkk1, was identified by its head-inducing activity [3]. While *Dkk3* was first identified by a homology-based cloning and database search [3,4], the human counterpart was independently isolated as a gene whose expression is reduced in human immortalized cells and tumor-derived cells, and thus also called *REIC* [5]. Anti-tumor effects of Dkk3 have been reported in mouse xenograft models of various tumors, such as prostate cancer and non-small-cell lung cancer [6,7]. Overexpression of human *Dkk3* via adenovirus vector (Ad-REIC) to treat these xenograft cancers [6] causes cell death due to excess ER responses. Phylogenetic analysis reveals that *Dkk3* is distinct from *Dkk1/Dkk2/Dkk4*, as exemplified

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by the fact that the cnidarian hydra has only two *Dkk*-related genes, *hyDkk3* and *hyDkk1/2/4*, indicating that these 2 subfamilies diverged far back in evolutionary history [8,9]. Moreover, while other members are known to act as a Wnt antagonist or agonist [3,4,10], neither Dkk3 nor Dkkl1 inhibited the Wnt-induced axis duplication activity in frog embryos [4]. These intriguing findings have inspired much research into the roles of Dkk3 in developmental or tumor-related contexts as well as under physiological and pathological conditions.

A number of these studies have been performed using abrogation of the Dkk3 function in mice. Even though it has been established that *Dkk3* is expressed during mouse development [11], there are no obvious morphological defects in the *Dkk3* mutant mice apart from some altered hematological and neurological findings [12]. However, an immunological analysis on *Dkk3*-deficient cells revealed that Dkk3 is required for tolerance of cytotoxic T-cells [13]. In mouse atherosclerosis models, Dkk3 was found to promote reendothelialization by inducing endothelial cell migration [14]. Finally, 2 studies showed that *Dkk3* expression is upregulated in muscles from aged human patients with sarcopenia and in those from mice with age-related atrophy [15,16]. These studies imply that the secreted protein of Dkk3 has crucial effects on its endogenous functions and may be applicable as a novel therapeutic intervention. To this end, we previously examined the expression patterns of Dkk3 by in situ hybridization and immunohistochemistry in various organs of the adult mouse. We found that *Dkk3* is expressed in endocrine and exocrine organs, including the adrenal glands and gastrointestinal tracts, together with spermatogenic cells and ovarian follicular cells [17].

Here, we focus on the role of Dkk3 in the adrenal gland, which has two distinct endocrine tissues, the cortex and medulla. The tissue distribution of Dkk3 in the human adrenal gland was previously reported by Suwa *et al*. [18]: *Dkk3* expression is higher in the zona fasciculata/zona glomerulosa than in the zona reticularis and is also higher in the medulla. In this study, we examined the glycosylation status of mouse endogenous Dkk3 in the adrenal gland. We compared the protein expression patterns in the adrenal gland between wild-type and *Dkk3*-deficient mice, which we generated as a third knockout strain here, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Based on the results from 2D-PAGE, we further examined the pathway of the unfolded protein response in which Dkk3 is involved.

Materials and Methods

Materials. Unless stated otherwise, reagents were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Ethics statement and generation of Dkk3 knockout mouse. All animal procedures were approved by the Okayama University Institutional Animal Care and Use Committee (approval numbers: OKU2014608, OKU2016530) and all efforts were made to minimize animal suffering. Heterozygous mice with a conditional knockout (KO) of *Dkk3* were generated at Ozgene (Bentley, Australia). Briefly, DNA that includes exons 4 to 8 of the mouse *Dkk3* gene was amplified by PCR from C57BL/6 genomic DNA (Fig. 1A). The phosphoglycerate kinase promotor (PGK)/*neomycin* (neo) cassette was inserted downstream of exon 6. The PGK-neo cassette was flanked by flippase recognition target (FRT) sites and can be deleted with flippase recombinase. Exons 5 and 6 were flanked by loxP sites and can be deleted with Cre recombinase. The Bruce-4 embryonic stem (ES) cells were electroporated and selected, and correct recombination events were verified by Southern blot analysis of genomic DNA (Fig. 1B). Injection of positive clones into blastocysts generated chimaeras, which transmitted the recombinant locus. Heterozygous male and female were bred to generate *Dkk3* KO mice [B6.Cg-Dkk3$^{tm1.1}$ [Dkk3 KO (wtfloxΔneoFlpE)]]. Genotyping of *Dkk3* mutant mice was performed by PCR on genomic tail DNA using forward (F) (5'-AGTGCTGGCCAGGCTGGCTTCTGGG-3') and reverse (R) (5'-AAACTGTGACTGTGAGATTGGGAGC-3') oligonucleotides to produce 1519-bp and 158-bp bands from the wild-type (WT) and targeted allele, respectively (Fig. 1C). *Dkk3* mutant mice were maintained in a C57BL/6 background. Mice were housed under a 12 : 12 light dark-cycle with food and water *ad libitum*. Organs were obtained from male WT and *Dkk3* KO mice. Each experiment was performed at least in duplicate and using at least 2 animals.

