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RESEARCH ARTICLE

Activation of the calcium sensing receptor increases claudin-14 expression via a PLC -p38-Sp1 pathway

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Abstract

Activation of the basolateral calcium sensing receptor (CaSR) in the renal tubular thick ascending limb (TAL) increases claudin-14 expression, which reduces paracellular calcium (Ca^{2+}) permeability, thus increasing urinary Ca^{2+} excretion. However, the upstream signaling pathway contributing to altered *CLDN14* gene expression is unknown. To delineate this pathway, we identified and then cloned the CaSR responsive region including the promoter of mouse *Cldn14* into a luciferase reporter vector. This 1500 bp sequence upstream of the 5′ UTR of *Cldn14* variant 1, conferred increased reporter activity in the presence of high extracellular Ca^{2+} (5 mM) relative to a lower (0.5 mM) concentration. Assessment of *Cldn14* reporter activity in response to increased extracellular Ca^{2+} in the presence or absence of specific inhibitors confirmed signaling through PLC and p38, but not JNK. Overexpression of SP1 attenuated *Cldn14* reporter activity in response to CasR signaling. SP1 is expressed in the TAL and phosphorylation was attenuated by CaSR signaling. Finally, activating mutations in the CaSR increased *Cldn14* reporter activity while a dominant negative mutation in the CaSR inhibited it. Together, these studies suggest that basolateral activation of the CASR leads to increased *Cldn14* expression via a PLC- stimulated p38 pathway that prevents Sp1 mediated repression.

KEYWORDS
calcium homeostasis, claudins, urine calcium excretion

1 | INTRODUCTION

Nephrolithiasis is a prevalent disease, which is most commonly the result of calcium (Ca^{2+}) precipitation within the renal tubule.[1,2] Kidney stones, when large enough, can lodge in narrower parts of the urinary tract, causing pain, a phenomenon known as renal colic. Not surprisingly, these episodes often result in emergency visits and if

Abbreviations: Ca^{2+}, calcium ion; CaSR, calcium sensing receptor; *CLDN14*/CLDN14, human claudin-14 gene/protein; *Cldn14*/Cldn14, mouse claudin-14 gene/protein; GPCR, G protein coupled receptor; JNK, c-Jun N-terminal kinases; MAPK36, Mitogen-activated Kinase Kinase Kinase 6; P38, p38 mitogen-activated protein kinase; PLC, phospholipase C; PTH, parathyroid hormone; SP1, transcription factor Sp1; TAL, thick ascending limb.

untreated can lead to serious complications including infections, ureteral stenosis or perforation, and acute kidney injury.[3] With annual treatment costs rising up to 5 billion dollars in the United States alone, a better understanding of this disease is needed to inform improved therapies.[4]

The etiology of kidney stone formation is complex, with genetic and environmental risk factors contributing. Though the exact mechanism of stone formation is unknown, the greatest risk factor for nephrolithiasis is hypercalciuria, with approximately 80% of kidney stones being composed of Ca^{2+}.[5] Consequently, successful management strategies aim to reduce urinary Ca^{2+} excretion.[6,7] The majority of Ca^{2+} reabsorption along the renal tubule occurs via the paracellular pathway driven by a transepithelial electrochemical gradient (a detailed renal handling of Ca^{2+} mechanisms is reviewed elsewhere).[8,9] This is mediated by paracellular pore forming complexes comprised of the tight junction protein family called claudins.[10,11] A large genome-wide association study (GWAS) linked SNPs in the human Claudin-14 gene (*CLDN14*) to an increased risk of hypercalciuric nephrolithiasis.[12] Follow up work demonstrated the importance of *CLDN14* in regulating paracellular cation transport in the thick ascending limb, a site of significant paracellular Ca^{2+} reabsorption. *CLDN14* was found to be pore blocking, i.e., reduces paracellular cation permeability, including permeability to Ca^{2+}.[13] Additionally, the role of *CLDN14* in Ca^{2+} homeostasis, and the formation of kidney stones, has been emphasized by multiple additional studies demonstrating that at least murine CLDN14 (*Cldn14*) is regulated transcriptionally by activation of the Ca^{2+} sensing receptor (CaSR), a process that involves NFAT and microRNAs.[12–16] Activation of the CaSR in the parathyroid, via increased extracellular Ca^{2+}, regulates Ca^{2+} homeostasis by reducing PTH secretion from the parathyroid glands.[17,18] However, CaSR activation in the TAL and intestine also directly inhibits transcellular Ca^{2+} absorption across these epithelia, i.e., independently of PTH and calcitriol.[15,19] We and others have shown that activation of the CaSR increases *Cldn14* expression in the TAL, which in turn blocks paracellular Ca^{2+} reabsorption and increases urinary Ca^{2+} excretion.[14,15] However, the signalling pathway downstream of CaSR activation, as well as the CaSR-sensitive promoter region of *CLDN14* are unknown.

We therefore set out to identify the *Cldn14* variant responsive to CaSR activation and examine the signalling pathway between CaSR activation and increased *Cldn14* expression. We hypothesized that the CaSR signals through its G-protein cascade to activate Sp1, a transcriptional repressor, which is known to be activated by the CaSR[20] and decrease both *Cldn3* and *Cldn4* expression.[21,22] To test our hypothesis, we first identified the promotor containing region in *Cldn14* transcript variant 1 to be between 1200 and 1500 bp 5′ to the 5′ UTR. Next, we confirmed that the 1500 bp fragment 5′ to the 5′ UTR of this transcript variant also contains a CaSR-responsive region by cloning it into a luciferase reporter construct and assaying activity in the presence and absence of the CaSR and altered extracellular Ca^{2+}. Using a combination of pharmacological and genetic methods, we delineate a CaSR-*CLDN14* signalling pathway, which involves Gq/11-protein—p38 and SP1 signaling.

2 | MATERIALS AND METHODS

2.1 | RNA isolation and quantitative PCR

Renal tissue was used from a previously reported experiment after animals were treated with vehicle or cinacalcet.[15] Isolated total mRNA was reverse transcribed into cDNA. To do so, 1 μg of RNA was reverse transcribed using random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). 5 μl of 1 in 10 diluted cDNA (approximately 25 ng cDNA) was used as template to determine the gene expression of *Cldn14* variants. A mixture consisting of TaqMan universal qPCR master mix (Applied Biosystems Inc, Foster City, CA, USA), primer, probe and RNase free water was prepared and added to the cDNA in a 384-well plate (Applied Biosystems Inc, Foster City, CA, USA). As an internal control, mRNA levels of the housekeeping gene 18s ribosomal RNA were determined. Expression levels were quantified with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA). Primers and probes were made by IDT (Integrated DNA Technologies Inc, San Diego, CA, USA) or ABI (Applied Biosystems Inc, Foster City, CA, USA). The sequences of all primers and probes utilized are listed in (Table 1).

