Osmoregulation of ceroid neuronal lipofuscinosis type 3 in the renal medulla

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Stein CS, Yancey PH, Martins I, Sigmund RD, Stokes JB, Davidson BL. Osmoregulation of ceroid neuronal lipofuscinosis type 3 in the renal medulla. Am J Physiol Cell Physiol 298: C1388–C1400, 2010. First published March 10, 2010; doi:10.1152/ajpcell.00272.2009.—Recessive inheritance of mutations in ceroid neuronal lipofuscinosis type 3 (CLN3) results in juvenile neuronal ceroid lipofuscinosis (JNCL), a childhood neurodegenerative disease with symptoms including loss of vision, seizures, and motor and mental decline. CLN3p is a transmembrane protein with undefined function. Using a Cln3 reporter mouse harboring a nuclear-localized bacterial β-galactosidase (β-Gal) gene driven by the native Cln3 promoter, we detected β-Gal most prominently in epithelial cells of skin, colon, lung, and kidney. In the kidney, β-Gal-positive nuclei were predominant in medullary collecting duct principal cells, with increased expression along the medullary osmotic gradient. Quantification of Cln3 transcript levels from kidneys of wild-type (Cln3+/+) mice corroborated this expression gradient. Reporter mouse-derived renal epithelial cultures demonstrated a tonicity-dependent increase in β-Gal expression. RT-quantitative PCR determination of Cln3 transcript levels further supported osmoregulation at the Cln3 locus. In vivo, osmoresponsiveness of Cln3 was demonstrated by reduction of medullary Cln3 transcript abundance after furosemide administration. Primary cultures of epithelial cells of the inner medulla from Cln3lacZ/lacZ (CLN3p-null) mice showed no defect in osmolyte accumulation or taurine flux, arguing against a requirement for CLN3p in osmolyte import or synthesis. CLN3p-deficient mice with free access to water showed a mild urine-concentrating defect but, upon water deprivation, were able to concentrate their urine normally. Unexpectedly, we found that CLN3p-deficient mice were hyperkalemic and had a low fractional excretion of K+. Together, these findings suggest an osmoregulated role for CLN3p in renal control of water and K+ balance.

Batten disease; juvenile neuronal ceroid lipofuscinosis; osmolytes; kidney; polyuria; potassium

THE JUVENILE FORM OF NEURONAL CEROID LIPOFUSCINOSIS

The juvenile form of neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is caused by recessive inheritance of mutations in the ceroid neuronal lipofuscinosis type 3 (CLN3) gene (reviewed in Refs. 25 and 32). Symptoms typically manifest in children between 5 and 7 yr of age and present with visual problems that proceed to blindness. Other manifestations include seizures and deterioration of cognitive and motor skills, with death commonly occurring in young adulthood. Progressive photoreceptor cell loss occurs (13), as does widespread neuronal cell loss in the brain, with prominent loss of neurons in parts of the cortex and hippocampus (4, 54). A diagnostic feature of JNCL is the cumulative buildup of autofluorescent lipopigment within lysosomes in many cell types (49).

CLN3p is a hydrophobic protein without significant homology to other proteins, and its molecular function remains elusive (reviewed in Ref. 40). CLN3p is predicted to contain six membrane-spanning regions and has been variously localized to lysosomes, endosomes, and Golgi in nonneuronal cells and in endosomes and vesicles along neurites and at synapses in neurons. Studies in CLN3p-deficient murine cells or patient cell lines or yeast cells deficient in Btn1p (the CLN3p ortholog) suggest that CLN3p may influence a variety of cell functions, including endocytosis (10, 18, 29), vesicular trafficking (9, 11), vacuolar (37) or lysosomal (21) pH homeostasis, arginine import (9, 42), galactosylceramide transport (45), bis(monoacylglycerol)phosphate synthesis (22), palmitoyl-protein desaturation (33), apoptosis resistance (39), and autophagy (8). From this diverse set of altered functions, it is not clear whether CLN3p performs a molecular function that impacts multiple pathways or performs distinct functions in different cell types and/or in different subcellular compartments.

Low endogenous expression levels and a lack of reliable CLN3p-specific antibodies have limited progress using traditional cell biology approaches. Our laboratory generated a Cln3 reporter mouse that allows us to acquire information regarding temporal and regional Cln3 expression (14). In this knock-in mouse, the recombinant allele contains a nuclear-localized bacterial β-galactosidase (β-Gal) transgene and SV40 polyadenylation sequences replacing Cln3 sequences from exon 1 to intron 8. β-Gal expression is thus directed by endogenous Cln3 promoter elements. CLN3p is not expressed from the recombinant allele, and homozygous reporter (Cln3lacZ/lacZ) mice are CLN3p-null. As previously reported (14), in adult brain we detect moderate levels of β-Gal expression in granule neurons of the dentate gyrus of the hippocampus and in endothelial cells of the microvasculature. In the present study, we find that, outside the central nervous system (CNS), reporter protein is evident in several tissues, notably skin, lung, colon, and, most strikingly, the inner medulla (IM) of the kidney. No kidney phenotype has, to our knowledge, been reported. However, it is very possible that mild phenotypes may go unnoticed. Moreover, while function may be readily compensated in the kidney, this may not be the case for the CNS. It is possible that neurons, because of their dependence on interconnectivity and lack of regenerative capacity, are more sensitive to effects of CLN3 deficiency.

The kidney medulla harbors a unique environment; nowhere else in the mammalian system does osmolality exceed 1,500 mosmol/kgH2O and P02 reach 10 Torr. The osmotic gradient owes largely to increasing concentrations of interstitial NaCl and urea from the corticomedullary border to the tip of the IM, or papilla (IM/papilla). This hypertonic medulla is absolutely essential for water reabsorption across the epithelial cells of the medullary collecting ducts and, thus, the excretion of concentrated urine. This hypertonic milieu is not static; the osmolality can change depending on the long-term state of hydration (48). With water deprivation or antidiuresis, interstitial osmolalities

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around the collecting duct cells of the IM can surpass 2,000 mosmol/kgH₂O in rodents (1). In contrast, animals undergoing water diuresis (or those given loop diuretics such as furosemide) have a substantially less hypertonic medulla. The osmolality of the renal cortex is similar to that of other tissues; osmolality is relatively constant (280–300 mosmol/kgH₂O), even under physiological extremes of water balance.