Glycosidase digestion. The human embryonic kidney (HEK) 293T cell line was maintained as previously reported [19] and transfected with a pIDT-CMV-mouse *Dkk3*-His-tag vector, which consisted of the CMV promoter and His-tagged mouse *Dkk3* cDNA in the pIDTSmart-Kan (Integrated DNA Technologies,

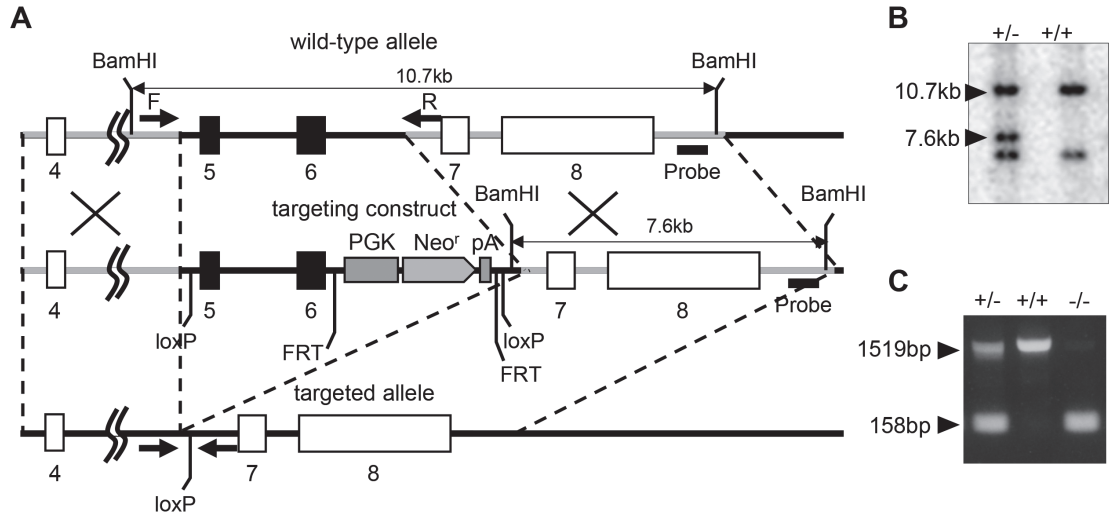

Fig. 1 Generation of *REIC/Dkk-3* knockout mice. **A**, Schematic diagrams of the wild-type allele, targeting construct and targeted allele. Exons (boxes) are numbered. In the targeted allele, the PGK/*neomycin* cassette and poly(A) sequences are highlighted, and regions of homology are shaded in grey. The positions of the wild-type (+/+) and mutant (−/−) allele-specific genotyping primer sets are indicated by arrows; **B**, Southern blot analysis used to confirm the genotypes of the embryonic stem cells used in this study; **C**, Genotyping PCR of DNA isolated from wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice using both wild type- and mutant-specific primer pairs showing detection of the wild-type (1519 bp) and mutant (158 bp) alleles.

Coralville, IA, USA) backbone, by the use of a NEPA21 electroporator and 2-mm gap cuvettes (Nepa Gene, Chiba, Japan) according to the manufacturer's instructions [poring pulse (voltage: 150 V; pulse length: 5 ms; number of pulses: 2)]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cat#043-30085; Fujifilm Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum for one day on a 35-mm dish, and then in serum-free media for 3 days. The conditioned media containing secreted Dkk3 protein were harvested. The lysates from mouse tissues were harvested as previously reported [17]. Samples were incubated at 37°C for 16 h in phosphate buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate (SDS) and 1% Triton X-100 with or without 0.2 U/ml N-glycosidase F (Roche, Mannheim, Germany) or 0.1 mU/ml O-glycosidase (Roche).

Western blot analysis. Western blotting was performed as previously reported [19]. Conditioned media were prepared according to the standard procedure. Thirteen-week-old male mice were anaesthetized and perfused with ice-cold phosphate-buffered saline (PBS). The heart, brain and adrenal gland were harvested, and lysates from these tissues were prepared using RIPA buffer [19]. The membrane was blocked by Blocking One and then incubated overnight with the rabbit anti-mouse Dkk3 antibody (1 : 30000) [17] or anti-His-tag antibody (1 : 30000) (Cat#PM032; Medical and Biological Laboratories, Nagoya, Japan) diluted in IMMUNO SHOT (Cosmo Bio, Tokyo) at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cat#7074; Cell Signaling Technology, Danvers, MA, USA), and then immunoreactive bands were visualized with Immunostar LD (Fujifilm, Tokyo) and a C-DiGit Blot scanner (LiCor, Lincoln, NE, USA).