2.2 | Cloning and sequencing

Gene fragments were amplified by PCR. The PCR reaction (94℃ for 1 min, 94℃ for 30 s, primer melting temperature (60–65℃) for 30 s, 72℃ for 90 s, 39 cycles, 71℃ for 10 min and 4℃ forever) was carried out with a C1000 Thermal cycler (BioRad, Mississauga, ON, Canada) from mouse gDNA using cloning primers (IDT, Oralville, IA, USA) with unique restriction enzyme sites (Tables 2 and 3). The PCR products were run on an agarose gel and visualized with ethidium bromide. PCR products were then digested with enzymes corresponding to the unique restriction sites KpnI and BglII (New England Biolabs, Ipswich, MA, USA) and ligated into the PGL3 Basic and Enhancer (Promega, Madison, WI, USA) vectors that were previously linearized using the same restriction enzymes. CaSR

TABLE 1 Real-time PCR primers and probes

	Sequences
Claudin-14	Forward: TGGCATGAAGTTTGAAATCGG
	Reverse: CGGGTAGGGTCTGTAGGG
	Probe: TGAGAGACAGGGATGAGGAGATGAAGC
Variant 1	Forward: GGAATGGCATGTTCTGAAAGG
	Reverse: GCCTGAGGCGCACCTAGTT
	Probe: CAGCGTTGATAGCTG
Variant 2	Forward: TCCGTGGTCTACCTGAGAGCAT
	Reverse: AGCCACTCCACTCACATACAGAAC
	Probe: AAGGTGGATGGGACTGG
Variant 3	Forward: CAGCCACAGGGACCCATTAG
	Reverse: TTTGGAACAAGAATGCCAGAGA
	Probe: CGGCCGTGTGTAGAT

TABLE 2 List of cloning primers for mouse Cldn14 5′ UTR segments

Construct	Forward primer
1500	GGGGTACCCCTGATGTAGGTGGCCTCATTTC
1200	GGGGTACCCCCTTGAAGTCGAACCACACTATTG
1000	GGGGTACCCCGTCGAGCTCCGGAATTTGT
750	GGGGTACCCCCAAAGTGTCTTGTGCATGTGG
500	GGGGTACCCCCCCCTGGCAATAAAGTCAT

Note: All reactions used the same reverse primer which was GAAGATCTTCCTATCAACGCTGCCCTTTC.

TABLE 3 List of cloning primers for human Cldn14 5′ UTR segments

	Sequence
Forward primer	GGG GTA CCC CCC TGC CTT CTG ATG
Reverse primer	GAA GAT CTT CAA TCA GAC CCC CTG CTT TAG

mutants were created by performing site directed mutagenesis (Quick Change Lighting kit from Agilent, Santa Clara, CA, USA) on the human CASR construct in pCMV (Origene, Rockville, MD, USA) with specific primers listed in Table 4, according to the manufacturer's instructions. The fragment in each construct was sequenced by Eurofins MWG Operon (Huntsville, Alabama, USA) using both the forward and reverse cloning primer (Table 2).

2.3 | Cell culture

Human Embryonic Kidney (HEK293) and HeLa cells (acquired from ATCC (Rockville, MD, USA)) were grown and maintained as previously described in Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin streptomycin glutamine (PSG).[23] Rat TAL cells were a kind gift of Dr N. Ferreri and were maintained in Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin streptomycin glutamine (PSG).

2.4 | Dual-luciferase assay

The assay was carried out as per the manufacturer's directions (Promega Corp.). Cells were transfected as previously described.[23] In short, cells were plated in a 10-cm petri-dish 3–6 h prior to transfection. The Ca^{2+} phosphate method was employed to co-transfect HEK293 cells with pGL3 constructs containing firefly luciferase downstream of the *Cldn14* promoter; and the pRL-TK vector containing renilla luciferase as an internal control. Rat TAL cells were transfected with Fugene 6 HD reagent (Promega, WI, USA) as per the manufacturer's directions (Promega Corp.) Rat Tal cell were seeded at 3×10^5 per well into a 6 well-plate and incubated overnight at 37℃ with 5% CO_2 incubator. The media was changed to Opti-MEM. After an hour, Rat TAL cells were transfected with Fugene 6 HD with a reagent/DNA ratio of 3 to 1. After an overnight incubation, the media was changed to Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin streptomycin glutamine (PSG). Following a 24-h incubation period, the media was vacuumed carefully off and 3 ml of essentially Ca^{2+} free DMEM with 10% FBS and 1% PSG were added. Then the appropriate amount of 2 M $CaCl_2$ was added to create either a low (0.5 mM) or high (5 mM) extracellular Ca^{2+} concentration. Additional drugs were also applied at this time point. We preferentially used this method to activate the CaSR, instead of a calcimimetic, as we observed stimulation of the reporter construct even in the absence of the CaSR when treated with cinacalcet. The cells were then incubated for another 24 h. Media was vacuumed and cells were washed 2× with PBS. Thereafter, cells were lysed with the manufacturer supplied lysis buffer and 5–20 µl of extract was used to determine expression in a luminometer (DLR Ready, TD-20/20 Luminometer, Turner Design).

2.5 | Immunoblotting

Transfected cells were treated initially in an identical fashion as for the dual-luciferase assay. However, after 24-h treatment, the cells were washed, and then the total protein was extracted using RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% lgepal CA-630, pH 7.4) containing 1% protease inhibitor cocktail

TABLE 4 CaSR site-directed mutagenesis primers Ssed to create the mutants

	Sequences
CaSR R185Q	Forward: CAAGAATCAATTCAAGTCTTTCTTCCAAACCATCCCCAATGATGAGC
	Reverse: GCTCATTGGGGATGGTTTGGAGGAAAGACTTGAATTGATTCTTG
CaSR C129S	Forward: GAACCTTGATGAGTTCAGCAACTGCTCAGAGCAC
	Reverse: GTGCTCTGAGCAGTTGCTGAACTCATCAAGGTTC
CaSR A843E	Forward: GATTGCCATCCTGGAAGCCAGCTTTGGC
	Reverse: GCCAAAGCTGGCTTCCAGGATGGCAATC
CaSR L727Q	Forward: GCTCAACCTGCAGTTCCTGCAGGTTTTCCTCTGCACCTTCATGC
	Reverse: GCATGAAGGTGCAGAGGAAAACCTGCAGGAACTGCAGGTTGAGC

(Calbiochem, Gibbstown, NJ) and 1 in 100 of 0.1 M phenylmethane sulfonyl fluoride (PMSF) (Thermo Fisher Scientific, MA, USA). Immunoblotting was carried out as previously described.[15] In short, protein lysate was subjected to 8% SDS-PAGE and electroeluted to 0.45 μm PVDF membrane (Millipore, Burlington, MA). Mouse primary anti-CaSR monoclonal antibody (Gentex, catalog# GTX19347, 2456 Alton Pkwy Irvine, CA, 92606, USA), or anti-Myc-tag (9B11, Cell signaling, catalog #2276, Beverly, MA, 01915, USA), or rabbit anti-SP1 (Thermofisher Scientific, catalog# PA5-95583, Waltham, MA 02 451, USA), was applied overnight at 4℃, followed by incubation with the appropriate secondary horseradish peroxidase-coupled secondary antibody (anit-mouse-HRP linked, catalog#7076, anti-rabbit -HRP linked, catalog# 7074, Cell signaling, Beverly, MA, 01915, USA). For internal control, blots were stripped and blotted for β-actin (Thermo Fisher scientific, catalog# MA5-15739, MA, USA). Proteins were detected with Immobilon Crescendo Western HRP substrate (Millipore, Burlington, MA) and visualized using a ChemiDoc Imaging System (Bio-Rad, Hercules, CA).