To maintain osmotic equilibrium with hypertonic surroundings, renal medullary cells upregulate the expression of proteins involved in the synthesis or import of nonperturbing organic osmolytes (reviewed in Refs. 19, 20, 34). These solutes allow cells to maintain a hypertonic intracellular environment, yet maintain normal concentrations of ions such as Na⁺ and K⁺. Renal medullary cells, which have adapted to their hypertonic environment, have an abundance of five organic osmolytes: sorbitol, inositol, glycerophosphorylcholine (GPC), betaine, and taurine. Sorbitol and GPC are synthesized intracellularly, betaine and taurine generally require transport, and inositol can be synthesized or transported. Cells outside the renal medulla, while not subjected to extreme fluctuations in osmolality, nonetheless utilize similar mechanisms for cell volume control. In the brain, taurine is present in substantial concentrations and plays critical roles as a neuroprotectant (47), as well as an osmolyte (23, 36). Moreover, taurine is crucial for photoreceptor development and maintenance in the retina (43).

The significance of Cln3 expression in non-CNS organs in mammals has not, to our knowledge, been explored. Given the remarkable gradient of β-Gal expression in the kidneys of our Cln3 reporter mice, we hypothesized that Cln3 expression is osmoregulated and that CLN3p plays a role in osmolyte accumulation. We found Cln3 expression to indeed correlate with osmolality. However, CLN3p-deficient cells displayed normal osmolyte accumulation. Interestingly, evaluation of mice for water intake and blood/urine chemistries showed enhanced water consumption, high serum K⁺, and reduced fractional excretion of K⁺ in CLN3p-deficient mice compared with controls. These findings suggest roles for CLN3p in water reabsorption and K⁺ excretion by the kidney.

**MATERIALS AND METHODS**

**Animal maintenance.** The Cln3 reporter mouse was generated as previously described using targeted recombination, and recombinant mice were backcrossed to C57/BL/6J for ≥17 generations before use in experiments (14). For this study, offspring of Cln3<sup>lacZ/−</sup> × Cln3<sup>lacZ/−</sup>, Cln3<sup>lacZ/−</sup> × Cln3<sup>lacZ/−</sup>, or Cln3<sup>1/+/−</sup> × Cln3<sup>1/+/−</sup> breeding pairs were used. All animal care and handling were approved by the University of Iowa Institutional Animal Care and Use Committee. Ratios of male to female mice were equal or similar for different groups in animal experiments and primary culture experiments.

**β-Gal activity in tissue and cell lysates.** Tissues were harvested from mice and homogenized in 0.1 M phosphate buffer (pH 7.8) containing 0.2% Triton X-100 (lysis buffer), frozen and thawed three times, and centrifuged at 4°C for 15 min at 12,000 g to remove debris. Protease inhibitors (EDTA-free Complete protease inhibitor tablets, used at 1×; Roche Applied Science, Indianapolis, IN) were added to the supernatants, and endogenous galactosidase activity was inactivated by 50 min of incubation at 48°C. β-Gal activity in lysates was determined using the FluoReporter lacZ/Galactosidase quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA), set up in triplicate in 96-well black plates, and detected using a FluorStar fluorometer and Fluo32 software (BMG Lab Technologies, Durham, NC).

β-Gal concentration in lysates was interpolated from a standard curve generated with purified β-Gal (Sigma, St. Louis, MO). Protein concentrations in lysates were determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). For each tissue, samples were assayed from three Cln3<sup>3lacZ/−</sup> and three Cln3<sup>1/+/−</sup> mice and expressed as nanograms of β-Gal per milligram of protein (means ± SE). For assay of β-Gal from cultured cells, lysis buffer was added to monolayers, and lysates were collected, frozen and thawed three times, and centrifuged at 4°C for 15 min at 12,000 g, and protease inhibitors were added to the supernatants as described above. β-Gal activity was determined using the Galacto-Link Plus assay (ABI, Foster City, CA), with samples read in duplicate using a FluorStar fluorometer and Fluor32 software (BMG Lab Technologies, Durham, NC). Protein concentrations in lysates were determined by Bio-Rad assay, and results are expressed as light units per milligram of protein (means ± SD) of duplicate cultures.

**Tissue processing and staining.** For X-Gal staining and immunohistochemistry (IHC), mice were perfused with 2% paraformaldehyde (PFA) in PBS (pH 7.4), and tissues were processed to 10-μm sections using a cryostat as previously described (14). For some sections, X-Gal staining was followed by IHC. For IHC, X-Gal-stained sections were washed in PBS, blocked at room temperature by incubation for 1 h in PBS containing 10% donkey serum and 0.3% Triton X-100, and then incubated overnight at 4°C with goat polyclonal anti-aquaporin 2 (AQP2; C-17 antibody, Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-Tamm-Horsfall protein (THP; G-20 antibody, Santa Cruz Biotechnology), or biotinated Dolichos biflorus agglutinin (DBA; Vector Laboratories, Burlingame, CA) diluted in PBS containing 1% donkey serum and 0.1% Triton X-100 (diluent). Sections with primary antibody were washed in PBS and incubated for 2 h at room temperature with biotinylated donkey anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:1,000 in diluent. Sections were washed in PBS and incubated for 2 h at room temperature with ABC complexes (Vectastain ABC Elite kit, Vector Laboratories) diluted 1:10 in PBS, washed in PBS, and developed with a diaminobenzidine kit (Vector Laboratories), dried overnight, passed twice through xylene for 10 min each, and mounted with Permount-xylene (1:1). Images were captured using Olympus IX70 and Olympus BX60 microscopes and an Olympus DP70 digital camera with associated DP software (Olympus, Center Valley, PA).

