**D-Serine Mediates Cellular Proliferation for Kidney Remodeling**

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**Key Points**
- D-serine has a physiologic activity, besides its reported function as a biomarker of kidney disease.
- The plasma level of D-serine increases due to reduced urinary excretion from kidney in human living kidney donors.
- The increased level of D-serine, in turn, promotes kidney remodeling through mTOR for the functional compensation of decreased kidney function.

**Abstract**

**Background** D-serine, a long-term undetected enantiomer of serine, is a biomarker that reflects kidney function and disease activity. The physiologic functions of D-serine are unclear.

**Methods** The dynamics of D-serine were assessed by measuring D-serine in human samples of living kidney donors using two-dimensional high-performance liquid chromatography, and by autoradiographic studies in mice. The effects of D-serine on the kidney were examined by gene expression profiling and metabolic studies using unilateral nephrectomy mice, and genetically modified cells.

**Results** Unilateral nephrectomy in human living kidney donors decreases urinary excretion and thus increases the blood level of D-serine. D-serine is quickly and dominantly distributed to the kidney on injection in mice, suggesting the kidney is a main target organ. Treatment of D-serine at a low dose promotes the enlargement of remnant kidney in mouse model. Mechanistically, D-serine activates the cell cycle for tissue remodeling through an mTOR-related pathway.

**Conclusions** D-serine is a physiologic molecule that promotes kidney remodeling. Besides its function as a biomarker, D-serine has a physiologic activity that influences kidney function.

**Introduction**

D-amino acids are enantiomers of amino acids that were recently detected in humans (1–4). Although D-amino acids are present only in trace amounts, unlike abundant L-amino acids, associations between D-amino acids and diseases have been reported in patients with kidney diseases (1–4). The blood level of D-serine reflects GFR and prognosis of CKD, whereas a combination of blood and urine levels of D-serine is useful in the assessment of the activity of kidney diseases (2–4). D-serine may solve the fundamental problem, early diagnosis, and prediction of prognosis, lying in CKD, a condition with chronically reduced kidney function and a global health problem for >850 million patients (5,6).

Despite these facts, the physiologic function of D-serine in the kidney is relatively unclear (7–12). D-serine is a neurotransmitter that works via the N-methyl-D-aspartate receptors in the neurons (7), although it is also supposed to function in the kidney. Treatment with extremely high doses of D-serine has caused AKI in rodents (8). This suggests a direct effect of D-serine on the kidney at least at the supraphysiologic level; however, the physiologic function...
of D-serine in human disease (2,3) remains unknown. Additionally, a lack of key information, such as tissue distribution and mechanisms of action, has obscured the physiologic functions of D-serine.

In this study, we examined the function of D-serine at the physiologic level, and elucidated the homeostatic role of D-serine in kidney.

Materials and Methods

Clinical Study

We enrolled ten living kidney donors who underwent inulin clearance (clearance of inulin, Cin) tests in a single center before and after nephrectomy. Inulin clearance is the golden standard of GFR, the key kidney function. In these patients, inulin clearance was performed twice: before nephrectomy to examine the precise kidney function, and approximately 1 year after nephrectomy as a follow-up for remnant kidney function. We performed sample size calculation using a type I error of 5% and 90% of power. Supposing average Cin before nephrectomy is 80 ml/min per 1.73 m$^2$ with 20% SD, which is reduced by about 30% after nephrectomy as reported (13,14), the number of participants needed is seven. Given that blood ratio of D-/L-serine correlates well with Cin (3), we included ten participants in this study. The characteristics of these donors were summarized in Supplemental Table 1. Written informed consent was obtained from all participants. The study protocol was approved by the central ethics committee (the Ethical Review Board of Osaka University Hospital, 16281). This study was conducted in compliance with the ethical principles of the Declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Kidney Clearance of Inulin and Substrate, and Estimation Equations

The Cin procedure was performed using clinically standardized methods as described previously (15). Cin was calculated from serum and urine inulin concentrations and urine volume. In brief, three sets of serum and urine samples were collected at three different time points, during a 2-hour continuous intravenous infusion of 1% inulin given under fasting, medication-suspended, and hydrated conditions. The participants received 500 ml of water orally 30 minutes before the infusion. To maintain hydration, 60 ml of water was given at 30, 60, and 90 minutes after the start of inulin infusion. The initial rate of infusion was 300 ml/h for the first 30 minutes, followed by 100 ml/h for 90 minutes. Blood samples were collected at 45, 75, and 105 minutes after the initiation of inulin infusion. Participants were instructed to empty the bladder completely at 30 minutes after initiation of infusion. Then, urine samples were collected between 30 and 60 minutes, between 60 and 90 minutes, and between 90 and 120 minutes. The mean of the three Cin values was used as the Cin by standard method (Cin-ST). Cin (ml/min) was calculated from total urinary inulin excretion per time (mg/min) divided by blood level of inulin (mg/ml). Blood inulin is filtrated in the kidney glomeruli without reabsorption by tubules, and thus, Cin is the gold standard of GFR.