2.6 | Immobilized iron affinity electrophoresis

Cell lysates were prepared, and gel electrophoresis was performed as above with slight modifications. The lysis buffer contained 1% phosphatase inhibitors (Calbiochem, Gibbstown, NJ, USA) and 1% protease inhibitor cocktail (Calbiochem, Gibbstown, NJ, USA) and 1 μM phenylmethane sulfonyl fluoride (Thermo Fisher Scientific, MA, USA). Prior to electroelution, half of the wells were preloaded with 20 mM $FeCl_3$ and half without $FeCl_3$, and left to polymerize. All samples were run on $FeCl_3$-free and $FeCl_3$-loaded wells on the same gel and immunoblotting was performed as above. The same anti-myc and secondary anit-mouse HRP antibodies were employed. ImageJ (US National Institutes of Health, Bethesda, MD) was used to semi-quantify SP1. Each sample was normalized to β-actin as a loading control. To calculate phospho-protein abundance, protein expression from the $FeCl_3$ loaded well sample was subtracted from the total protein abundance (i.e., non $FeCl_3$ loaded lane) and then normalized to total protein abundance.

2.7 | siRNA transfection

HEK293 cells were co-transfected with pGL3 constructs containing firefly luciferase downstream of the *Cldn14* promoter; and the pRL-TK vector containing renilla luciferase as an internal control, by the Ca^{2+}-phosphate method. After a 24-h incubation, the medium was changed to Opti-MEM, then an hour later, cells transfected with 40 nM of p38 siRNA or scrambled siRNA (p38 siRNA: #6564, scrambled siRNA: #6568, cell signaling, Beverly, MA, USA) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. After an overnight incubation, the media was changed to DMEM with 10% FBS and 1% PSG with either a low (0.1 mM) or high (5 mM) extracellular Ca^{2+} concentration. The cells were then incubated for another 24 h. Preparation of cell lysate, the DLA, and immunoblots was carried out as above.

2.8 | Immunohistochemical staining of tissue for light microscopy

C57BL/6 mice were anesthetized using isoflurane and perfusion fixed through the left ventricle using 10% formalin. Kidneys were stored in 10% formalin overnight, dehydrated in graded ethanol and tissueclear and then embedded in paraffin. Staining of formalin-fixed paraffin-embedded kidney tissue was done as previously described.[24] Sections were incubated in tissueclear and rehydrated in a series of graded ethanol before heat-induced antigen retrieval using 10 mM Tris and 0.5 mM EGTA buffer (TEG, pH 9.0). Endogenous peroxidase enzymes were blocked using 0.6% H_2O_2 and free aldehyde groups blocked using 50 mM

NH_4Cl in PBS. Sections were incubated overnight at 4℃ with primary anti-SP1 antibody (#PA5-95583, Invitrogen) in 0.1% Triton X-100 in PBS, subsequently washed and incubated with secondary horseradish peroxidase-conjugated secondary antibodies (DakoCytomation). SP1 immunoreactivity was visualized using the Liquid DAB+ Substrate Chromogen System (K3467, DakoCytomation). Thereafter, the sections were boiled again in TEG buffer to remove bound antibodies and a second round of incubation with rabbit polyclonal antibodies against NKCC2 was performed (SLC12A1, HPA014967, Sigma-Aldrich). Following secondary horseradish peroxidase-conjugated antibody incubation, the second antigen was visualized using the Vector SG chromogen substrate (Vector Laboratories). Sections were counterstained with hematoxylin and mounted using aquamount. Light microscopy was carried out using an Olympus BX51 microscope.

2.9 | In silico analysis

To predict putative transcription factor binding sites for the Sp1 transcription factor upstream of *Cldn14* gene transcript variant 1, we used the JASPAR vertebrate database.[25] We queried a region 1500 bp 5′ to the 5′ UTR of the mouse *Cldn14*transcrpit variant 1 using a relative threshold score of 85%.

2.10 | Statistics

All luciferase reporter assay results were normalized to the luminescence from the internal control, pRL-TK, prior to analysis. Data are presented as means ± SEM, with all data reported being based on measurements made on at least 4 different independent samples. Data were tested for normal distribution with a Shapiro-Wilk test. Data sets with normal distribution were subjected to Students' *t*-tests (GraphPad, La Jolla, CA), One-way ANOVA or Friedman test to determine statistical significance as appropriate. Data sets that were not-normally distributed were subjected to nonparametric statistical analyses as described in the figure legends. Values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Mouse *cldn14* transcript variant 1 contains a promoter and a CaSR-responsive element 1500 bp upstream of exon 1

The mouse *Cldn14* gene consists of 4 different splice variants, which when transcribed produce different 5′ untranslated regions. However, the coding regions are identical and hence code for the same protein. To determine which of the *Cldn14* transcript variants are subjected to regulation by the CaSR in the kidney, i.e., which variant is responsive to CaSR regulation, we performed quantitative real-time PCR on mRNA extracted from mice treated with vehicle or cinacalcet hydrochloride, a positive allosteric modulator of the CaSR (1 mg/g body weight for 5 days). We have previously shown that *Cldn14* expression is markedly upregulated in these mice.[15] Using primers and probes specific for the 5′ untranslated regions (UTR) of the different murine *Cldn14* variants (Table 1), we assessed their expression after treatment with vehicle or cinacalcet. *Cldn14* transcript variant 1 displayed a cinacalcet-sensitive increase in expression similar to that of the coding region, whereas variants 2 and 3 did not increase their expression after treatment with cinacalcet (Figure 1B). These results indicate that mouse *Cldn14* transcript variant 1 is responsive to CaSR activation.

To identify the promoter region of this variant, we cloned increasingly longer lengths of DNA from the 5′ end of the 5′ UTR of *Cldn14* Variant 1 into the PGL3 basic vector, upstream of the firefly luciferase gene (Figure 2A). We co-transfected these constructs with the PRL-TK control vector, containing the renilla luciferase gene as an internal control for variation in transfection. We found that the 1500 bp fragment produced significantly greater luciferase activity compared to the empty vector, PGL3 basic vector (Figure 2B). None of the other constructs showed significantly different luciferase activity when compared to the empty vector. These results are consistent with the promoter being present within the 1500 bp fragment 5′ to the 5′ UTR of the *Cldn14* transcript variant 1, specifically between 1200 and 1500 bp.

Although the results above indicate that 1500 bp 5′ to the 5′ UTR of mouse *Cldn14* variant 1 contains the promoter region, whether this region also contains an element sensitive to CaSR activation is not clear. To assess if this region also contains a CaSR-sensitive element, we transfected the 1500 bp construct and PRL-TK control vector into HEK293 cells with either a human *CASR* containing vector or the empty vector (pCMV6) and then incubated them with varying concentrations of extracellular Ca^{2+} (Figure 3A). A minimum of a 2-fold increase in luciferase activity was observed in cells co-transfected with the *CASR* relative to cells co-transfected with the empty vector. This is consistent with the 1500 bp fragment of DNA, 5′ to the 5′ UTR of mouse *Cldn14* variant 1, containing an element sensitive to CaSR activation. Moreover, increasing extracellular Ca^{2+} concentrations ($[Ca^{2+}]$) demonstrated a dose-dependent increase in luciferase activity that was highest at 5 mM $[Ca^{2+}]$ and lowest at 0.1 mM $[Ca^{2+}]$ (Figure 3B).