For immunofluorescent staining, mice were perfused with 4% PFA, and cryostat sections (40 μm floating or 6 μm on slides) were prepared. Floating sections were blocked at room temperature for 2 h in PBS containing 10% goat serum plus 10% rabbit serum and 0.3% Triton X-100, stained at 4°C overnight with polyclonal rabbit anti-*Escherichia coli* β-Gal (Biodesign International, Kennebunk, ME) conjugated to Alexa 488 (A488, Alexa Fluor 488 protein labeling kit, Invitrogen), and diluted in PBS containing 0.1% Triton X-100 and 1% goat serum. Sections were stained for 25 min at room temperature with the nonspecific nuclear dye TO-PRO-3 (0.5 μM; Invitrogen) to label all cells, washed in PBS, and then mounted with Vectashield (Vector Laboratories) and viewed via confocal microscopy using a Zeiss LSM 510 laser scanning microscope and associated LSM software (Carl Zeiss MicroImaging, Thornwood, NY). For assessment of autofluorescent inclusions in unstained sections, 40-μm kidney sections from Cln3<sup>3lacZ/−</sup> or Cln3<sup>1/+/−</sup> mice were mounted with Vectashield, and confocal images were captured in the red channel. Confocal images within experiments were captured using identical settings. Sections (6 μm) on slides were blocked for 30 min with 10% normal donkey serum and 0.05% Triton X-100 in PBS and incubated with primary antibodies to AQP2/3 (goat polyclonal; Santa Cruz Biotechnology), V-ATPase B1 and B2 (rabbit polyclonal, Santa Cruz Biotechnology), and A488 anti-β-Gal (rabbit polyclonal, as described above) at 2 μg/ml each in PBS for 2 h at room temperature. After they were washed with PBS, the slides were incubated for 30 min with Alexa 568 (A568) anti-rabbit IgG and Alexa 647 (A647) anti-goat...
IgG secondary antibodies from donkey (Invitrogen). Slides were washed with PBS, stained for 5 min with Hoechst nuclear dye, and mounted with Vectashield, and images were captured on a Zeiss LSM 710 laser scanning microscope and analyzed with Zeiss Zen Light-associated software. AQP2 and AQP3 reside on the surface membrane or cytoplasmic vesicles of intercalated (IC) cells. β-Gal signal appeared in green and red channels due to staining with rabbit A488 anti-β-Gal and A568 anti-rabbit antibodies and appeared yellow in merged images. The yellow β-Gal signal was clearly distinguishable from red V-ATPase staining, which was outside cell nuclei and did not overlap with A488 or Hoechst signals.

For immunofluorescent staining of cultured cells, monolayers were fixed with 4% PFA in PBS for 10 min at room temperature, blocked for 1 h in PBS with 10% goat serum and 0.1% Triton X-100, and then incubated overnight at 4°C with primary antibodies. Primary antibodies included combinations of rabbit A488-anti-β-Gal, mouse monoclonal anti-pan cytokeratin (clone C-11, Sigma), rat monoclonal anti-CDS1 (BD Biosciences, San Jose, CA), and rabbit anti-ZO-1 (Invitrogen), each at 1–2 µg/ml. After they were washed with PBS, the monolayers were incubated for 1 h with appropriate A568- or A488-conjugated goat polyclonal secondary antibodies (Invitrogen) and washed again. Cell nuclei were stained by 5 min of incubation with Hoechst dye, and images were captured using an inverted Olympus IX70 fluorescent microscope and an Olympus DP70 digital camera (Olympus). Images were captured using identical settings for each staining condition.

Cln3 transcript abundance in kidney regions. Mice were perfused with 20 ml of cold PBS, and samples of liver, spleen, and lung were immediately homogenized in TRIzol (Invitrogen). Kidneys were placed in ice-cold PBS, and renal cortex samples, outer medullas (OM), and IM/papillla were dissected out and homogenized in TRIzol. RNA was isolated from tissue samples using TRIzol as directed by the manufacturer (Invitrogen). One microgram of RNA was reverse-transcribed to cDNA in 50-µl reactions using the High-Capacity cDNA RT kit and random primers (ABI), and DNA was removed using a DNA-Free kit (ABI). Quantitative PCR (qPCR) were set up in triplicate using Universal Master Mix (ABI) and primer-probe sets for murine Cln3 (Mm00477972_ml, exon 2–3) and eukaryotic 18S rRNA (Hs99999901_s1) and run using a 7900HT Fast real-time PCR system (ABI). No significant amplification was observed in “no-RT” controls. The comparative CT method (ABI Bulletin 2) was applied to determine the relative abundance of Cln3 transcripts in the various tissues and kidney regions, with 18S rRNA as the endogenous control and a liver sample as the calibrator (equal to 1). Samples were taken from two mice for liver, spleen, and lung and from three mice for kidney regions. Results are expressed as means ± SD, and significant differences between kidney regions were established by unpaired t-tests.

Primary IM cultures. Primary renal IM cell cultures were generated using methods similar to those described in previous studies (31, 60). The starting medium for primary cultures was a 50:50 mixture of low-glucose DMEM (Invitrogen) and F-12-Coons (Sigma) containing 100 U/ml penicillin, and 100 µM sodium selenite (Sigma), 10% fetal bovine serum, 100 U/ml hyaluronidase (Worthington Biochemical, Lakewood, NJ) and 2 mg/ml collagenase B (Roche), with trituration every 15 min. At the halfway point, DNase I (Promega, Madison, WI) was added to a final concentration of 10 U/ml to reduce clumping. After digestion, the desired small clusters were diluted in prewarmed HT-DMEM/F-12 and collected by centrifugation for 2 min at 230 g and further washed by two more centrifugations in HT-DMEM/F-12. Pellets were suspended in HT medium and seeded into dishes at a density corresponding to about one 60-mm dish per mouse. The culture medium was replaced with fresh HT medium every 2 or 3 days. At confluence (5–7 days), the medium was changed to HT medium without serum (HT medium-0). For experiments, cultures were subjected to further changes in osmolality as described below.