Fractional excretion (FE, %) was calculated from the clearance of substrate divided by creatinine clearance, as follows:

\[
FE \text{ of Substrate} = \frac{\text{Substrate clearance}}{\text{Creatinine clearance}} = \frac{Us \times Pcre}{Ucre \times Ps}
\]

where Us and Ps represent urine and plasma levels of substrate, respectively. FE is the ratio of a substrate filtered by the kidney glomeruli that is excreted in the urine. Serum and urine creatinine were measured enzymatically, and D-/L-serine was measured as aforementioned using the same sample. Low and high FE indicates tubular reabsorption and excretion, respectively.

Kidney clearance of substrate (ml/min) was calculated from total urinary substrate excretion per time (mg/min) divided by blood level of substrate (mg/ml). Kidney clearance of substrate is estimated by multiplying FE and inulin clearance on the basis of the FE formula considering the concept that creatinine clearance is a representative of GFR and is strongly associated with inulin clearance. Kidney clearance reflects the excretion of substrates into the urine, the result of glomerular filtration, tubular reabsorption, and/or secretion.

Sample Preparation for Two-dimensional High-performance Liquid Chromatography

Sample preparation for two-dimensional high-performance liquid chromatography (2D-HPLC) was performed as previously described with modification (16,17). In brief, 20-fold volumes of methanol were added to the sample and an aliquot (10 μl of the supernatant obtained from the methanol homogenate) was placed in a brown tube and used for 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD) derivatization (0.5 μl of the plasma was used for the reaction). After drying the solution under reduced pressure, 20 μl of 200 mM sodium borate buffer (pH 8.0) and 5 μl of fluorescence labeling reagent (40 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole in anhydrous acetonitrile [MeCN]) were added, then heated at 60°C for 2 minutes. An aqueous 0.1% (v/v) trifluoroacetic acid solution (75 μl) was added, and 2 μl of the reaction mixture was subjected to 2D-HPLC.

Determination of Amino Acid Enantiomers by Two-dimensional High-performance Liquid Chromatography

The enantiomers of serine were quantified using the 2D-HPLC platform, as previously described (16,17), with the shape-fitting algorithm (18). Briefly, the NBD-derivatives of the amino acids were separated from numerous intrinsic substances using a reversed-phase column (Singularity RP column, 1.0 mm inner diameter ×50 mm; provided by KAGAMI Inc., Ibaraki, Japan) with the gradient elution using aqueous mobile phases containing MeCN and formic acid. To separately determine the D- and L-forms, the fractions of the target amino acids were automatically collected using a multiloope valve, and transferred to the enantioselective column (Singularity CSP-001S, 1.5 mm i.d.×75 mm; KAGAMI Inc.). The mobile phases are the mixed solution of methanol-MeCN containing formic acid, and the fluorescence detection of the NBD-amino acids was carried out at
530 nm with excitation at 470 nm. The fluorescence detector utilizes two photomultiplier tubes to cover high and low ranges and enables simultaneous and accurate measurement of both abundant t-serine and trace d-serine in human samples. The blood level of d-serine was determined as μM or as a ratio over t-serine (2,10).

**Antibodies and Reagents**

Antibodies against phos pho-p70 S6 Kinase (Thr389) (9234; 1:2000 IB), p70 S6 Kinase (2708; 1:2000 IB), phospho-S6 ribosomal protein (56R; Ser235/236) (4858, 1:2000 IB), S6R (2217, 1:2000 IB), phospho-AKT (Ser473) (9271; 1:2000 IB), AKT (9272; 1:2000 IB), p18 LAMTOR1/C11orf59 (8975; 1:2000 IB), mTOR (2983; 1:1000 IF), HRP-labeled anti-mouse IgG (7076; 1:5000 IB), and HRP-labeled anti-rabbit IgG (7074; 1:5000 IB) were from Cell Signaling Technology (Danvers); Rheb (sc-271509; 1:2000 IB) and LAMP1 (sc-19992; 1:1000 IF) were from Santa Cruz Biotechnology (Dallas); β-actin (A-5316; 1:20,000 IB) was from Sigma-Aldrich (St. Louis); Ki67 (718071; 1:1 IHC) was from Nichirei Biosciences (Tokyo, Japan); and LAMP2 (ab25631; 1:200 IF) and 594-labeled secondary antibodies (A-21207, 1:1000 IF) were from Invitrogen (Carlsbad); and Alexa Fluor 488 (A-32790; 1:1000 IF) was from Invitrogen (Carlsbad); and Alexa Fluor 488 (A-32790; 1:1000 IF) was from Invitrogen (Carlsbad); and Alexa Fluor 488 (A-32790; 1:1000 IF) was from Invitrogen (Carlsbad); and Alexa Fluor 488 (A-32790; 1:1000 IF) was from Invitrogen (Carlsbad).