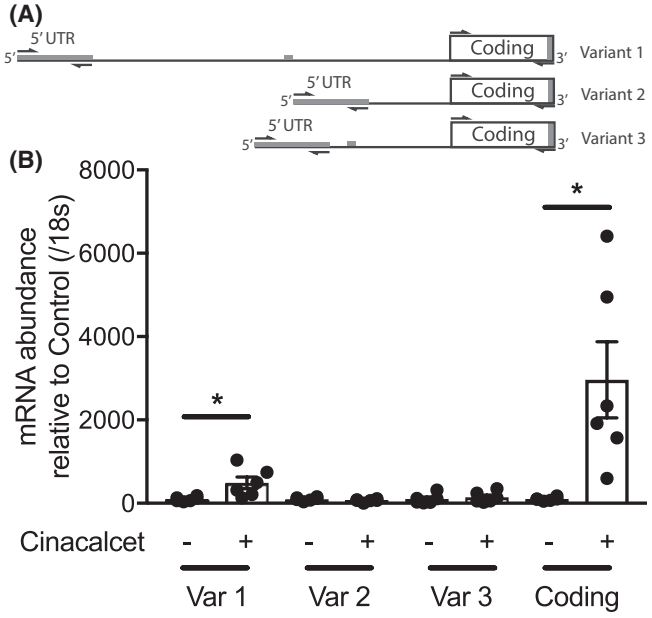

FIGURE 1 (A) A cartoon depicting the mouse *Cldn14* variants. Variants not to scale; introns have been truncated. (B) mRNA abundance of the mouse *Cldn14* variants as well as the coding region isolated from kidneys of mice treated with vehicle or cinacalcet (1 mg/g body weight for 5 days). Mouse *Cldn14* variant 1 showed significantly greater expression after cinacalcet treatment. Expression was normalized to 18s (One-way ANOVA; $*p < .05$)

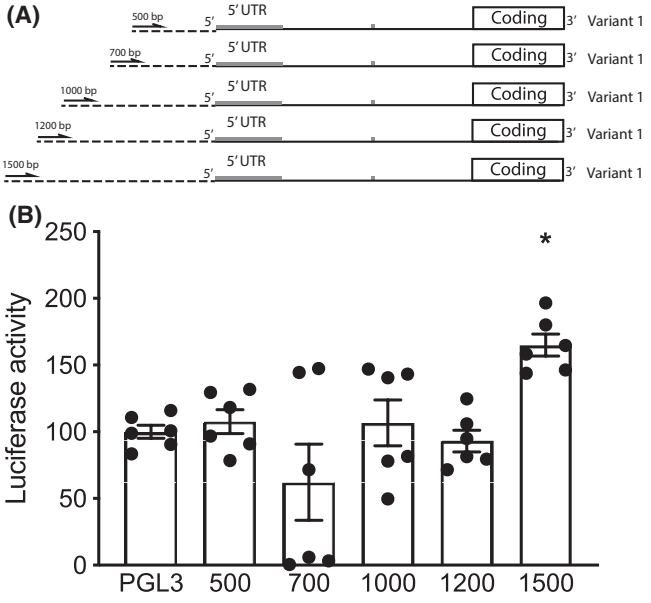

FIGURE 2 (A) Cloning strategy used to identify the *Cldn14* variant 1 promoter. (B) Luciferase activity from different lengths of DNA 5′ to the 5′ UTR of mouse *cldn14* variant 1 cloned into pGL3 basic vector. The 1500 bp fragment displayed significantly greater expression compared to the empty vector (PGL3), difference (One-way ANOVA; $*p < .05$)

To identify the homologous claudin-14 variant in humans we performed a basic logical alignment search (BLAST) with the 1500 bp fragment 5′ to mouse claudin-14 variant 1 against the human genome (GRCh38.p13 reference assembly). This revealed a high degree of homology to the region 5′ to human claudin-14 transcript variant 3. We then cloned the 1500 bp segment 5′ to the human claudin-14 transcript variant 3 into the pGL3 basic vector and performed dual luciferase assays as previously for the mouse constructs. This fragment revealed a significant increase in luciferase activity relative to the empty PGL3 basic vector (p-value = .018), consistent with claudin-14 transcript variant 3 in humans being CaSR responsive as per variant 1 in mice.

To translate our results to a potentially more physiologically relevant cell model, we transfected the 1500 bp construct and PRL-TK control vector into rat mTAL cells, a model that likely expresses the CaSR,[26,27] and exposed them to vehicle or the calcimimetic, cinacalcet, or increased extracellular [Ca^{2+}] which resulted in significantly increased promoter activity (Figure S1A,B). We also transfected the 1500 bp construct and PRL-TK control vector into rat mTAL cells with either a human *CASR* containing vector or the empty vector (pCMV6) and then incubated them with varying concentrations of extracellular Ca^{2+}. This yielded a similar response as in HEK293 cells with significantly increased reporter activity observed when the CaSR expressing cells were exposed to increased extracellular [Ca^{2+}] (Figure S1C).

3.2 | CaSR-mediated increases in *Cldn14* expression occur via a Gαq/11 mediated activation of the PLC pathway, not via Gαi or Gαs

Following the generation of a construct containing both the promoter of the renal regulated *Cldn14* transcript variant and the CaSR responsive element upstream of firefly luciferase, we next sought to employ this tool to delineate the cellular pathway by which CaSR activation modulates *Cldn14* expression. To this end, we first pharmacologically modulated the known Gα-proteins coupled to the CaSR and measured luciferase activity of HEK293 cells co-transfected with this construct, PRL-TK, and empty or a CaSR containing vector. These cells were exposed to either low or high extracellular Ca^{2+} (0.1 or 5 mM). Exposing the cells to different extracellular [Ca^{2+}] did not alter luciferase activity in cells not transfected with the CaSR (Figure 4). However, as expected, luciferase activity significantly increased when exposed to high vs low extracellular [Ca^{2+}] when co-transfected with the CASR (Figure 4A–D).

Pertussis toxin (PTX) is a known Gαi inhibitor.[28] When cells were exposed to this toxin, luciferase

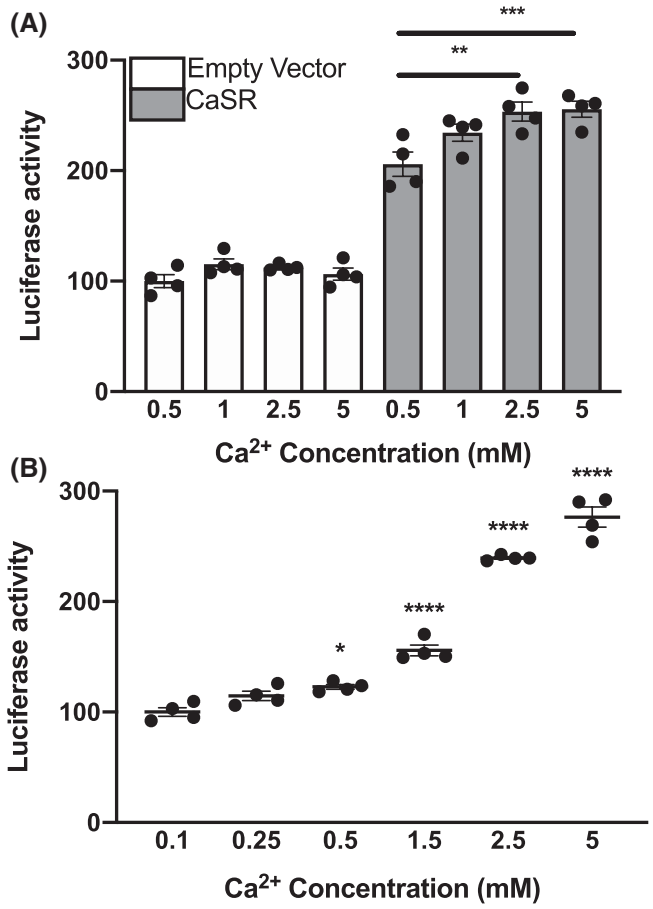

FIGURE 3 (A) Luciferase activity of HEK293 cells co-transfected with the 1500 bp *Cldn14*-reporter construct and either empty vector or the CaSR incubated with varying concentrations of extracellular Ca^{2+}. CaSR transfected cells had significantly greater luciferase activity compared to the empty vector (***$p < .001$; **$p < .01$). (B) Luciferase activity of cells expressing the CaSR and the 1500 bp Cldn14 reporter construct exposed to varying amounts of extracellular Ca^{2+} (Kruskal-Wallis Test; ****$p < .0001$; *$p < .05$)