Osmoregulation of reporter protein. For testing of osmoregulation of β-Gal, primary IM cultures from Cln3lacZ+/+ mice were generated as described above in 24-well dishes. After confluence and culture in HT medium-0 for 48 h, the osmolality was lowered (dropped to isosmolar, 300 mosmol/kgH2O, maintained at 600 mosmol/kgH2O, or increased at the rate of 100 mosmol/kgH2O per day to 800, 1,000, 1,200, or 1,400 mosmol/kgH2O by the addition of NaCl plus urea (1.5:1 molar ratio) in duplicate wells. At 24 h after the last addition, the cells were harvested into lysis buffer and analyzed for β-Gal activity by Galacto-Light assay as described above.

In vitro osmoregulation of Cln3 transcript abundance. Primary cultures of IM from Cln3lacZ+/+ mice were generated as described above, and after change to HT medium-0, osmolality of the cultures was maintained at 600 mosmol/kgH2O or lowered to isosmolar (300 mosmol/kgH2O) in duplicate wells. After 48–72 h, RNA was isolated using an RNeasy Plus kit (Qiagen, Valencia, CA), which includes a DNA removal column. RNA was reverse transcribed to cDNA, and qPCR was performed using primer-probe sets for murine Cln3 (Mmm01163353_ml, exon 3–5), eukaryotic 18S rRNA, and murine Pax2 (Mmm00477972_ml; ABI). qPCR reactions were performed as described above. No significant amplification was detected in no-RT controls. Results are expressed as hypertonic transcript abundance relative to isosmotic.

Osmoregulation of Cln3 in vivo. Cln3lacZ+/+ mice were injected with furosemide (3 mg/100 g body wt ip; Sigma) at time 0 and again at 3 h and were killed 3 h after the second dose. Control Cln3lacZ+/+ mice were injected with saline vehicle alone. At the time of death, kidneys were removed to dishes on ice containing RNAlater (Qiagen). IM and samples of renal cortex were dissected out, and RNA was isolated using RNeasy Plus kits (Qiagen). RT and qPCR were performed using ABI reagents as described above. The qPCR was set up in triplicate using ABI primer-probe sets for eukaryotic 18S rRNA and murine Cln3, murine aldose reductase (AR; Mmm01135578_g), and murine β-actin (Mmm0607939_s). Relative transcript abundance was determined as described above using 18S rRNA as endogenous control. Results are expressed as means ± SE of samples from four mice, and t-tests were applied to establish P values.

Quantification of intracellular osmolytes. Primary cultures of IM from Cln3lacZ+/+, Cln3lacZ−/−, or Cln3+/+ mice (6 mice pooled per group) were generated in 100-mm dishes as described above. A few days after change to HT medium-0, the medium was lowered to isosmotic (300 mosmol/kgH2O) or raised to a final osmolality of 1,300 mosM at a rate of 100 mosmol/kgH2O per day. Medium was made hypertonic by addition of NaCl plus urea (at 1.5:1 molar ratio). Hypertonic and isosmotic media were also supplemented with the osmolytes betaine, myo-inositol, and taurine (Sigma) at 200 µM each. At 48 h after the final increase in osmolality, the monolayers were rinsed twice with the corresponding medium without osmolyte supplements and then three times with PBS or HT-PBS (made hypertonic with NaCl to match the osmolality of the medium). Perchloric acid (7%) was added to monolayers to precipitate total proteins. Cells were scraped and collected, and precipitates were pelleted by centrifugation for 6 min at 10,000 g at 4°C. Protein pellets were dissolved in 0.25 M NaOH and kept at 4°C until protein determination (Bio-Rad). Super-
nates were neutralized with KOH, incubated for 10 min on ice, and centrifuged again for 15 min. Supernatants were delipidated by passage over a Sep-Pak Light C18 cartridge (Waters, Milford, MA) and then passed over 0.22-μm filters. Samples were evaporated and dissolved in the volume of water to give a 2 mg/ml total protein equivalent, and 100 μl were analyzed by HPLC for osmolalities as previously described (44, 58). Osmolalities are expressed as millimoles per milligram of total cellular protein.

Taurine uptake/efflux. Primary cultures from Cln3+/+ and Cln3lacZlacZ mice were established as described above in 24-well dishes with removal of serum and change to isotypic cultures at 1 wk. After 2 days, cultures were transitioned to 900 mosmol/kgH2O by three additions of NaCl plus urea (1.5:1 molar ratio) for induction of the Cln3 (OSMOREGULATION IN THE KIDNEY

RESULTS

Cln3 expression in extraneous tissues. We used our phenotypically normal heterozygous reporter (Cln3lacZlacZ+/+) mice (14) to assess relative Cln3 expression. In these recombinant mice, a nuclear-localized β-Gal reporter gene is inserted in place of Cln3 sequences and driven by native Cln3 promoter elements. Nuclear-targeted β-Gal, but not Cln3, is transcribed from the recombinated allele, such that Cln3lacZlacZ mice are CLN3p-null and provide a CLN3p-deficient mouse model. Our previous study localized reporter to neuronal subtypes and vascular endothelia in the brain. In the present study, we examined extraneural β-Gal activity in Cln3lacZlacZ mice. Of the tissues examined, kidney medulla displayed the highest activity, followed by skin and lung (Fig. 1A). X-Gal staining revealed reporter-positive nuclei in skin epidermal cells (Fig. 1B), epithelial cells within the large conducting airways of the lung (Fig. 1C), and epithelial cells within colonic crypts (Fig. 1D). In addition, weakly positive nuclei were often detectable within the vascular endothelial cells of most tissues (not shown). The kidney stained very strongly with X-Gal (Fig. 1E): positive nuclei were detected in the glomeruli of the cortex (Fig. 1F), in some tubules in the outer medulla (OM; Fig. 1G), and in increasing density in most to all cell types in the IM/papilla (Fig. 1H).

Immunofluorescent staining for β-Gal illustrated increasing intensity of nuclear β-Gal staining with progression to the tip of the papilla (Fig. 1J).

To complement reporter detection with Cln3 expression in the kidney, we performed RT-qPCR analysis for Cln3 transcript abundance in kidney regions, as well as in lung, spleen, and liver tissues dissected from Cln3+/+ mice. Similar to reporter expression, transcript levels in the kidney followed the osmotic gradient, with IM/papilla > outer medulla > cortex (Fig. 1J); expression in the IM was threefold higher than in the renal cortex. In addition, the expression level was almost sixfold higher in the IM than in the liver and threefold higher than in the lung and spleen. Transcript quantity in the renal cortex was similar to that in the lung and spleen. Thus Cln3 transcript levels in vivo show regional differences consistent with the β-Gal reporter protein.