**Rodent Experiments**

C57BL/6 and BALB/c mice were purchased from SLC (Tokyo, Japan) and fed with normal food (FR-2, Funabashi Farm, Funabashi, Japan) before the experiments. In the d-serine treatment experiments, rodents were fed with a serine-free diet generated on the basis of either the FR-2 or serine treatment experiments, rodents were fed with a serine-free diet to exclude the effects of D-serine contained in a normal animal diet (10) and to assess the effects of D-serine directly (19).

**Autoradiographic Experiments**

t-serine and 3H-labeled d-serine were purchased from PerkinElmer (NET1092 and NET248, respectively, Waltham, MA) with specific radioactivities of 0.555–1.48 TBq/mmol. The 12-week-old Balb/c male mice were intravenously injected with 260 kBq (7.0 μCi) of 3H-labeled d-/t-serine dissolved in 0.1 ml normal saline. The mice were sacrificed and dissection was performed at the time indicated in the figure (Figure 2 and Supplemental Figure 2). The following organs were collected after perfusion with normal saline and frozen in liquid nitrogen: cerebrum, lung, heart, liver, pancreas, and kidney. The radioactivity count (cps) of each homogenized tissue was measured in 10 ml of Ecosint XR (National Diagnostics, Atlanta, GA) with a liquid scintillation counter LSC 5100 (Hitachi, Tokyo, Japan). The radioactivity concentrations were converted to kBq/g unit of measurement using the crosscalibration factor. Results were expressed as standardized uptake value, corrected for the injected dose (MBq) and body weight (g).

**Cell Culture**

HK-2 (CRL-2190, American Type Culture Collection, Manassas) and human RPTEC (CC-2553, Lonza, Basel, Switzerland) were cultured in the recommended media. HeLa cells (JCRB9004, JCRB Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Japan) and TIG-1 (spontaneously developed diploid fibroblast cell lines of fetal lung; JCRB0501, JCRB Cell Bank) cells were cultured in DMEM (08458–16, Nacalai Tesque, Kyoto, Japan) with 10% FCS (10270–106, Gibco, Carlsbad), p18-deficient mouse embryonic fibroblasts (MEF) (p18 knockout [KO]) and their revertants (p18 wt), and Rheb and p18 double-deficient MEF expressing p18 (Rheb KO) were described previously (21,22). In experiments using Rheb KO cells, p18 revertants were used as control (Rheb wild type). Cells were maintained at 37°C in a humidified chamber supplemented with 5% CO2.

For the proliferation assay, cells were seeded on 96-well plates using a culture medium. The numbers of cells per well were 1.5–6.0 × 103 for HK-2 and RPTEC, 1.25–3 × 103 for MEF, 6–9 × 103 for TIG-1, and 6–8 × 103 for HeLa. The next day, the culture medium was replaced with a serum-free medium (amino acid–free DMEM [048–33579], supplemented with MEM essential amino acids [132–15641], glycine [073–00732], and GlutaMax [L-Alanyl-L-Glutamine] [016–21841, Fujifilm Wako]) with 1% dialyzed FCS (26400–036, Gibco) with or without rapamycin (100 nM). After overnight incubation otherwise indicated in the legends, d- and/or t-serine were treated for the time indicated with or without Ly294002 (50 μM), and relative cell number was measured using a WST-8 kit (CK04, Dojindo Laboratories, Kumamoto, Japan) or counted manually. We preferred to examine them for a shorter treatment period for two reasons: (i) the reaction with d-serine was seen quickly, within 6 hours in cells and 1 day in kidney, and (ii) secondary reactions in response to primary reaction with t-serine may affect results after a longer observational period. For experiments using MEF, type I collagen-coated microplates (4860–010, AGC Techno Glass, Haibara, Japan) were used.

**Immunoblotting and Histology Study**

Immunoblots were performed as described previously (23). Briefly, cells were lysed in Radioimmunoprecipitation
buffer (89901, Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (4693132001, Roche, Basel, Switzerland) with or without phosphatase inhibitor (4906837001, Roche) for 1 hour on ice and centrifuged at 15,000 × g for 10 minutes. Supernatants were boiled for 3 minutes in SDS-PAGE gel loading buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were subjected to western blot analysis.