activity was further increased in the presence of high [Ca^{2+}] 5 mM (Figure 4A). This suggest that a GαI independent signaling pathway is driving CaSR dependent *Cldn14* expression (and that Gαi signaling attenuates CaSR mediated increases in *Cldn14*). Further, forskolin, which increases cAMP levels (mimicking Gαs activity), did not alter luciferase activity in the presence of either a 0.5 or 5.0 mM extracellular Ca^{2+} concentration (Figure 4B), excluding Gαs as the coupled G protein, driving *Cldn14* expression. It also supports the PTX data, as decreased CAMP is downstream of Gαi activation. In contrast, U73122, an inhibitor of Gαq/11 signaling,[19] attenuated the increased luciferase activity of HEK293 cells expressing the *Cldn14* promoter and the CaSR when exposed to high extracellular Ca^{2+} but not to low extracellular Ca^{2+} (Figure 4C). Together, these results are consistent with CaSR mediated increased *Cldn14* expression occurring, at least in part through a Gαq/11 pathway, as at high extracellular [Ca^{2+}], where the CaSR is activated, U73122 attenuated reporter activity.

To delineate the downstream signaling pathway after Gαq/11, we employed U0126, a MKK1/2 inhibitor in HEK293 cells transfected with the *Cldn14* 1500 bp construct, PRL-TK, and empty or a CaSR containing vector. U0126 did not alter reporter activity in the presence or absence of the CaSR, nor did it alter the response to increased extracellular [Ca^{2+}], indicating that CaSR signaling mediated increase in *Cldn14* expression does not occur through MKK1/2 (Figure 4D).

3.3 | CaSR activation signals through a p38 pathway to increase *Cldn14* expression

The CaSR has been shown to phosphorylate and activate the mitogen-activated protein kinases (MAPKs) including JNK and p38, in various cell lines and tissues, including epithelia.[29,30] Therefore, we set out to identify which downstream MAPKs are involved in this signaling pathway by employing SP600125, a JNK inhibitor, in our model system. Similar to U0126, SP600125 did not alter *Cldn14* reporter activity in the presence or absence of the CaSR, regardless of the extracellular Ca^{2+} concentration (Figure 5A). To ascertain whether p38 signaling is downstream of CaSR activation, we knocked down p38 and repeated the studies (Figure 5B,C). p38 knockdown prevented the increase in Cldn14 reporter activity induced by high Ca^{2+} in the presence of the CaSR. Together, these results are consistent with CaSR mediated increased *Cldn14* expression not occurring through JNK, but instead being downstream of p38.

3.4 | SP1 acts as a repressor of *Cldn14* expression

SP1 is a transcription factor known to be activated by the CaSR, that transcriptionally represses the expression of some claudins.[20–22] To examine if SP1 is a downstream transcriptional repressor of *Cldn14* expression, regulated by CaSR activation, we co-transfected empty vector or *Sp1* into our experimental model system and assayed *Cldn14* reporter activity. In cells not expressing the CaSR, *Cldn14* reporter activity of cells co-transfected with *SP1* was significantly lower in the presence of either low, or high, extracellular Ca^{2+} relative to cells co-transfected with empty vector (EV) (Figure 6A). This was also observed in cells expressing the CaSR, albeit only under high extracellular [Ca^{2+}] (Figure 6A). These results support a role for SP1 in

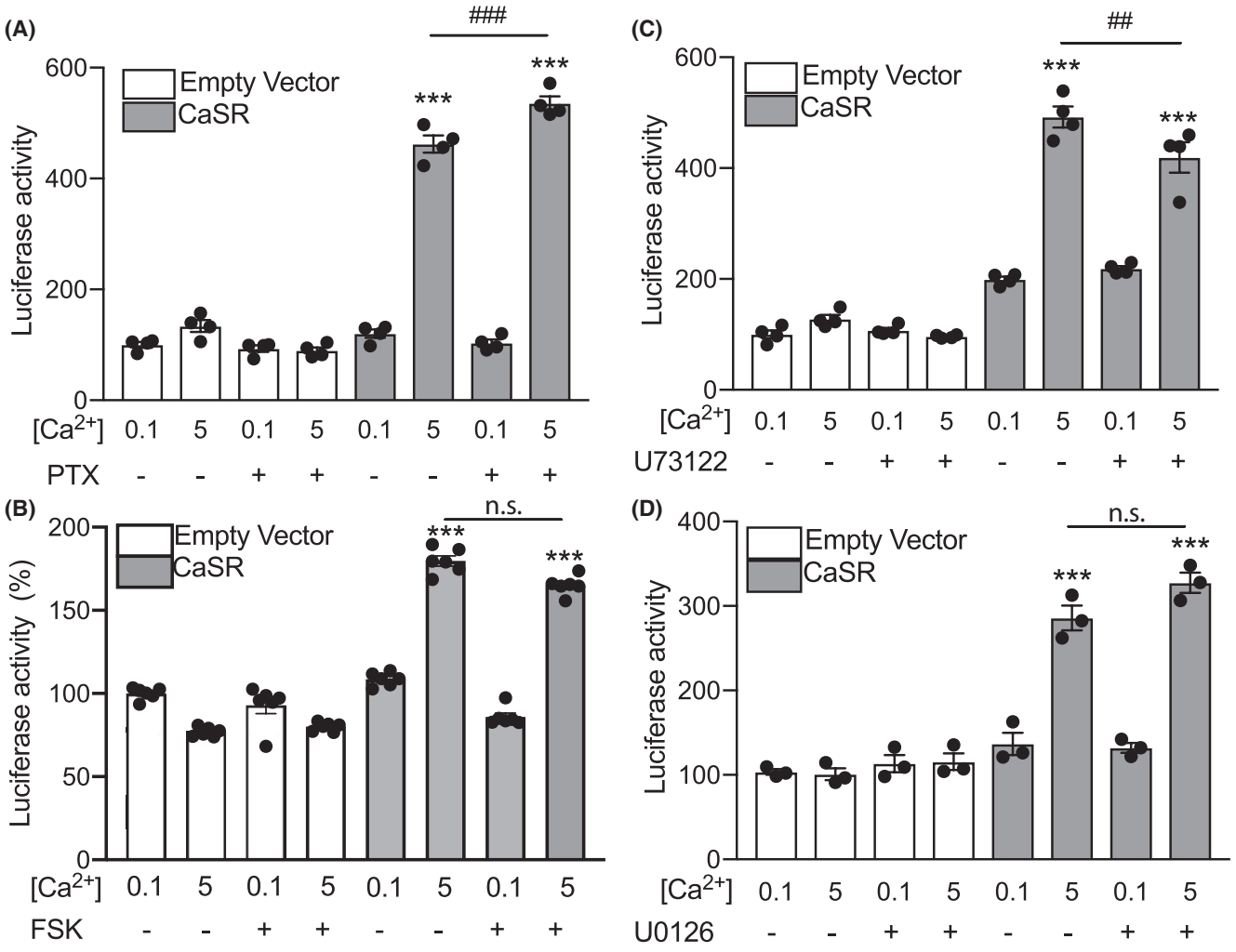

FIGURE 4 Luciferase activity from the *Cldn14* reporter construct after treatment with pertussis toxin (PTX), forskolin, U73122 or U0126. (A) PTX further increases luciferase activity after exposure to high extracellular Ca^{2+} concentration. (B) Forskolin does not alter luciferase activity. (C) U73122 attenuates reporter activity when cells are exposed to high extracellular Ca^{2+}. (D) U0126 did not significantly alter luciferase activity in the presence of either low or high extracellular Ca^{2+} concentration. (One-Way ANOVA; $^{***/###}p < .001$; $^{##}p < .01$)

repressing *Cldn14* expression, regardless of whether the cells were co-transfected with empty vector or CaSR.