To ascertain the major β-Gal-positive cell types in the kidney, we stained tissue sections with X-Gal followed by IHC staining for cell type-specific markers. In the OM, reporter-positive nuclei were found within DBA-lectin-positive tubules (Fig. 2A, left). DBA-lectin selectively binds collecting duct cells in the rodent kidney (53). In contrast, no β-Gal-positive cells were localized within thick ascending limbs (TAL), which stained strongly on their luminal surface for THP (Fig. 2A, middle) and could be seen running adjacent to β-Gal-positive tubules. Confocal analyses after staining for AQP2/3, β-Gal, and V-ATPase B (Fig. 2B) indicated that, within medullary collecting ducts, the β-Gal-positive nuclei belonged to AQP2/3-positive principal cells, and not V-ATPase B-positive IC cells. Rare, weakly β-Gal-positive IC cells were encountered (not shown). In the cortex, β-Gal-
positive nuclei localized to glomeruli and vasculature (Fig. 2C). Both X-Gal and immunofluorescent staining gave the appearance of an increasing proportion of β-Gal-positive cells from superficial to deep IM. We addressed this impression more quantitatively by double-staining for β-Gal (using anti-β-Gal antibody) and cell nuclei (using TO-PRO-3, a nonspecific nuclear dye). The proportion of dual-positive nuclei was approximated by confocal microscopy. In the superficial IM (near the OM), 40% of the cells were β-Gal-positive (Fig. 2D, left). In the deep IM of the same sections, near the tip of the papilla, nearly all the cells were β-Gal-positive (Fig. 2D, middle). Together, reporter analyses indicate a pattern of increased Cln3 expression from the OM to the tip of the papilla, wherein most cells express reporter, and a pattern of preferential expression in principal cells, rather than IC cells, in the OM region of the kidney.

Cln3 expression is osmoregulated. The pattern of Cln3 expression in the kidney is reflective of the natural osmotic gradient in the kidney. Proteins such as heat shock protein (HSP70) (2) and Pax2 (7) and proteins necessary for osmolyte import or synthesis (6) display an expression gradient in the kidney. As these proteins are osmoregulated, we hypothesized that Cln3 would be similarly responsive. To determine reporter expression relative to osmolality, we generated primary cultures from dissected IM of Cln3lacZ/+ mice. We enriched for tonicity-adapted medullary cells by initiation and early maintenance of the cultures in hyperosmotic (600 mosmol/kgH2O) medium. Cultures generated in this manner comprise predominantly IM epithelial cells (60).

Immunofluorescent staining for pan cytokeratin or CD31 was performed to assess relative contributions of epithelial and endothelial cells, respectively, to culture monolayers. Cln3++/+
and Cln3<sup>β-lacZ/lacZ</sup> cultures were generated and maintained in hypertonic medium. Cytokeratin-positive epithelial cells were clearly prevalent (see supplemental Fig. S1A in the online version of this article), with a minority of CD31-positive endothelial clusters (see supplemental Fig. S1B). Many cytokeratin-positive cells in the Cln3<sup>β-lacZ/lacZ</sup> cultures expressed β-Gal (see supplemental Fig. S1C), as expected. In addition, ZO-1 staining at intercellular boundaries indicated formation of tight junctions.
of tight junctions between neighboring epithelial cells (see supplemental Fig. S1D).

When primary Cln3lacZ/H11001IM cultures reached confluence (~1 wk), serum was removed from the medium. The osmolality of the culture medium was subsequently reduced to 300 mosmol/kgH2O or increased, in 100 mosmol/kgH2O increments once or twice per day, by the addition of NaCl plus urea at a ratio approximating the in vivo environment (1.5:1 molar ratio of added NaCl to urea). Figure 3, A and B, shows that reporter expression correlated with medium osmolality. Reporter levels were 6.5-fold higher at 1,400 than at 300 mosmol/kgH2O. An O2 gradient also exists in the renal medulla. Specialized arterioles, the vasa recta, extend from the corticomedullary border to the tip of the papilla and loop back up, creating a hypoxic gradient (34). Thus we considered that PO2 might additionally regulate reporter expression. However, reporter levels in primary renal medullary cells were similar after culture in reduced PO2 (2%) compared with normoxia (20% O2), regardless of osmolality (Fig. 3B). These results suggest that osmolality, rather than PO2, is a significant regulator of Cln3 expression.

In separate experiments, we utilized primary cultures from Cln3lacZ/H11001/H11001 mice to assess the effect of hypertonicity on Cln3 transcript levels. Primary cultures were generated as described above, with hypertonic changes achieved upon addition or removal of NaCl plus urea. Significant transcript increase was also determined in cultures made hypertonic (final 600 mosmol/kgH2O) by addition of NaCl, mannitol, or raffinose (Fig. 3C, right). Raffinose was particularly effective (33-fold increase above isotonic), suggestive of additional, toxicity-independent regulatory mechanisms. Pax2, a previously described tonicity-regulated protein (7), was used as a positive control; accordingly, Pax2 expression was found to increase with hypertonicity imparted by NaCl plus urea, NaCl, and raffinose (Fig. 3C). These results demonstrate in vitro osmoregulation of Cln3 in primary renal medullary cells.

To assess in vivo osmoregulation of Cln3, we compared Cln3 transcript abundance in kidneys of untreated vs. furosemide-treated heterozygous mice. By inhibiting Na+/H11001 reab...
sorption in the TAL, furosemide effectively reduces interstitial osmolality in the renal medulla. RT-qPCR analysis of dissected renal cortex and IM tissue showed that furosemide profoundly diminished \(Cln3\) transcript abundance in the IM, with little influence in the cortex (Fig. 3D). AR was used as an osmo-regulated control. AR catalyzes the synthesis of the non-perturbing osmolyte sorbitol and displays tonicity-sensitive expression along the renal medulla (51). Furosemide triggered a significant reduction of AR transcript abundance, while levels of \(\beta\)-actin (a non-osmo-regulated control) were unaltered (Fig. 3D). These results indicate that interstitial osmolality regulates \(Cln3\) expression in the renal medulla.