Histologic analysis was performed by a nephrologist, blinded for experimental conditions as previously described (20). Briefly, mice were perfused using saline, and removed kidneys were sectioned, postfixed with 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were stained with Periodic acid-Schiff (PAS) and at least ten fields (×400) of PAS-stained sections for each mouse were reviewed. After counting the numbers of tubules and glomeruli manually, the glomerular area was measured by the algorithm (24), and the tubular area and cell numbers were measured outside the glomerular area (25). Tubular area and cell numbers were demonstrated per tubule. Kidney section was also immunostained with an anti-Ki67 antibody and Histofine Simple Stain Rat MAX PO(R) (414181F, Nichirei Biosciences). For counting Ki67-positive nuclei in proximal tubules per view, at least ten fields (×200) were reviewed. For immunofluorescence, paraffin sections were incubated with Ki67 antibody and Alexa594-labeled secondary antibody. FITC-conjugated Lectin from Trichium vulgaris (L4895–5MG, Sigma-Aldrich) were used to stain the proximal tubules, and nuclei were counterstained with DAPI. Images were captured using a fluorescence microscope (Axio Observer) and digital cameras (AxioCam MRC, ZEISS, Oberkochen, Germany). All images were processed using ZEN pro software (ZEISS) and Image J (National Institutes of Health).

**High-content Imaging Analysis**

Immunofluorescence staining was performed as previously described with modification (23,26). Briefly, HK-2 cells were seeded on SCREENSTAR microplates (655866, Greiner Bio-One, Kremsmunster, Austria) and stimulated using serine for 10 minutes in an amino acid-free assay medium. Cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Fixed specimens were incubated in methanol at room temperature for 10 minutes, then treated with 50 μg/ml digitonin (D141, Sigma-Aldrich) in PBS (digitonin-PBS) at room temperature for 10 minutes for permeabilization. After a 1 hour block with Blocking-One (Nacalai Tesque), specimens were incubated with primary antibodies at room temperature for 1 hour, washed three times with digitonin-PBS, incubated with Alexa488- or Alexa594-labeled secondary antibodies at room temperature for 1 hour, and washed four times with digitonin-PBS. Finally, specimens were incubated with CellMask, deep red plasma membrane stain (fluorescence excitation/emission maxima: 649/666 nm, C10046, Invitrogen, Carlsbad), at 37°C for 10 minutes, washed three times with PBS, and immersed in PBS for observation.

High-content imaging analysis was performed using CellVoyager CV7000 (Yokogawa electric corporation, Tokyo, Japan). Confocal images were collected automatically from 32 fields per well (>1500 cells for HK-2 and >500 cells for MEF) using a 60× objective. CellMask was excited at 640 nm, and fluorescence was recorded at 676/29 nm; Alexa Fluor 488 was excited at 488 nm, and fluorescence was recorded at 525/20 nm; Alexa Fluor 594 was excited at 561 nm, and fluorescence was recorded at 600/37 nm. CellMask staining was used to detect cellular outlines. The overlapping area corresponding to colocalization between mTOR and LAMP1 was analyzed using CellPathFinder (Yokogawa). Briefly, we set cutoff values for each fluorescence signal intensity. Then, the colocalized area was identified as both LAMP1- and mTOR-positive regions.

**RNA Sequencing**

RNA was extracted using TRIzol (15596018, Thermo Fisher Scientific, Waltham). Library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA) according to the manufacturer’s instructions. Sequencing was performed on an Illumina HiSeq 2500 platform in a 75-base single-end mode. Illumina Casava1.8.2 software was used for base calling. The sequenced reads were mapped to the mouse reference genome sequences (mm10) using TopHat version 2.0.13 in combination with Bowtie2 version 2.2.3 and SAMtools version 0.1.19. The fragments per kilobase of exon per million mapped fragments were calculated using Cufflinks version 2.2.1. Raw sequencing data are available from Gene Expression Omnibus under accession number GSE155475.

**Enrichment Analysis of Biologic Pathways and Gene Ontology Terms**

To interpret RNA sequencing data and elucidate the pathways involved, we utilized TargetMine (27), an integrated warehouse of human and mouse biologic data, etc. from data sources such as Reactome and KEGG. TargetMine features an ontology-based data model and a user-friendly web interface that facilitates the identification of characteristic pathways and biologic processes. The genes upregulated in D-serine treated mice over vehicle-treated mice with a two-fold cutoff changes in average values were uploaded to TargetMine. Enrichment of the Reactome pathway and Gene Ontology terms (28) was estimated by hypergeometric distribution and the inferred P values, which were further adjusted for multiple test corrections to control the false discovery rate using the Benjamini and Hochberg procedure (29). Heatmap was drawn using R.