To confirm whether SP1 is a repressor activated by the CaSR, we first conducted in silico studies, where we attempted to predict with JASPAR software possible SP1 binding sites within the 1500 bp DNA fragment upstream of the 5′ UTR of *Cldn14* variant 1. This identified a likely binding sequence in the 1355–1346 bp region at a relative threshold score of 87%. Secondly, we performed immunohistochemistry. Renal sections were stained with antibodies against both SP1 and the furosemide sensitive cotransporter NKCC2 to identify the thick ascending limb. SP1 was identified primarily in the nuclear compartment in the majority of tubular epithelial cells throughout the section, including cells of the TAL expressing NKCC2. However, there was no visible differences in nuclear Sp1 localization in TAL cells versus others (Figure 6C,D). Next, we performed immobilized iron affinity immunoblots to examine the relative phospho-SP1 protein abundance in the presence and absence of the CaSR and low or high extracellular Ca^{2+}. Activation of the CaSR by high extracellular Ca^{2+} decreased the amount of phospho-SP1 (Figure 6E,F), an effect not observed in the absence of the CaSR. This is consistent with the activation of the CaSR decreasing the amount of phosphorylated SP1, an effect that affects the binding of SP1 to DNA and thus the ability to regulate gene expression.[31] Finally, we treated rat mTAL cells expressing the CaSR and the Cldn14 reporter construct with either vehicle or the SP1 inhibitor mithramycin. The SP1 inhibitor increased reporter activity in the low extracellular Ca^{2+} exposed cells, which was not further enhanced by increasing extracellular Ca^{2+} (Figure S1D). Together this work collectively supports CaSR activation regulating claudin-14 expression via preventing SP1 mediated repression.

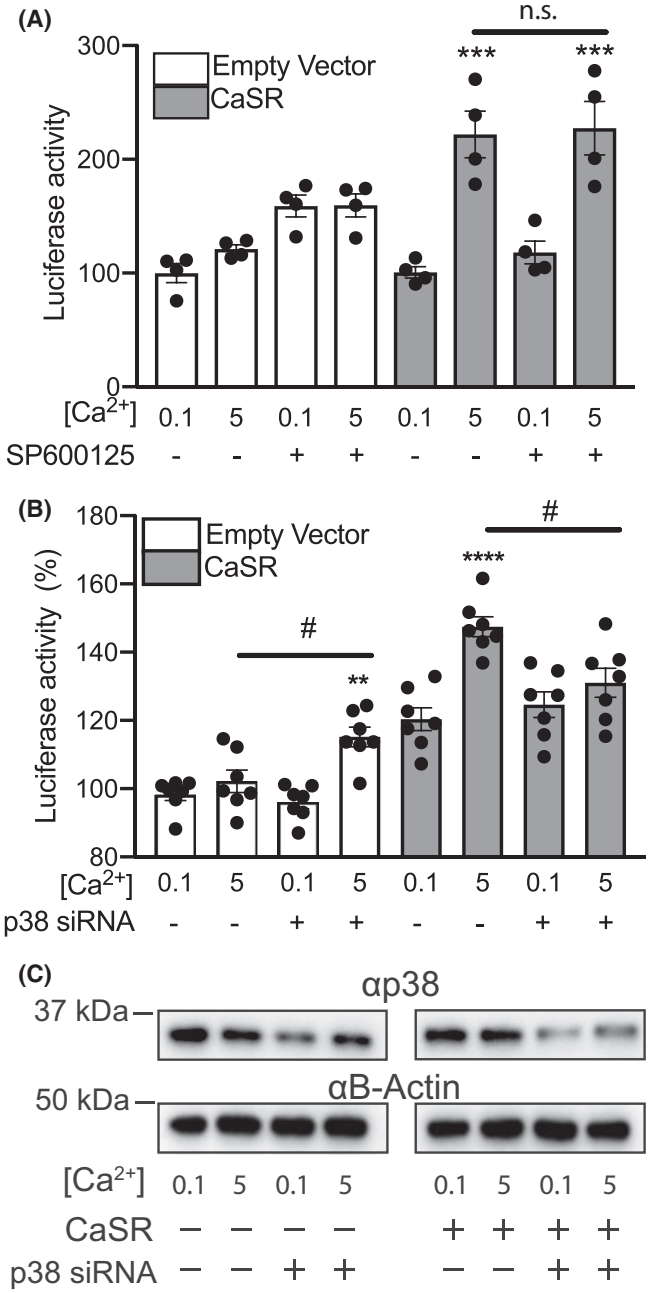

FIGURE 5 Luciferase activity from the *Cldn14* reporter construct after treatment with MAPK inhibitors. (A) SP600125, a JNK inhibitor, does not alter luciferase activity. (B) Transfection of p38 siRNA prevented increased Cldn14 reporter activity induced by high [Ca^{2+}]. Ordinary one-way ANOVA were used to determine statistical difference ($^{***/\#\#\#}p < .001$; $^{\#\#}p < .01$; $^{\#}P < .05$). (C) Representative immunoblots demonstrating knock-down of p38, by p38 siRNA but not scrambled siRNA

3.5 | *Cldn14* transcription is enhanced by overactivating CaSR mutations and repressed by inactivating CaSR mutations

There are various pathological mutations of the CaSR that result in altered Ca^{2+} homeostasis in humans. Using site-directed mutagenesis, we made an inactivating CaSR mutant (R185Q) and several over-activating CaSR mutants (C129S, A843E, L727Q). We proceeded to perform *Cldn14* reporter assays in cells expressing the *Cldn14* 1500 bp reporter construct and PRL-TK with these mutants, exposed to either low extracellular Ca^{2+} (0.5 mM) or high extracellular Ca^{2+} (5 mM). Reporter activity of the wild-type CaSR was greater than the empty vector as expected (Figure 7). When incubated with low extracellular Ca^{2+}, reporter activity from the cells also expressing the inactivating CaSR mutant R185Q was significantly lower than that of the wild-type CaSR. Further the activating CaSR mutants C129S, A843E, L727Q all contributed significantly greater reporter activity in the presence of low extracellular Ca^{2+} (Figure 7). When incubated with high extracellular Ca^{2+}, reporter activity from the inactivating CaSR mutant R185Q was lower than that of the wild-type. However, the over-activating CaSR mutants C129S, A843E, L727Q were not significantly different from the wild-type. These results further support a role for *CaSR* mediated alterations in renal *Cldn14* expression, where an inactivating mutation decreases *Cldn14* expression and activating mutations increase *Cldn14* expression.