CLN3p deficiency does not limit osmolyte accumulation. The regulation of \(Cln3\) expression by osmolality suggests that CLN3p might function in pathways affecting intracellular osmolyte accumulation. To examine this possibility, we established primary IM cultures from control (\(Cln3^{+/+}\) or \(Cln3^{lacZ/lacZ}\)) and CLN3p-deficient (\(Cln3^{lacZ/lacZ}\)) mice and measured intracellular osmolyte concentrations after maintenance in isotonic or hypertonic conditions. Intracellular osmolalities were quantified by HPLC and normalized to total cellular protein. For control and CLN3p-deficient cells, intracellular concentrations of the dominant osmolalies (inositol, taurine, GPC, sorbitol, and betaine) were manyfold higher after growth in hypertonic than isotonic medium (Fig. 4A), and there was no apparent effect of genotype. Intracellular levels of the less prominent osmolyte proline also increased under hypertonic conditions, while glycine showed no accumulation. Urea also rose to high intracellular levels. Unlike osmolalies, urea is relatively cell-permeant and equilibrates across cell membranes, explaining this intracellular rise. GPC and betaine, in addition to assisting in osmotic equilibrium, have been shown to protect intracellular proteins from the denaturing actions of urea (59). These results indicate that CLN3p-deficient cells acquire normal levels of osmolalies upon exposure to a hypertonic environment.

Our data indicate that CLN3p is dispensable for osmolyte accumulation. However, CLN3p could affect the rate of transport or synthesis of an osmolyte, without changing the final steady-state level achieved after several days. Along with its role as an osmolyte in the kidney, taurine is also an important osmolyte in the hippocampus (36) and as a neurotrophin in the retina (43), two areas of neurodegeneration in JNCL. To determine whether the rate of taurine import is regulated by CLN3p, we measured \(^{3}H\)taurine uptake in CLN3p-deficient vs. wild-type primary renal medullary cells. No significant differences in uptake were detected at 15 or 30 min (Fig. 4B), indicating that CLN3p absence does not disturb the kinetics of taurine uptake. In a separate experiment, we assessed taurine efflux. Primary IM cells were loaded with \(^{3}H\)taurine, and efflux was measured 10 min after change from hypertonic (600 mosmol/kgH\(_2\)O) to isotonic (300 mosmol/kgH\(_2\)O) medium. \(^{3}H\)taurine efflux was substantial for \(Cln3^{lacZ/lacZ}\) and \(Cln3^{+/+}\) cells and similar for each genotype (65 ± 0.3 and 68 ± 0.1\%, respectively), indicating that taurine efflux is also intact in CLN3p-deficient cells.

Enhanced water consumption and urine output in knockout mice. In the kidney, interstitial osmolality in conjunction with selective expression and translocation of aquaporins along nephron segments and vasa recta permits osmotic flow of water for maintenance of body fluid. To assess whether CLN3p deficiency might affect renal functions related to water balance, we compared water consumption, urine output, and urine osmolality for \(Cln3^{lacZ/lacZ}\) and \(Cln3^{+/+}\) mice after free access to water or water deprivation. CLN3p-deficient mice consumed significantly more water over 24 h and had higher urine volume and lower urine osmolality than controls (Fig. 5). However, with 24 h of water deprivation, urine output was diminished and was similar to \(Cln3^{+/+}\) controls with respect to osmolality (Fig. 5B) and volume (0.4 ± 0.33 and 0.46 ± 3 ml for wild-type and \(Cln3^{lacZ/lacZ}\), respectively). Thus, in the absence of CLN3p, mice display mild polyuria. However, water deprivation stimulates water conservation, such that deficient mice are able to concentrate their urine to levels similar to those observed in controls.

Abnormal K\(^+\) excretion in CLN3p-deficient mice. Changes in water balance could reflect underlying defects in electrolyte transport across renal tubules. We measured serum and urine creatinine, electrolytes, blood urea nitrogen, and other indicators of kidney function (Tables 1 and 2). We found no significant differences between \(Cln3^{lacZ/lacZ}\) and \(Cln3^{+/+}\) mice in urine pH or serum total CO\(_2\), suggesting that pH regulation is
not impaired in CLN3p-deficient mice. Upon analysis of 24-h total and fractional excretion of ions, we determined that, with free access to water, serum K⁺ tended to be higher and fractional excretion of K⁺ was significantly lower in CLN3p-deficient than control mice (Tables 1 and 2). With water deprivation, fractional excretion of Mg²⁺ was significantly higher in CLN3p-deficient than control mice. This tendency to higher fractional excretion of Mg²⁺ was also observed in mice with free access to water. However, because serum Mg²⁺ was not different (tended to be higher in CLN3p-deficient mice), it is unlikely that the higher fractional excretion of Mg²⁺ represents a primary renal defect in the ability to conserve Mg²⁺. In contrast, the K⁺ abnormalities provide clear evidence for a defect in K⁺ excretion consequent to CLN3p deficiency.

**Autofluorescence accumulation in Cln3lacZ/lacZ kidneys.** Lysosomal accumulation of autofluorescent lipofuscin-like material is a diagnostic pathological feature of JNCL (32, 49). We found significant autofluorescent material in all regions of Cln3lacZ/lacZ kidney from 8-wk-old mice but little or no such autofluorescent material in an age-matched Cln3⁻/⁻ kidney (Fig. 6). The appearance of inclusions was not restricted to cells expressing reporter protein. For example, in the cortex, we observed deposits in cells within proximal convoluted tubules (Fig. 6, bottom left), despite the fact that tubules were consistently reporter-negative by X-Gal and immunofluorescent staining. Indeed, in sections immunofluorescently stained for β-Gal, autofluorescent material was evident in the cytoplasm of cortical epithelial cells lacking β-Gal-positive nuclei (Fig. 2C, arrowheads). At 6 mo, auto fluorescence accumulation was more extensive in all kidney regions (not shown), as expected by the progressive nature of this pathology.