**Quantitative RT-PCR**

Quantitative PCR was performed as previously described (30). In brief, samples were reverse transcribed into cDNA using PrimeScript RT Reagent Kit (RR037A, Takara Bio, Kusatsu, Japan). Quantitative PCR was performed using GeneAce SYBR qPCR Mix α Low ROX (316-07693, Nippon gene, Tokyo, Japan) and QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Carlsbad). mRNA levels of target genes were normalized to β-actin RNA and data were presented as fold-change relative to control levels. The sequences of the following primers were used: Cyclin A2: 5'-GCCTGAGTCATTGGCA-CAAC-3' (forward), 5'-TGTTGTCGCCTTGAAGTA-3' (reverse), Cyclin B1: 5'-TCTCCAAGCGGATGGAAAC-3'.
Blood level of d-serine increases in living kidney donors after nephrectomy. (A–D) Inulin clearance (A), plasma levels of d- (B) and l-serine (C), and plasma ratio of d-serine to total serine (D) of living kidney donors were measured before (pre) and after nephrectomies (post). n=10; statistics, paired two-tailed Student’s t test. *P<0.05, **P<0.01, ***P<0.001. D-Ser, d-serine; L-Ser, l-serine.

Results

Blood Level of d-serine Increases in Living Kidney Donors after Nephrectomy

To examine the effects of d-serine in a clinically relevant situation, we focused on UNX, a surgical procedure to remove a kidney. UNX is often performed in living kidney donors, and preserving GFR after nephrectomy is very important to avoid kidney failure of the donors (31). After UNX, the remnant kidney enlarges itself to compensate partially for reduced GFR (14). Expectedly, UNX reduced inulin clearance, gold standard of GFR, and increased the blood levels of creatinine and cystatin C (Figure 1A, Supplemental Figure 1, A and B).

We measured levels of d-serine in the blood and urine of living donors using the 2D-HPLC system (16,17) with a multichannel fluorescence detector. After nephrectomy, the plasma level of d-serine increased with marginal statistical significance (P=0.057), and the ratio of d-serine over total serine, a frequently used index that also reflects kidney function (2,32), increased (Figure 1, B–D). The plasma ratio of d-serine correlated well with GFR, as expected, like creatinine and cystatin C (Figure 1A, Supplemental Figure 1, C and D).

d-serine reaches the kidney through the blood flow, then about half is excreted into urine whereas the rest is reabsorbed in the proximal tubules (3,33,34). The kidney clearance of d-serine (i.e., the speed of urinary excretion of d-serine) was greatly reduced after UNX (median, 42.6, interquartile range, 36.9–53.7 before UNX, versus 27.2, interquartile range, 20.9–37.7 after UNX; Figure 1, F–H,
Supplemental Figure 1, E and F). The blood level of D-serine increases in living kidney donors due to decreased urinary clearance of D-serine.

D-serine Accumulates in The Kidney

We then aimed to determine the D-serine tropic organs. We hypothesized that D-serine exerts its key function in organs that uptake more D-serine. We injected mice with 3H-labeled D- or L-serine intravenously and followed its chiral-selective tissue distribution. The 3H–D-serine was broadly distributed in the tissues studied. Each tissue showed a specific uptake of 3H–D-serine. Among tissues, the kidney accumulated 3H–D-serine most abundantly and quickly on injection (Figure 2, A and B, Supplemental Figure 2). These dynamics were chiral selective, and 3H–L-serine accumulated in the kidney less abundantly. The kidney was deemed the potential target organ of D-serine.

D-serine Promotes Kidney Enlargement and Cellular Proliferation

We wondered whether D-serine exerts physiologic function in the remnant kidney after UNX. To examine this, we used the UNX mice model. Nephrectomy induces enlargement of the remnant kidney in mice (Figure 3A, Supplemental Figure 3A), as seen in humans (14), whereas the plasma D-serine ratio increased in UNX mice compared with sham-operation mice (Figure 3, B–D).

Next, we examined whether increased level of D-serine affects the process of kidney enlargement after UNX. To examine this, we treated UNX mice with a low dose of D-serine to accelerate its effects on the kidney. We chose a
low dose of d-serine for this purpose, because this level of d-serine, treated through free access to water containing d-serine, was neither lethal nor toxic to the kidney (Supplemental Figure 3, B–E), unlike higher doses of d-serine treated orally or intraperitoneally (Supplemental Figure 3, F–I) (8). Also, d-serine accelerated the nephrectomy-induced enlargement of the remnant kidney (Figure 4A). Under this condition, the blood level of d-serine moderately increased (mean, 19.05 ± 2.28 and 49.91 ± 14.12 μM for d-serine–treated sham and UNX mice, respectively; Figure 4, B and C) without adverse effects on the kidney (Figure 4, D and E). Thus, a low dose of d-serine had a potency to mediate kidney enlargement.