Immunohistochemistry. Ten-week-old male mice were fixed with 4% paraformaldehyde (PFA) in PBS via transcardial perfusion. Subsequently, the tissues were collected and fixed in 4% PFA on ice for 6 h, then equilibrated with 20% sucrose in PBS until they sank down. The fixed tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan, Tokyo) and then frozen with liquid nitrogen. The frozen blocks were sectioned (14 μm) with a cryostat. The tissue sections were placed on CREST coated glass slides (Matsunami, Osaka, Japan) and stored at −20°C until further use. Immunohistochemistry was performed as previously described [17]. Briefly, the tissue sections were incubated with PBS containing 0.3% Triton X-100 (PBST) 3 times for 5 min each at room temperature (RT). The sections were then incubated with blocking

reagent (PBST containing 5% normal goat serum) for 1 h, followed by a primary antibody diluted in blocking reagent [anti-mouse Dkk3 antibody, 1 : 1000 (5 μg/ml); anti-tyrosine hydroxylase antibody, 1 : 250, normal goat IgG (negative control), 5 μg/ml] for 3 h at RT and then overnight at 4°C. The next day, the sections were rinsed with PBST and incubated with Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) at RT for 1.5 h. After washing, the sections were treated with 4', 6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan) containing PBS and then mounted with polyvinyl alcohol-glycerol (10% polyvinyl alcohol, 25% glycerol in 0.1 M Tris-HCl [pH 8.0]). We collected fluorescent images using an LSM 780 confocal laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany).

Sample preparation and 2D-PAGE. Sample preparation and 2D-PAGE were performed with an ANATECH Cool Phorestar (Anatech, Tokyo) according to the manufacturer's instructions. The littermate male *Dkk3* WT and KO mice (11 weeks old) were anaesthetized and then perfused transcardially with PBS. Adrenal glands obtained from the mice were subjected to frost shattering and then lysed in 2D-PAGE lysis buffer (2 M thiourea, 7 M urea, 3% CHAPS and 1% Triton X-100) containing a protease inhibitor cocktail. The lysates were sonicated and cleared by centrifugation. The isoelectric focusing (first-dimension) separation was performed on nonlinear immobilized pH gradients using Immobiline DryStrip gels (18 cm in length, pH 3-11; GE Healthcare, Chicago, IL, USA). After isoelectric focusing, the gel strips were equilibrated to the SDS-treatment solution [25 mM Tris-HCl (pH 6.8), 6 M urea, 5 mg/ml dithiothreitol, 2% SDS, 0.0025% bromophenol blue and 30% glycerol] for 30 min and then treated with alkylating solution [25 mM Tris-HCl (pH 6.8), 2% SDS, 0.0025% bromophenol blue, 45 mg/ml iodoacetamide (Fujifilm Wako Pure Chemical) and 30% glycerol] for 20 min. The 2D SDS-PAGE was performed using 10% polyacrylamide gel. Gels were stained with SYPRO Ruby (Lonza, Basel, Switzerland), and the protein spots were detected with an AE-6932GXES Printgraph (ATTO, Tokyo). The gel images were rendered in RGB pseudo-color with Photoshop software (Adobe Systems, San Jose, CA, USA).

In-gel digestion, LC-MS/MS of tryptic peptides. In-gel digestion was performed using an In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Protein spots on the SYPRO Ruby-stained 2D gel were excised, and the proteins in the pieces were digested with 10 μg/ml trypsin in 25 mM ammonium bicarbonate overnight at 30°C. After digestion, peptide fragments in the supernatant were subjected to analysis with a mass spectrometry (MS) system (MDS SCIEX 4800 MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA, USA). Protein identification was performed using the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine and the Mascot MS/MS ions search engine (Matrix Science, Boston, MA, USA).

Luciferase assay for activation of unfolded protein response (UPR) pathways. Activation of the UPR was detected by using HEK293A cells stably transfected with the pGL4-3xAARE luciferase reporter vector for the protein kinase R-like ER kinase (PERK) pathway, pGL4-UPRE-luc2P luciferase reporter vector for the inositol-requiring enzyme (IRE) 1 pathway, pGL4-ERSE-I-luc2P luciferase reporter vector for the activating transcription factor (ATF) 6 pathway, and pGL4-ERSE-II-luc2P luciferase reporter vector for the IRE1 pathway [20, 21]. The cells were seeded onto 96-well plates and treated with tunicamycin (2 μg/ml), or transfected with the pcDNA-*Ins2*^{C96Y} [22] or the pFLAG-mouse *Dkk3* vector (in the backbone of pFLAG-CMV5.1; Sigma-Aldrich, St. Louis, MO, USA), in the presence of beetle luciferin (Promega, Madison, WI) for 16 h. Luciferase activity was measured with an EnVision reader (Perkin Elmer, Waltham, MA, USA).