4 | DISCUSSION

The majority of Ca^{2+} filtered at the glomerulus is reabsorbed under normal physiological conditions.[8,32] This process is subject to regulation by hormones including PTH and 1,25-dihydroxyvitamin D_3. Tubular Ca^{2+} reabsorption is also directly regulated by extracellular Ca^{2+}, independent of these hormones. However, hormone-independent regulation of renal Ca^{2+} transport is not as well understood. The CaSR localizes to the basolateral membrane of the thick ascending limb,[33–35] where we and others have previously demonstrated that its activation results in increased *Cldn14* expression, thereby increasing urinary Ca^{2+} excretion.[14,15] It was therefore our objective to delineate the cellular pathway between CaSR activation and increased *Cldn14* expression. Here, we report that the *Cldn14* gene has a CaSR-responsive region in the segment 1500 bp 5′ to the 5′ UTR of variant 1. This region is sensitive to CaSR signaling through a Gq/11-protein-PLC coupled pathway, which activates p38 to inhibit Sp1, a transcriptional repressor of *Cldn14*. This is predominantly based on three observations. (1) Only in the presence of the CaSR was a *Cldn14* reporter construct, containing this 1500 bp fragment, able to yield increased activity when incubated with increased extracellular Ca^{2+}. (2) Reporter activity was attenuated by PLC inhibition, p38 knock-down and SP1 co-expression, and (3) activation of the CaSR by high extracellular Ca^{2+} reduced SP1 phosphorylation. Taken together, these results suggest a signaling

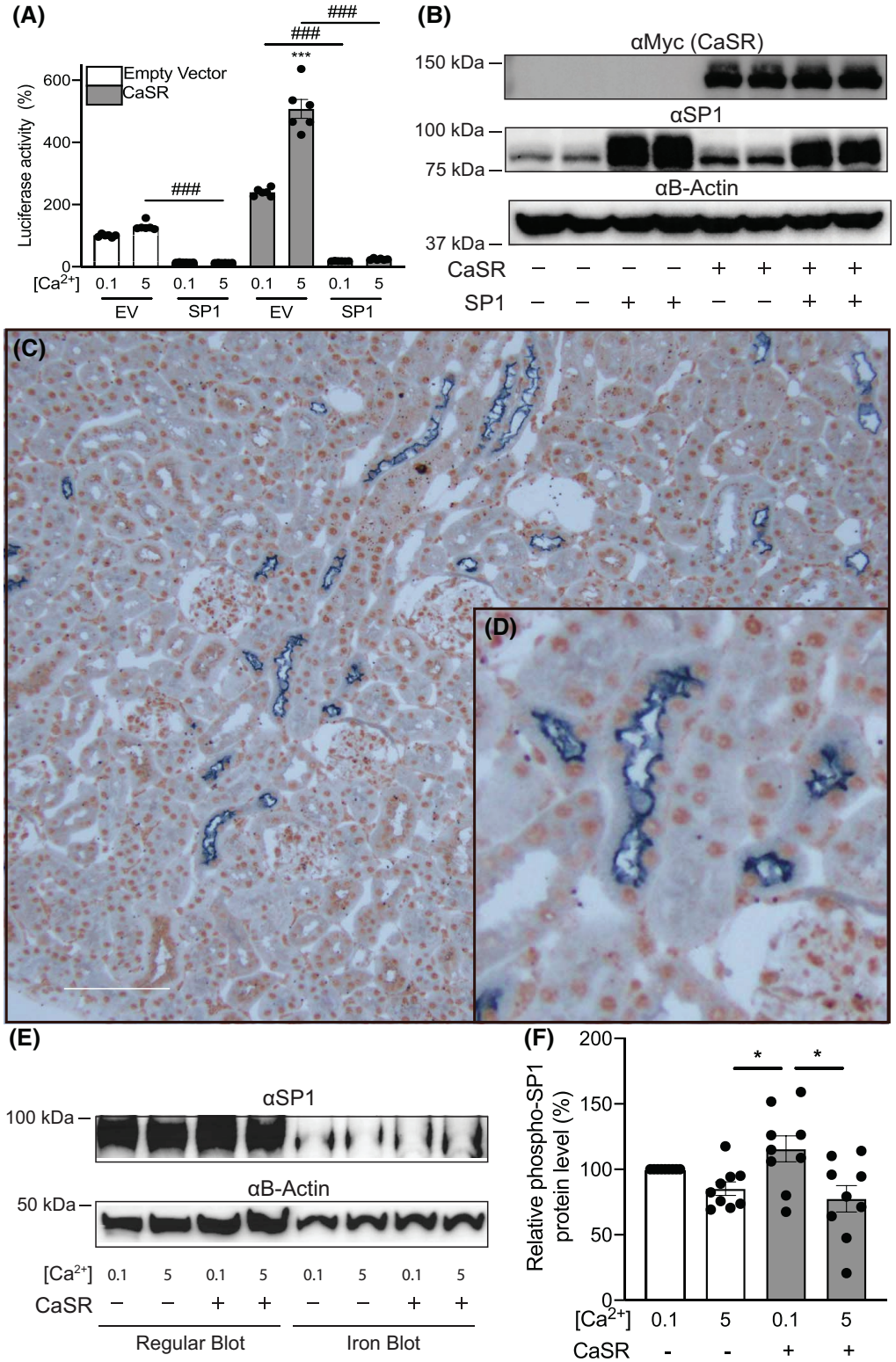

FIGURE 6 SP1 inhibits expression of the 1500 bp cldn14 promoter reporter construct. (A) Luciferase activity from the *Cldn14* reporter construct after transfection with the transcription factor SP1. SP1 cotransfection significantly decreased luciferase activity of cells cotransfected with Empty Vector (EV) or the CaSR under low and high Ca^{2+} concentrations. Ordinary one-way ANOVA was used to determine statistical difference ($^{***/\#\#\#}p < .001$; $^{\#\#}p < .01$; $^{\#}p < .05$). (B) Immunoblots demonstrating co-expression of the CaSR with SP1. (C) Immunohistochemical staining of a murine renal section for SP1 (brown) and NKCC2 (blue). (D) high magnification image of thick ascending limb with nuclear localization of SP1. (E) Representative immunoblots of myc tagged SP1 run on regular or $FeCl_3$-loaded gels. (F) Relative phospho-SP1 protein abundance decreased when cells were incubated in with high $[Ca^{2+}]$ and expressed the CaSR. Friedman test with Dunn's multiple comparisons test were used to determine statistical differences ($^{*}p < .05$)

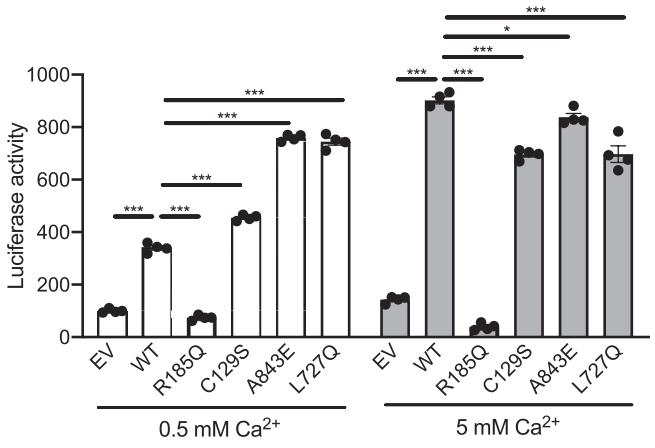

FIGURE 7 (A) *Cldn14* reporter activity after cotransfection with either wild-type, an inactivating (R185Q) or overactivating (C129S, A843E, L727Q) CaSR mutants and cultured with either low or high extracellular Ca^{2+}. The inactivating mutation R185Q showed significantly lower luciferase activity compared to WT. In the presence of low extracellular Ca^{2+} the overactivating mutations showed significantly greater luciferase activity compared to WT. However, overacting mutations did not confer significantly greater reporter activity when incubated with high extracellular Ca^{2+} conditions. Ordinary one-way ANOVA used to determine statistical difference (***$p < .001$; *$p < .05$)

pathway, where the basolateral CaSR in the TAL increases *Cldn14* expression through a PLC-p38-Sp1 pathway.