We further examined the effects of d-serine on kidney remodeling. Profiling of gene expression using RNA sequencing and pathway enrichment analysis in the kidney of UNX mice revealed dominant activation of the cell cycle–related pathways by d-serine treatment (Figure 5, A–E).

![Figure 3](image-url)

**Figure 3.** Blood level of d-serine increase in mice after unilateral nephrectomy. (A–D) 10-week-old mice were fed with a serine-free diet for 1 week, subjected to either unilateral nephrectomy (UNX) or sham operation, and then sacrificed 2 days after operation. Kidney per body weight (A), plasma ratio of d-serine to total serine (B), plasma levels of d- (C) and l-serine (D). n = 6 (A) and 8 (B–D); statistics, unpaired two-tailed Student’s t test. **P < 0.01. Data, mean ± SEM.

![Figure 4](image-url)

**Figure 4.** d-serine promotes kidney enlargement after unilateral nephrectomy. (A–E) Mice were fed with a serine-free diet and water with or without 0.1% d-serine for 1 week, subjected to either UNX or sham operation, and then sacrificed 2 days after operation. Kidney weight per body weight (A), plasma levels of d- (B), and l-serine (C), and plasma levels of urea nitrogen (D) and creatinine (E). n = 6–7; statistics, two-way ANOVA. (A) *P < 0.05 for main effect of d-serine; ***P < 0.001 for main effect of operation; (B) *P < 0.05 for interaction effect; (C) *P < 0.05 for main effect of operation; (D) *P < 0.05 for main effect of operation. *P < 0.05, **P < 0.01, ***P < 0.001. Data, mean ± SEM.
and B). Upregulated genes included cyclins, the cell cycle–activating family proteins, and Cdc20, the key regulator of mitotic exit through the degradation of cyclins (35) (Figure 5, C and D), and these results were replicated by quantitative PCR (Figure 5E, Supplemental Figure 4A). Histologically, although the tubular changes were difficult to detect in the analysis of PAS-stained kidney, d-serine promoted cellular proliferation as indicated by the increased number of Ki67-positive nuclei in kidney tubules of d-serine-treated UNX mice compared with vehicle-treated mice (Figure 6, A and B, Supplemental Figure 4, B and C). Cellular proliferation was seen in the proximal tubules of the kidney (Figure 6C), the main regions of d-serine reabsorption (33,34). The glomerular area tended to be enlarged on d-serine treatment, although statistically insignificant (Supplemental Figure 4D). Overall, d-serine promotes...
kidney enlargement and cellular proliferation after nephrectomy.

D-serine Activates Cellular Proliferation via an mTOR-related Pathway

We wondered whether D-serine’s capacity of cellular proliferation is chiral selective. We tested this hypothesis in cell culture, with the culture medium including human blood D-serine levels. Blood contains about 100 μM of serine, and the reported median values of blood D-serine levels were 1.56 and 2.39 μM in non-CKD and CKD, respectively, with the maximum value in patients with CKD <17 μM (2,3). Unneglectable levels of D-serine were detected in reagents (Figure 7, A–C). The tested L-serine reagent contained 1% D-serine (Figure 7C). Several D-amino acids were found in quantities up to 10 μM in FCS and were below the detection limit after dialysis (Figure 7A).