Quantitative PCR (qPCR) assay for activation of UPR pathways. Activation of the UPR was examined by using PC12 cells transiently transfected with pFLAG-mouse Dkk3, or pFLAG-GFP as a negative control, by using TransIT-X2 Reagent (Mirus Bio, Madison, WI, USA). At 36 h post-transfection, cells were harvested and processed for RNA extraction (RNeasy Mini Kit; Qiagen, Hilden, Germany), reverse transcription (ReverTra Ace qPCR RT Master Mix; Toyobo, Osaka, Japan) and DNase treatment (TURBO DNA-free Kit; Thermo Fisher Scientific). qPCR was performed by using FastStart Essential DNA Green Master (Roche), a LightCycler Nano System (Roche), and the following primers: for rat *Bip* (*Hspa5*), 5'-GTGCAGCAGGACATCAAGTTC-3' (forward) and 5'-GGCAGTTTCCTTCATTTTAGTGAG-3' (reverse);

for rat *Atf4*, 5'-GGCCACCATGGCGTATTAGA-3' (forward) and 5'-GACATTAAGTCCCCCGCCAA-3' (reverse); for rat *Xbp1*, 5'-GGCCACCATGGCGTATTAGA-3' (forward) and 5'-GACATTAAGTCCCCCGCCAA-3' (reverse); for rat *Atf6*, 5'-AGTCCACGTTGTTTGCTGAG-3' (forward) and 5'-TGCTGGGGCTCCATATGTCT-3' (reverse); and for rat *beta-actin*, 5'-AGCACCCTGTGCTGCTCAC-3' (forward) and 5'-CAGTGGTACGACCAGAGGCATA-3' (reverse). We confirmed that treatment of the cells with tunicamycin (300 ng/ml) induced increases in the expressions of *Bip* (approximately × 10), *Atf4* (× 2), and *Atf6* (× 2) relative to those in untreated cells. *Xbp1* mRNA was not detectable under our experimental conditions.

Results

Dkk3 protein in the adrenal gland is N-glycosylated. Previously, we reported that the Dkk3 protein in various mouse tissues had a higher molecular weight compared to that calculated from the mouse Dkk3 amino acid sequence [17]: 40 kDa for isoform a (366 amino acids [aa]), 38 kDa for isoform b (349 aa), and 35 kDa for isoform c (321 aa). This suggested that mouse Dkk3 might be subjected to post-translational modification. A prediction tool showed that there are 4 N-linked glycosylation sites and 11 O-linked glycosylation sites (Fig. 2A) [23]. Because the secreted form of human Dkk3 is an N-glycosylated protein [4, 24], we tested whether mouse Dkk3 is also modified by N-linked glycans. To assess the levels of overexpressed mouse Dkk3 and endogenous mouse Dkk3, the conditioned medium of HEK293T transfected with pIDT-CMV-mouse *Dkk3*-His-tag and lysates of mouse tissues were treated with either N-glycosidase, which cleaves all types of asparagine-bound N-glycans, or O-glycosidase, which releases the tetragalactose N-acetylgalactosamine unit from O-glycans at serine/threonine residues. Western blot analysis showed that the lower molecular weight of mouse Dkk3 appeared after treatment with N-glycosidase but was hardly detected after treatment with O-glycosidase (Fig. 2B, C), indicating