**DISCUSSION**

In this study, we used a recombinant Cln3 reporter mouse, wherein β-Gal is transcribed from the native Cln3 promoter, to investigate the expression of Cln3 outside the CNS. We demonstrate that 1) Cln3 is expressed in nonneural tissues and is prevalent in IM cells of the kidney, 2) expression of Cln3 in renal medullary cells is osmoregulated, 3) CLN3p is not necessary for accumulation of major renal osmolites, 4) CLN3p-null mice display polyuria, 5) K⁺ excretion is impaired in CLN3p-deficient mice, and 6) kidney cells in CLN3p-deficient mice accumulate autofluorescent inclusions.

As JNCL is largely a CNS disorder, it might be expected that CLN3p operates primarily in cells of the nervous system. However, on the basis of reporter expression, we find that Cln3 expression is not restricted to the nervous system. We detected reporter in many tissues, with very high expression in the kidney medulla. Reporter expression in the kidney paralleled the natural corticomedullary osmotic gradient, with the intensity and proportion of positive cells increasing with progression to the tip of the IM/papilla. Using primary renal medullary cultures derived from heterozygous reporter mice, we found that reporter protein levels correlated directly with the osmolality of the culture medium. Importantly, our in vivo data also support osmoregulation of Cln3.

Our results suggest that toxicity-responsive cis elements may function in the 5'-flanking region in the Cln3 locus. A number of genes expressed in the renal medulla are osmoregulated and are involved in response to marked changes in extracellular toxicity. Many of these genes, including the Na⁺-myo-inositol transporter (SMIT), the betaine transporter (BGT1), AR, and HSP70 are positively regulated by the tonicity-responsive element-binding protein (TonEBP) transcription factor (5, 15). Upon hypertonic stress, TonEBP is activated and translocates to the nucleus, where it acts at cis Ton/EORE (tonicity-responsive element/osmoresponsive element) sites in the 5'-flanking regions of osmoresponsive genes (16). Analysis of the 5'-flanking region sequence of the murine Cln3 gene reveals an 11-bp site (5'-Cln3 GENES are highly prevalent in IM cells of the kidney, 1

**Table 1. Blood and serum chemistries**

<table>
<thead>
<tr>
<th></th>
<th>Free Access to Water</th>
<th>Water-Deprived</th>
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<tbody>
<tr>
<td></td>
<td>++/+</td>
<td>lacZ/lacZ</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>lacZ/lacZ</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.35 ± 0.06</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>2.3 ± 0.0</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Hematocrit, %vol</td>
<td>45.2 ± 1.0</td>
<td>48.7 ± 0.6</td>
</tr>
<tr>
<td>Osmolarity, mmol/kgH₂O</td>
<td>315 ± 3</td>
<td>318 ± 1.4</td>
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<tr>
<td>Total CO₂, mmol/l</td>
<td>17.8 ± 0.7</td>
<td>21.0 ± 0.8</td>
</tr>
<tr>
<td>Na⁺, mmol/l</td>
<td>150 ± 2</td>
<td>154 ± 1</td>
</tr>
<tr>
<td>K⁺, mmol/l</td>
<td>6.0 ± 0.7</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>Mg²⁺, mmol/l</td>
<td>3.1 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>Ca²⁺, mg/dl</td>
<td>9.0 ± 0.2</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus, mg/dl</td>
<td>10.0 ± 0.4</td>
<td>9.5 ± 0.2</td>
</tr>
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</table>

Values are means ± SE from 4-10 mice per group. *P < 0.005 vs. +/+. †P < 0.001 vs. ++/+.


The results of the in vivo analysis of water balance indicate a mild defect in the ability to concentrate urine. When permitted free access to water, CLN3p-null mice were polyuric and polydipsic. The serum data do not support primary polydipsia (i.e., a thirst-regulating defect) as the cause of the polyuria. CLN3p-deficient mice had a tendency to higher serum osmolality, a higher hematocrit, and higher serum albumin than controls. These differences implicate polyuria as the main defect, causing the plasma hypertonicity, which in turn triggers increased drinking. The basis for the polyuria is not completely obvious, but we suggest two possibilities. First, there could be an abnormality in protein trafficking within the collecting duct. CLN3p has been implicated in endocytic processes and vesicular trafficking (18, 29), and one might envision a role for CLN3p in aquaporin (or other protein) trafficking to or from the plasma membrane.

A second possibility is that the polyuria is secondary to the defect in K⁺ excretion. Acute and chronic K⁺ loading causes accumulation of K⁺ in the renal medulla interstitium (26). This

### Table 2. Urine chemistries

<table>
<thead>
<tr>
<th></th>
<th>Free Access to Water</th>
<th>Water-Deprived</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>lacZ/lacZ</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>lacZ/lacZ</td>
</tr>
<tr>
<td>Creatinine, µg</td>
<td>530 ± 46</td>
<td>675 ± 59</td>
</tr>
<tr>
<td>Na⁺, µmol</td>
<td>205 ± 23</td>
<td>243 ± 23</td>
</tr>
<tr>
<td>K⁺, µmol</td>
<td>331 ± 35</td>
<td>335 ± 38</td>
</tr>
<tr>
<td>Mg²⁺, µg</td>
<td>836 ± 101</td>
<td>1,069 ± 102</td>
</tr>
<tr>
<td>Ca²⁺, µg</td>
<td>49 ± 7</td>
<td>74 ± 7.8*</td>
</tr>
<tr>
<td>Phosphorus, µg</td>
<td>4,950 ± 522</td>
<td>5,154 ± 507</td>
</tr>
<tr>
<td>Fractional excretion, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.95 ± 0.11</td>
<td>1.12 ± 0.14</td>
</tr>
<tr>
<td>K⁺</td>
<td>37.6 ± 2.5</td>
<td>25.8 ± 2.9*</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.16 ± 0.02</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.0031 ± 0.0005</td>
<td>0.0074 ± 0.0029</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.33 ± 0.04</td>
<td>0.31 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9–12 for 24 h total excretion and n = 4–6 for fractional excretion. Urine and serum were collected after 24 h of free access to water or after 24 h of water deprivation. *P < 0.05 vs. +/+.