To ascertain the low levels of D-serine in the culture medium, we used serine-free culture media and conducted experiments by adding corresponding levels of D-serine. On treatment, D-serine promoted cellular proliferation at low concentrations in human kidney tubular cell lines and primary cells (Figure 8, A and B, Supplemental Figure 5, A–F). The chiral-selective cellular proliferative effects of serine varied depending on cell lines and passages, and kidney cells were likely to favor D-serine (Supplemental Figure 5, G–I).
Then, we examined how kidney responses to D-serine with proliferation. Some studies suggested that mTOR plays a role in remnant kidney enlargement (36). The mTOR pathway senses L-amino acids and regulates cellular proliferation and metabolism through the kinase activity targeting such as S6K, which subsequently phosphorylates S6RP (37,38). Using p18/LAMTOR1 as an essential anchor (26), the mTOR-raptor-containing protein complex (mTORC1) localizes at lysosomes and integrates signals from amino acids and insulin/PI3K/AKT/Rheb (37), although the potential effect of D-serine on the mTOR pathway remain uncertain. We examined these effects in the presence of other amino acids, because we observed the proliferative effects of D-serine under this condition. Addition of D-serine on other amino acids augmented Thr389-phosphorylation of S6K in HK-2 cells (Figure 8C). In line with this, treatment with D-serine induced Ser235/236-phosphorylation of S6RP in the kidney of UNX mice (Figure 8D). Of note, D-serine alone was insufficient for Thr389-phosphorylation of S6K in the presence of other amino acids, because we observed the proliferative effects of D-serine under this condition. Addition of D-serine on other amino acids augmented Thr389-phosphorylation of S6K in HK-2 cells (Figure 8C). In line with this, treatment with D-serine induced Ser235/236-phosphorylation of S6RP in the kidney of UNX mice (Figure 8D). Of note, D-serine alone was insufficient for Thr389-phosphorylation of S6K in the absence of other amino acids (Supplemental Figure 6, A and B), suggesting D-serine augments signals from L-amino acids to the activation of mTORC1. The effect of D-serine on the mTOR pathway is crucial for cellular proliferation, as demonstrated in p18 deficiency, where D-serine–induced cellular proliferation was suppressed (Figure 8, E and F, Supplemental Figure 6, C and D). The effect of D-serine was also mediated through insulin/PI3K signaling, because Rheb deficiency suppressed the proliferative effects of D-serine (Figure 8, E and G, Supplemental Figure 6, E and F).

Inhibition of mTOR using rapamycin or Ly294002, a PI3K inhibitor, also suppressed D-serine–induced cellular proliferation (Supplemental Figure 6, G and H). Consistently, treatment with D-serine induced Ser473-phosphorylation of AKT in UNX mice kidneys (Supplemental Figure 6I). We examined the effect of D-serine on lysosomal localization of mTOR. Because D-serine alone was insufficient to activate mTORC1, we chose wash-out conditions of amino acids, and examined if D-serine can suppress the dissociation of mTOR from lysosome. Treatment with D-serine under amino acid starvation suppressed the dissociation of mTOR from the lysosome (Figure 8H, Supplemental Figure 6J) (26), suggesting that D-serine assists the lysosomal localization of mTOR for activation. Overall, D-serine and mTOR cooperate for cellular proliferation (Figure 8I, Supplemental Figure 6K).

**Discussion**

In this study, we identified the physiologic functions of D-serine (Figure 8I). The kidney is a target organ of D-serine, and a regulator of D-serine levels in the body, as observed in human living kidney donors. Increased level of D-serine after nephrectomy promoted the remodeling of the remnant kidney, which is a compensatory process for reduced kidney function. D-serine exerted these functions through the activation of the mTOR pathway. These findings provide guidance for the application of D-serine in the treatment of kidney diseases.
It is plausible the kidney is the target and the regulator of d-serine. Given the level of d-serine reflects kidney function, elevated levels of d-serine can mediate information about decreased kidney function to the remnant kidney. d-Serine promotes kidney remodeling, and the enlarged remnant kidney can increase the urinary clearance of d-serine. This, in turn, suppresses the further increase of d-serine level in the blood and remodeling. Thus, d-serine may promote kidney remodeling, and the enlarged remnant kidney can increase the urinary clearance of d-serine.

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promote and regulate the hypertrophic response of the kidney. D-serine mediated kidney enlargement through the proliferation of Ki67-positive tubular cells and potentially through glomerular enlargement. The tissue-remodeling effect of D-serine may not be limited to living kidney donors. Increased level of D-serine in patients with kidney diseases can promote kidney remodeling to support the damaged or ailing parts of the kidney. D-serine emerged as a physiologic molecule and a biomarker of kidney diseases.

D-serine transmits cellular signaling and modifies tissue functions. The mode of D-serine’s action identified in this study include cellular proliferation and mTOR activation. These functions of D-serine have not been studied, given D-serine has long been deemed nonexistent. The mTOR pathway is regarded as a sensor of L-amino acids, and from this study we now know the mTOR pathway also senses D-amino acids. Because genetic ablation of either p18 or Rheb was sufficient to suppress D-serine–dependent mTOR activation, D-serine activates mTORC1 through signals from both L-amino acids and PI3K/Rheb. The combination of amino acids is necessary for the full activation of mTORC1 (39), and D-serine is likely to assist mTORC1 activation as a key constituent of amino acids.