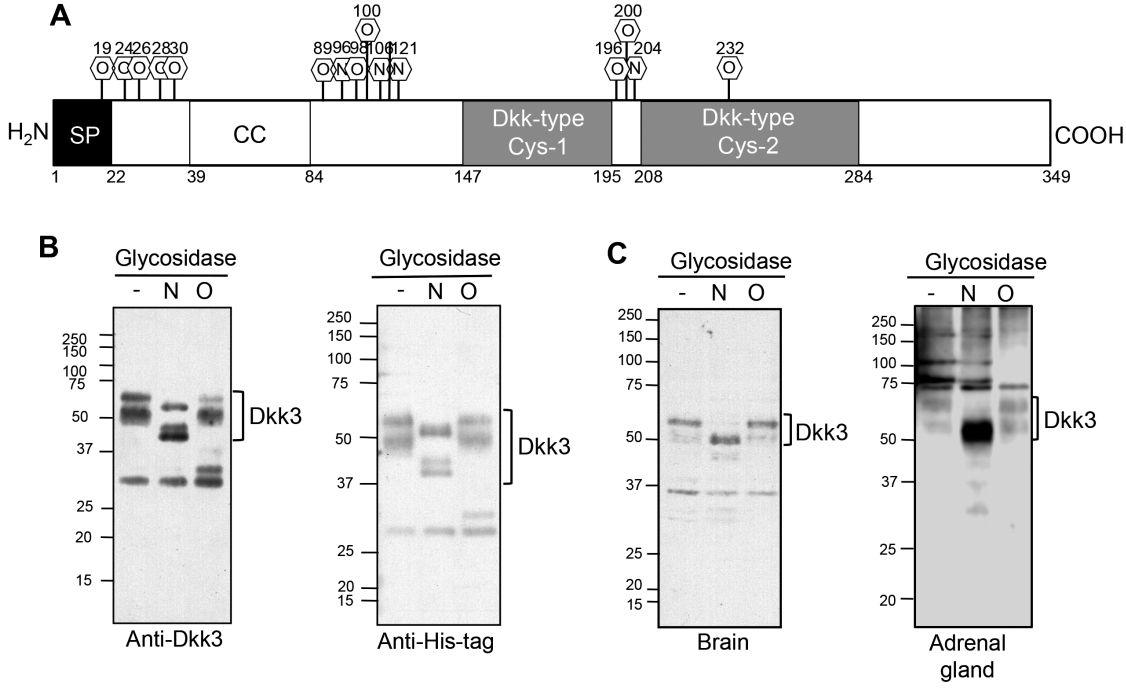

Fig. 2 Characterization of mouse Dkk3 protein glycosylation. **A**, Diagram of the mouse Dkk3 protein showing the signal sequence (SP), putative coiled-coil region (CC), cysteine-rich domains (Dkk-type Cys-1 and Dkk-type Cys-2), and putative N- and O-glycosylation sites (N and O) predicted by GlycoEP (http://crdd.osdd.net/raghava/glycoep/). Dkk3 isoform b of 349 amino acids (NP_001347186.1) is shown; **B**, **C**, Conditioned medium of *Dkk3*-overexpressing HEK293T cells (**B**) and lysates from the brain and adrenal gland (**C**) were treated with glycosidases, PNGase F (N) for *N*-glycosylation or Endo H (O) for *O*-glycosylation, and then Dkk3 was detected by western blotting using anti-Dkk3 or anti-His-tag antibodies.

that mouse Dkk3 is glycosylated by N-linked glycans in the adrenal gland (Fig. 2C) as well as in the brain (Fig. 2C) and transfected cells (Fig. 2B). In the case of *Dkk3*-overexpressing cells, a band of approximately 30 kDa appeared after treatment with O-glycosidase (Fig. 2B), while it was not detected in using lysates from the brain and adrenal gland (Fig. 2C). This implies that endogenous O-glycans of the Dkk3 protein could not be cleaved under the present experimental condition or that *in vitro* overexpressed Dkk3 protein was cleaved artificially, which should be examined in the next study.

Dkk3 protein is lost in Dkk3 KO mouse tissues. As mentioned in the Materials and Methods section, we generated *Dkk3*-deficient mice, in which exons 5 and 6 in the *Dkk3* gene were deleted, using a different method than used in the previous studies [12, 17]. To confirm the loss of Dkk3 protein in the adrenal glands of *Dkk3* KO mice, western blot analysis was performed. The bands were detected in the adrenal gland of WT mice as well as in the heart and brain, while these bands were undetectable in the liver and tissues from the KO mice (Fig. 3A). This indicates that the Dkk3 protein is present in the adrenal gland of the WT, while it is lost in the KO mouse tissues. We also confirmed using immunohistochemistry that the Dkk3 protein was detected in the heart (atrium), brain (cerebral cortex) (Fig. 2B), and adrenal gland (medulla) of the WT mice (Fig. 2C) but not in those of the *Dkk3* KO mice.

Protein expression profiling of the adrenal gland from WT and Dkk3 KO mice. To determine whether protein expression profiling was altered in the mouse adrenal gland due to the absence of Dkk3, we generated 2D-PAGE profiles for the proteins from the WT and *Dkk3* KO adrenal glands and compared them. The