The CaSR can signal through small G-proteins including G_i and $G_{q/11}$.[36] Previous studies in parathyroid hormone producing chief cells, endothelial cells and intestinal epithelium cells have reported a role for phospholipase C (PLC) in the downstream activation of the CaSR.[18,19,37,38] Consistent with these studies, we found that CaSR activation in our experimental model increases *Cldn14* expression via the $G_{q/11}$—PLC pathway. Consistent with this being a major signaling pathway for the maintenance of Ca^{2+} homeostasis, loss-of-function and gain-of-function mutations in the *GNA11* gene, which encodes the $G\alpha_{11}$-protein, cause familial hypocalciuric hypercalcemia (FHH) type 2 and autosomal dominant hypocalcemia (ADH) type 2, respectively.[39] The pathway we delineate here may therefore contribute to the pathophysiology of FHH and ADH. During periods of elevated serum Ca^{2+}, loss-of-function mutations in *GNA11* would prevent signaling and render TAL cells unable to increase *Cldn14* expression, leading to hypocalciuria and hypercalcemia. Conversely, gain-of-function mutations in *GNA11* would cause enhanced signaling and thus increase *Cldn14* expression. Together, these findings support CaSR-mediated increased *Cldn14* expression via a $G_{q/11}$—PLC pathway.

Previously, we performed a microarray on whole kidney RNA isolated from mice treated with cinacalcet for 5 days.[15] Of the renal genes upregulated in mice treated with cinacalcet, we identified *Map3k6*, a component of a protein kinase signal transduction cascade.[40] It has been reported that MAP3K6 activates the c-Jun N-terminal kinase (JNK) signaling pathway.[41,42] Interestingly, we were not able to observe any changes in *Cldn14* promoter activity with administration of a JNK inhibitor. However, knockdown of p38 (a similar mitogen-activated protein kinase pathway to that of JNK) prevented the CaSR mediated increase in Cldn14 expression, consistent with p38 being a downstream regulator of CaSR signaling.[29,30]

We also identified SP1 as a transcription factor likely responsible for constitutive repression of *Cldn14*, with CaSR activation decreasing SP1 repression. This is consistent with previous studies that found Sp1 to be one of the transcription factors downstream of CaSR activation via a MAPK pathway.[20] Moreover, Sp1 is a key repressor of *CLDN3* and *CLDN4* expression.[21,22] Using immunohistochemistry, we were unable to detect differences in SP1 expression in whole kidney slices, even after conditions causing CaSR activation (data not shown). However, we did find that mithramycin, a SP1 inhibitor, increased reporter activity in the presence of low extracellular Ca^{2+} that was not further increased by high extracellular Ca^{2+}, further implicating SP1 in this pathway. Moreover, we observed reduced SP1 phosphorylation upon CaSR activation. These results are thus consistent with CaSR activation ultimately leading to decreased SP1 phosphorylation via the MAPK pathway.[43–45] This likely in turn prevents SP1 DNA binding and thus reduces *Cldn14* repression.

It should be noted that CLDN14 expression is constitutively suppressed by microRNAs (specifically miR-9 and miR-374) which bind to the 3′ UTR of the gene.[13,16] However, the reporter construct generated for our studies only contains non-coding intronic DNA 5′ to the 5′ UTR of mouse claudin-14 variant 1. Therefore, repression of reporter activity by this pathway will not be observed. This likely accounts for the higher baseline activity of the reporter in the presence of the CaSR as opposed to the empty vector, even when cells were incubated with lower extracellular Ca^{2+}.

Mutations in the *CASR* gene results in significantly altered Ca^{2+} homeostasis. Familial hypocalciuric hypercalcemia (FHH) results from a loss-of-function CaSR mutation, with patients often presenting with mild hypercalcemia, hypocalciuria and either normal or mildly elevated parathyroid hormone levels (PTH).[46] Conversely, autosomal dominant hypocalcemia (ADH1) results from a gain-of-function *CaSR* mutation, where patients often present with hypocalcemia and low or mildly suppressed PTH levels.[47] We were able to create some of these mutations and express them in our experimental model. We found that loss-of-function mutations in the *CASR* gene decreases *Cldn14* reporter activity regardless of the extracellular [Ca^{2+}],

whereas gain-of-function mutations increases *Cldn14* promoter activity only under low extracellular [Ca^{2+}]. These results are consistent with the respective phenotypes of FHH and ADH and may help explain their pathophysiology. In FHH, loss-of-function mutations in the CaSR prevent signaling in the presence of high plasma Ca^{2+} and thus would prevent increased *CLDN14* expression and the resulting decreased paracellular Ca^{2+} reabsorption from the TAL and thus reduced urinary Ca^{2+} excretion, leading to hypercalcemia with inappropriate hypocalciuria. In contrast, ADH1 gain-of-function mutations would lead to inappropriately increased *CLDN14* expression, reduced Ca^{2+} reabsorption from the TAL, and hypercalciuria, thereby exacerbating hypocalcemia. Our study highlights the possible renal pathophysiological mechanism contributed by CaSR mutations. However, future studies employing animal models of kidney-specific CaSR mutations would help further delineate the pathophysiology of these diseases.

In summary, we demonstrate a cell signaling pathway that contributes CaSR mediated *Cldn14* expression. We identified the promoter region of *Cldn14* is contained within the 1500 bp 5′ of 5′ UTR of mouse variant 1 and found that this DNA sequence is under constitutive repression by Sp1. Activation of the CaSR ultimately reduces Sp1 activity by limiting nuclear entry. This occurs through a $G_{q/11}$-PLC-p38 pathway, resulting in increased *Cldn14* expression. These studies contribute to our understanding of Ca^{2+} homeostasis, providing evidence of hormone-independent regulation of Ca^{2+} transport in the TAL, while providing a possible explanation for altered Ca^{2+} homeostasis observed in FHH and ADH due to *CASR* and *GNA11* mutations.

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DISCLOSURES

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Justin J. Lee, Jawad Alzamil, Saba Rehman, Henrik Dimke, and R. Todd Alexander conception and design of research; Justin J. Lee, Jawad Alzamil, Saba Rehman, Wanling Pan, Henrik Dimke and R. Todd Alexander performed experiments and analyzed data; Justin J. Lee, Jawad Alzamil, Saba Rehman, Henrik Dimke and R. Todd Alexander interpreted results of experiments; Justin J. Lee and R. Todd Alexander prepared figures and drafted the manuscript; all authors edited, revised, and approved final version of manuscript.

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SUPPORTING INFORMATION

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