Of note, these reactions of D-serine were biphasic; higher levels of D-serine did not show these effects (for example, Supplemental Figure 5D). Instead, D-serine was toxic at higher doses (Supplemental Figure 3, F–I), corresponding with the reported toxic effect of D-serine at higher dose (8,11). D-serine also possessed affinity for certain organs and cell lines. As observed in this study, kidney and kidney-derived cell lines favored D-serine to L-serine, whereas cell lines such as HeLa (epithelial cell derived from cervical cancer cells) and TIG-1 cells (normal diploid fibroblast from lung that was established naturally without artificial immortalization) (40) favored L-serine but still responded to D-serine (Supplemental Figure 5, C–I). Cellular senescence affected the reaction to D-serine, as observed in old TIG-1 cells (Supplemental Figure 5, H and I). Additionally, non-negligible levels of D-amino acids were present in reagents and serum used for cell culture (Figure 7, A–C). The fact that low doses of D-serine was enough to provoke cellular response, some of the scientific findings reported up to now may be, at least in part, explained by the contents of D-amino acids in the reagents. Our findings may explain why the physiologic effects of D-serine has been overlooked.

D-serine may provide a therapeutic option for several diseases. Because D-serine mediates compensatory remodeling of the kidney, kidney living donors and patients with CKD may benefit from elevated D-serine levels. The fact that the addition of D-serine in UNX model mice promoted kidney enlargement may suggest the therapeutic potential of D-serine in this condition. Basically, D-serine is an endogenous molecule synthesized through a racemic reaction in brain (41) and by gut microbiota (10). D-serine is also present in food, and the dynamics of D-serine in the body is balanced between oral intake, synthesis, and urinary excretion (4). Because D-serine is a physiologic molecule, it may be applicable in kidney diseases as far as attention is paid to the biphasic reaction of D-serine, namely, the toxic effect of D-serine at extremely high doses.

In conclusion, this study revealed that D-serine has a dual function as a physiologic molecule and a biomarker of kidney function, and promotes tissue homeostasis through the regulation of kidney function. A trace amount of D-serine was sufficient to mediate a tremendous effect on tissue remodeling. Investigating the unexplored functions of D-amino acids will shed light on a new concept of pathophysiology and therapy.

Disclosures
A. Hesaka reports having an ownership interest in KAGAMI Inc. M. Mita and M. Nakane are co-founders of KAGAMI Inc., a startup company working on chiral amino acids analysis and research for medical application. T. Kimura reports having an ownership interest in KAGAMI Inc.; reports receiving research funding from Kyowa Hakko Kirin Co., Ltd., Shiseido Co., Ltd., and KAGAMI Inc. T. Kimura and M. Mita are preparing patent application which is related with this work. Y. Isaka reports receiving research funding from Bayel Yakuhin Ltd., Chugai Pharmaceutical Co. Ltd., Kissei Pharmaceutical Co. Ltd., Kyowa Kirin Co. Ltd., Mitsubishi Tanabe Pharma, Otsuka Pharmaceutical Co. Ltd., and Teijin Ltd.; and reports speakers bureau from Bayel Yakuhin Ltd., Chugai Pharmaceutical Co. Ltd., Kissei Pharmaceutical Co. Ltd., Kyowa Kirin Co. Ltd., Mitsubishi Tanabe Pharma, Otsuka Pharmaceutical Co. Ltd., and Teijin Ltd. All remaining authors have nothing to disclose.

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Author Contributions
T. Kimura conceptualized the study; A. Hesaka, T. Kimura, D. Okuzaki, M. Nakane, and Y. Tsukamoto were responsible for data curation; A. Hesaka, T. Kimura, M. Mita, M. Nakane, and D. Okuzaki were responsible for formal analysis; A. Hesaka, N. Ichimaru, T. Kimura, M. Mita, S. Nada, S. Sakai, and Y. Tsukamoto were responsible for the investigation; A. Hesaka N. Ichimaru, M. Kawamura, T. Kimura, M. Mita, D. Okuzaki, and Y. Tsukamoto were responsible for the methodology; Y. Isaka, T. Kimura, and M. Okada were responsible for project administration; T. Kimura and S. Nada were responsible for the resources; Y. Isaka and T. Kimura provided supervision; A. Hesaka, N. Ichimaru, M. Kawamura, T. Kimura, Y. Isaka, S. Nada, M. Okada, and S. Sakai were responsible for validation; and A. Hesaka and T. Kimura were responsible for visualization, wrote the original draft, and reviewed and edited the manuscript.

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Supplemental Material
This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl?doi:10.34067/KID.0000832021/-/DCSupplemental.

Supplemental Table 1. Characteristics of the participants.
Supplemental Figure 2. d-serine accumulates in the kidney.
Supplemental Figure 3. Effects of d-serine on unilateral nephrectomy in mice.
Supplemental Figure 4. d-serine activates cell cycle in kidney.
Supplemental Figure 5. d-serine activates cellular proliferation.
Supplemental Figure 6. d-serine activates mTOR-related pathway.

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