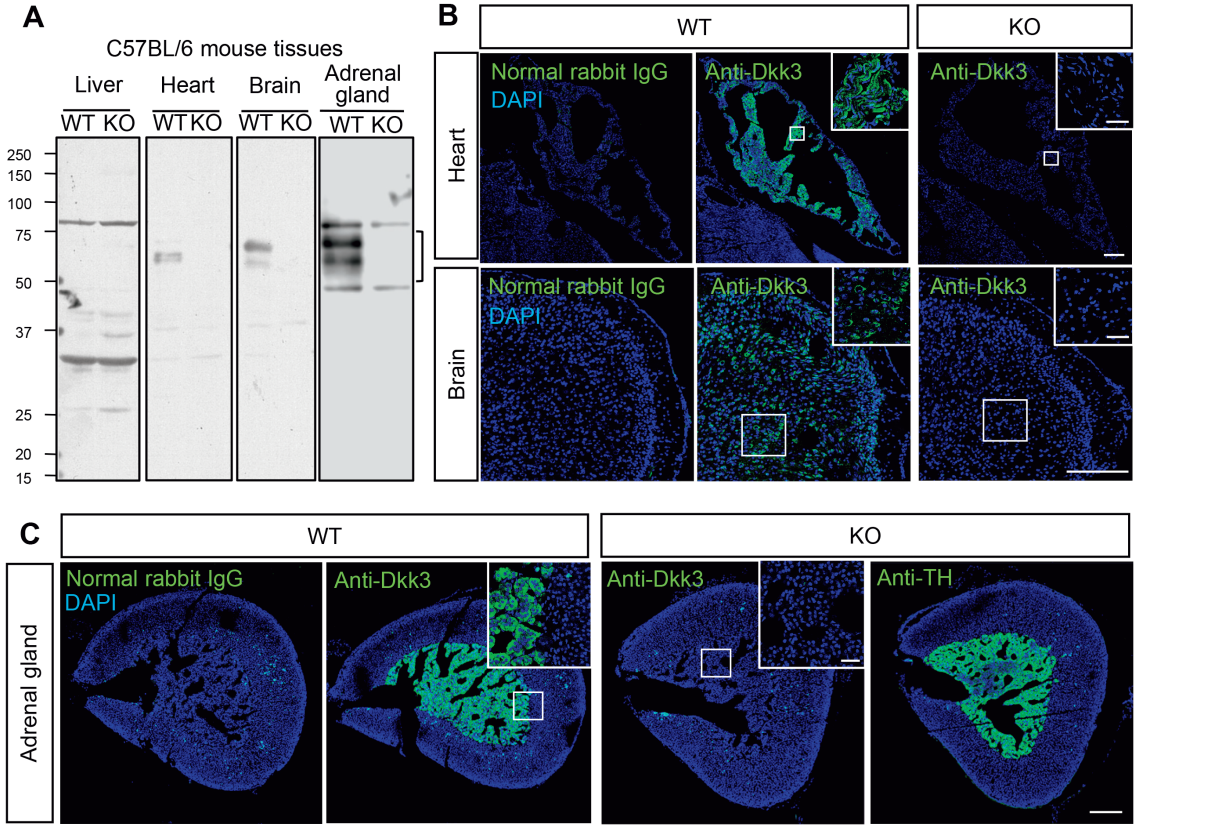

Fig. 3 Dkk3 protein is lost in the *Dkk3*-knockout mouse tissues. **A**, Dkk3 protein in the liver, heart, brain, and adrenal gland of wild-type (WT) and *Dkk3* knockout (KO) mice was examined by western blotting using an anti-mouse Dkk3 antibody; **B**, **C**, Localization of Dkk3 protein in the heart (atrium), brain (cerebral cortex) (**B**), and adrenal gland (**C**) of WT and *Dkk3* KO mice was examined by fluorescent immunostaining using anti-mouse Dkk3 antibody. Normal goat IgG was used as a negative control, and anti-tyrosine hydroxylase (TH) antibody was used to visualize adrenal medulla. Scale bars: 250 μm in (**B**, **C**); 50 μm in the insets of (**B**, **C**).