### Fig. 6. Intracellular autofluorescent deposits are present in all kidney regions of CLN3-deficient mice. Kidney sections (40 µm) from 8.5-wk-old Cln3<sup>−/−</sup> and Cln3<sup>lacZ/lacZ</sup> mice were mounted with Vectashield, and 0.45-µm confocal images were captured in the red channel and transformed to gray scale. Abundant autofluorescent storage is apparent in cortex, OM, and IM/papilla of Cln3<sup>lacZ/lacZ</sup>, but not Cln3<sup>+/+</sup>, kidneys.

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**C1397**

**OSMOREGULATION IN THE KIDNEY**

**Cln3**
accumulation causes inhibition of NaCl absorption from the medullary TAL with, consequently, less hypertonicity of the renal medulla (52). This effect, similar to that of loop diuretics, could produce a modest concentrating defect. Whatever the cause, it is apparent that extreme water deprivation can overcome this defect. The precise nature of the concentrating defect in these Cln3p-deficient mice requires further investigation.

Our serum/plasma chemistry analyses uncovered an unexpected phenotype in the CLN3p-deficient mice. These mice were hyperkalemic and had a lower fractional excretion of K+ than control mice. Virtually all the K+ appearing in urine is secreted by the distal nephron. Thus, since the Cln3p-null mice had a relatively normal glomerular filtration rate (as evidenced by a normal serum creatinine and urea nitrogen), they had a significant impairment in their ability to secrete K+. The major mediators of K+ secretion in the distal nephron are renal OM K+ channel (Romic) and the large-conductance Ca2+-activated K+ (BK) channels, apically situated in principal cells of the connecting tubule, cortical collecting duct, and, to a lesser extent, the OM collecting duct (56). Normal K+ secretion requires that these apical K+ channels be functional. In addition, normal K+ secretion requires normal Na+ transport by these nephron segments. Na+ must enter the cell via the apical membrane epithelial Na+ channels and be pumped out of the basolateral membrane via the Na+-K+-ATPase. CLN3p could be involved in functional regulation or trafficking of one or more of these channel or transporter complexes. A role for CLN3p in the localization of some Na+-K+-ATPase subunits has recently been reported (55) and may have relevance to distal nephron K+ excretion. Abnormalities in water intake and K+ balance have not, to our knowledge, been described for JNCL patients. However, mild defects could go unnoticed because of wide normal ranges and uncontrolled presampling conditions. K+ is critical to modulation of neuronal membrane potential and excitability. Defective regulation of K+ concentration could have adverse CNS effects, contributing to seizure phenotype and neuronal excitotoxicity in JNCL.

Accumulation of autofluorescent deposits in a variety of cell types is a hallmark feature of the neuronal ceroid lipofuscinosis (NCL) diseases (reviewed in Ref. 49). These lysosomal inclusions are also referred to as lipopigments or ceroid. They contain protein, carbohydrate, and lipid and are distinct from the lipofuscin that accumulates with age in nondisease states. In most NCL forms, subunit c of the mitochondrial ATP synthase is the dominant protein component of inclusions. In JNCL mouse models, ceroid is evident in neuronal populations of the brain and retina (12, 28), as well as in hepatocytes (12). We observed significant autofluorescent inclusions in the kidneys of Cln3lacZ/lacZ mice, but not in age-matched controls. Inclusions were evident in all regions, in contrast to a more restricted expression pattern for β-Gal reporter. Renal inclusions in our JNCL mouse model are consistent with previous reports of renal autofluorescent deposits and subunit c detection in urine samples in JNCL patients (57). The origin of these lipopigments in JNCL is not known, and the relationship between these deposits, CLN3p expression, and cell viability is unclear (30). It is possible that renal phenotypes arise as an indirect consequence of cellular dysfunction related to storage buildup. However, most in vitro studies use actively proliferating cell lines from JNCL mouse models or patients and uncover functional abnormalities in the absence of autofluorescence buildup.

Studies in mammalian cells support roles for CLN3p in a variety of seemingly diverse intracellular processes, including apoptosis resistance (39, 41), lipid metabolism or transport (22, 45), and vesicular trafficking and endocytosis (18, 29). An ability of CLN3p to protect against apoptosis would be consistent with a need for upregulation under high-salt conditions and is reminiscent of the antiapoptotic state associated with HSP70 expression under chronic hypertonicity (46). In addition, a function for CLN3p in localization of galactosylceramide to the plasma membrane (45) corresponds with a reported hypertonicity-induced increase in galactosylceramide and sulfatide synthesis in polarized Madin-Darby canine kidney cells (35). Interestingly, 1H-NMR spectroscopic analysis of brain tissues from CLN3p-deficient mice revealed elevated levels of myo-inositol, creatine, N-acetyl-aspartate, and taurine compared with controls (38), all of which have postulated roles in osmoregulation (3, 24).

The expression of CLN3p in epidermis, colonic epithelia, and lung epithelia, together with the very strong expression in the renal IM, adds to the evidence that it may be involved in defending against changes in cell volume. Epithelial cells, and particularly these epithelial cells, are especially susceptible to changes in cell volume by virtue of their role in regulating solute and water transport through the cell. The ability of an epithelial cell to regulate its volume while transporting large amounts of solute and water requires a delicate balance of transport activity and, possibly, metabolism of osmoles. The expression of CLN3p in specific kidney cells is consistent with this idea. Whereas principal cells of the collecting duct can transport relatively large amounts of water and Na+, IC cells of the collecting duct and cells of the TAL are water immepermeant. Thus the expression of large amounts of CLN3p in cells that must invoke robust defense against perturbations in cell volume supports the general idea that it is involved in osmoregulation. It is conceivable that CLN3p performs a toxicity-related function in the CNS, as well as the kidney and other epithelia.

In summary, our findings implicate CLN3p in renal regulation of water balance and K+ secretion. The contribution of CLN3p to these pathways, particularly in the setting of JNCL, requires further testing. The novel information from this study brings us closer to elucidating the molecular function of CLN3p and understanding JNCL pathogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
REFERENCES