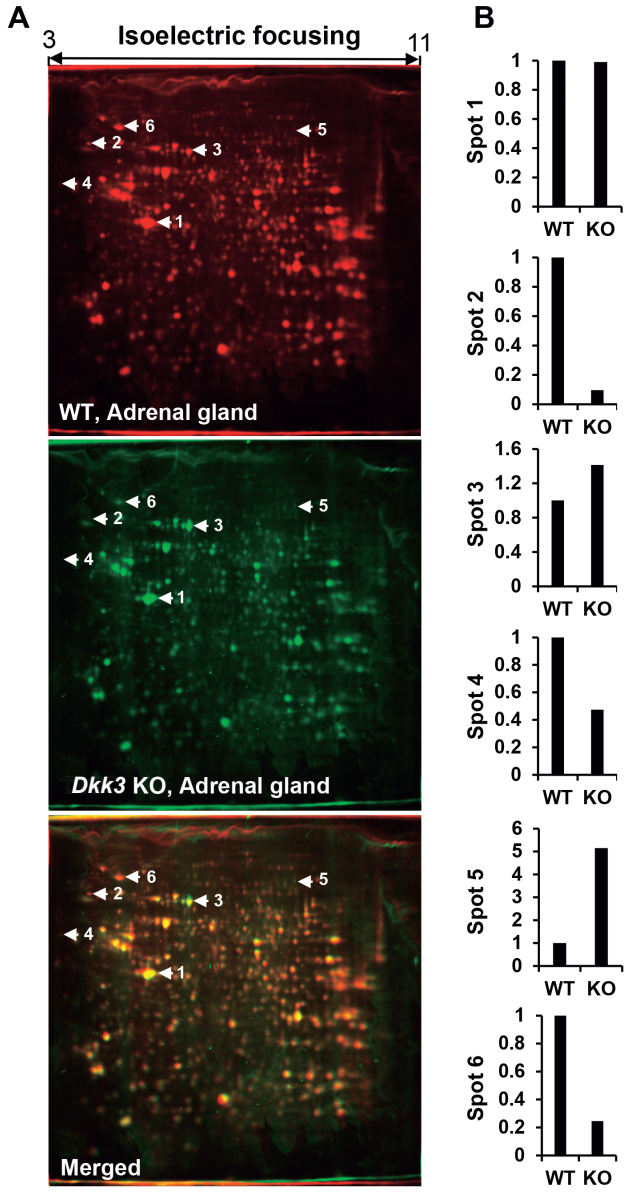

Fig. 4 Protein expression profiling in the adrenal glands of WT and *Dkk3* KO mice. **A**, Pseudo-colored images of adrenal gland proteins separated by 2D-PAGE in WT and KO mice. Protein extracts from the adrenal glands of WT and *Dkk3* KO mice were separated by 2D-PAGE and stained with Sypro Ruby. The images were generated and merged by using a gel documentation system and Photoshop software. Numbered arrows indicate spots differentially expressed in the adrenal glands of *Dkk3* KO mice; **B**, The ratios of protein spot density.

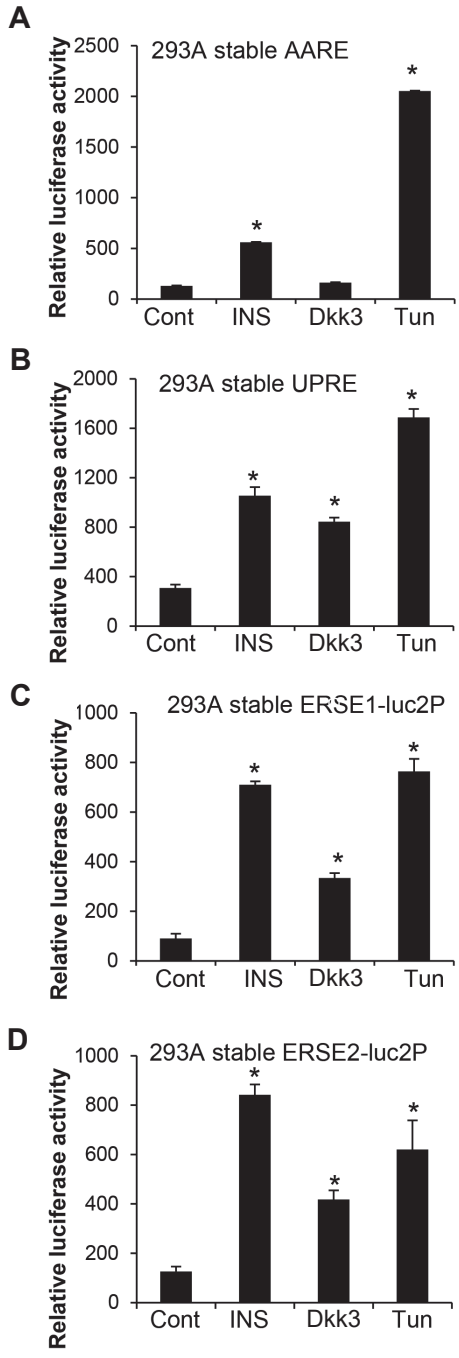

Fig. 5 Effect of *Dkk3* expression on the activities of ER stress-responsive elements. Reporter gene-stably expressing HEK293A cells were transfected with the pFLAG-mouse *Dkk3* vector 24 h prior to the assay. The activities of AARE (**A**), UPRE (**B**), ERSE1 (**C**), and ERSE2 (**D**) were measured. Data are shown as means ± standard deviation (n=4). Asterisks (*) indicate that the relative luciferase activities are significantly higher compared to those in the controls ($*p < 0.05$). Insulin mutant $Ins2^{C96Y}$ (INS) and tunicamycin (Tun) were used as positive controls of ER stress inducers.

Table 1 Summary of protein identification

	Protein Name	SWISS-PROT accession No.	pI	Mr	Score	Coverage
Spot 1	Actin, cytoplasmic 1	P60709	5.29	42052	432	34%
Spot 2	78 kDa glucose-regulated protein	P20029	5.07	72492	214	24%
Spot 3	Serum albumin	P07724	5.75	70700	376	24%
Spot 4	Tensin-3	Q5SSZ5	6.19	157031	19	1%
Spot 5	No hit	–	–	–	–	–
Spot 6	Heat shock protein HSP 90-beta	P11499	4.97	83571	266	19%

Protein spots indicated with arrows in Fig. 4A were excised from the gels and subjected to peptide mass fingerprinting. The identification was performed using a Mascot database search.

resultant 2D-PAGE profiles showed that the fluorescent intensities of 5 protein spots in *Dkk3* KO mice were changed compared to those in WT mice (Fig. 4A, B). These spots were selected, and their corresponding proteins were identified by mass spectrometry (Table 1). We found that the spot fluorescence intensities for the molecular chaperones 78 kDa glucose-regulated protein (GRP78/BiP) and heat shock protein (HSP) 90β were decreased in the adrenal gland of *Dkk3* KO mice.

Mouse Dkk3 expression induces ER stress via cis-acting elements UPRE, ERSE1 and ERSE2. GRP78/BiP is known to dislocate misfolded proteins for ubiquitination and degradation though the ER-associated degradation (ERAD) pathway [25, 26]. Therefore, we next sought to clarify whether and to what extent mouse Dkk3 induces ER stress. A luciferase assay for the activation of unfolded protein response (UPR) pathways was performed using stable transformants of HEK293A with luciferase reporter vectors containing *cis*-acting elements for either the amino-acid-regulatory element (AARE), the UPR element (UPRE), endoplasmic reticulum stress element 1 (ERSE1), or ERSE2, which are known to be activated in the ER stress response. As shown in Fig. 5, the UPRE, ERSE1 and ERSE2 *cis*-acting elements were activated by mouse *Dkk3* expression, and the levels of activation were comparable to those in the positive controls treated with tunicamycin and insulin mutant $Ins2^{C96Y}$ [22]. These results suggest that mouse Dkk3 induces ER stress and that the UPR pathways in ER stress induced by mouse Dkk3 are predominantly those involving ATF6 and IRE1. We also examined whether the UPR was activated after overexpression of mouse Dkk3 in the rat pheochromocytoma-derived cell line PC12 at the mRNA level (see Materials and Methods). Thus far, however, we have been unable detect significant mRNA expression for proteins involved in the ATF6 and IRE1 pathway (data not shown), possibly because of the low transfection efficiency, which should be optimized in the next study.

Discussion

Dkk3/REIC is currently utilized as an anti-tumor intervention for certain types of cancers and tumors. Although numerous studies have considered the anti-tumor actions of Dkk3 from a mechanistic point of view, since *Dkk3* is expressed in a regionally restricted manner during development and in adult tissues, studies on the physiological roles of this protein could provide additional clues toward its optimal medicinal use.

We here showed that, even in the physiological state, Dkk3 exerts effects on the protein expression of an ER stress-related molecule, GRP78/BiP, in the adrenal gland, and forced expression of mouse *Dkk3* in HEK293 cells affects two UPR pathways. It has previously been reported that ER stress inducers can cause apoptosis in PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla [27]. We therefore suggest that *Dkk3* may prevent tumor progression in the adrenal medulla by inducing ER stress-mediated apoptosis. Specifically, since Dkk3 is N-glycosylated *in vivo*, as shown in this study, disturbed glycosylation of Dkk3 may occur when its expression level becomes high and likely results in the UPR and cell death.

Recently, evidence of the importance of physiological ER stress has accumulated [26]. The pancreas, which produces large amounts of endocrine and exocrine secretory proteins, exhibits physiological ER stress [28]. It was also reported that physiological ER stress mediates the differentiation of fibroblasts [29]. IRE1α regulates the expression of the ER stress-related genes *Grp78/BiP*, *Grp94* and *Calreticulin* as well as the adap-

tive thermogenesis-related gene *Ucp1* in brown adipocytes [30]. Regulation of ER stress is essential for bone formation and astrocyte differentiation [31,32]. Thus, we suggest that Dkk3 could act as an intrinsic ER stressor regulating the physiological functions of mouse adrenal glands.

Physiological ER stressors such as glucagon-like peptide 1 (GLP-1) induce mild ER stress and an unfolded protein response without cell death in normal cells [33]. In contrast, overexpression of human *Dkk3* using an adenovirus vector strongly induces ER stress in cancer cells, resulting in cell death [6,7]. Additionally, the $Ins2^{C96Y}$ mutant, which cannot form disulfide bonds, has been shown to induce ER stress, leading to cell death in mouse insulinoma MIN6 cells [22]. As depicted in Fig. 2A, Dkk3 has a number of conserved cysteine residues that are essential for disulfide bond formation [8]. Therefore, overexpression of *Dkk3* mutants that cannot form disulfide bonds might induce a high level of ER stress and thereby be more effective for killing cancer cells, which should be examined in future studies.

In conclusion, a third *Dkk3*-knockout mouse line lacking exons 5 and 6 was reported herein. Although the resulting mice have residual exons encoding an N-terminal region of 145 amino acids in Dkk3, none of the Dkk3 proteins are present. The endogenous mouse Dkk3 is N-glycosylated in the adrenal gland as well as in the brain. The 2D-PAGE analysis showed that the ER stress-related protein GRP78/BiP is regulated by Dkk3 in the mouse adrenal gland. Dkk3 may act as a physiological ER stress inducer by way of the ATF6 and IRE1 pathways.

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