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Article

Development and Applications of a Novel High Throughput Screening Assay to Identify a New First-In-Class Therapy for Kidney Disease

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SUMMARY

Chronic kidney disease (CKD) is a slowly progressing chronic illness with no cure. Recent discovery by Gyarmati et al identified a kidney tissue regenerative mechanism controlled by a specific kidney cell type, named macula densa (MD). MD activation is linked to intracellular calcium signaling. This project aimed to develop a novel high throughput assay and screen (HTS) prototype using MD cells to facilitate much needed CKD drug discovery.

Gene transfer was performed on the immortalized macula densa cell line, MDgeo using a viral vector, AVV9/GCaMP8f for the expression of the calcium-sensitive fluorescent protein GCaMP8f. MDgeo-G cells were subcultured in microplates and differentiated before calcium measurements using fluorescence microplate reader, or confocal microscopy.

Confocal fluorescence microscopy of MDgeo-G cells confirmed the high expression of GCaMP8f indicated by the 5-fold increase in the GCaMP8f fluorescence intensity compared to background. HTS assay validation was performed using tool compounds that are known to increase MD calcium in vivo. AVP or isoproterenol treatment of MDgeo-G cells triggered approximately 2-fold increases of GCaMP8f fluorescence intensity compared to baseline. Screening a small test compound library of 28 small molecules identified a hit compound that can be developed into a lead compound in future work.

In conclusion, the present study successfully developed a new, live-cell-based HTS prototype using gene technology, and targeting a key kidney cell type that is linked to kidney regeneration. The first real-world application of the new HTS identified a new potential lead compound that is a promising small molecule drug candidate for CKD.

INTRODUCTION

About thirty-seven million adults in the United States have been diagnosed with chronic kidney disease (CKD), meaning more than one in seven U.S. adults have CKD (Saran et al, 2020)(Kovesdy, 2022)(Prevention, 2023). The kidneys of CKD patients are not able to sufficiently filter wastes and excess fluids from the blood which would normally be removed in urine. Advanced chronic kidney disease can cause dangerous levels of fluid, electrolytes, and wastes to build up in your body, and lead to comorbidities, such as cardiovascular diseases and cognitive impairment (Go et al, 2004). CKD can be inherited or caused by other diseases. The leading comorbidities being diabetes and hypertension. Complications that affect all body systems can also be a result of CKD (Go et al, 2004). When left untreated, kidney damage progresses and chronic kidney disease can lead to end-stage kidney failure. As of today, there is no existing cure for CKD. Therapies such as dialysis and kidney transplants are available which focus on replacing lost kidney function in end-stage kidney disease. These therapies are imperfect, highly risky, and are associated with high rates of mortality and morbidity.

Macula densa (MD) is a group of highly specialized epithelial cells in the renal tubules right at the entrance of the kidney filter, the glomerulus (Peti-Peterdi, 2010). Traditionally, MD cells are salt sensors that generate paracrine chemical signals in the juxtaglomerular apparatus to control vital kidney functions, including renal blood flow,

glomerular filtration, and renin release (Peti-Peterdi, 2010). A novel function of MD cells is suggested by a new scientific discovery which identified an endogenous kidney tissue repair program, a mechanism that helps to regenerate kidney tissue when augmented (Gyarmati et al, 2021). MD cell activation, specifically calcium signaling, plays a central role in this newly identified kidney tissue repair program (Gyarmati et al, 2021).

Drug development is a tedious process with which scientists have discovered new therapies for diseases. High throughput screening assays (HTS) are a standard method in drug discovery that improves the understanding of interactions between molecules of interest and biological systems. HTS is a method that rapidly tests thousands to millions of samples for biological activity at the model organism, cellular, pathway, or molecular level with the use of automated equipment. Commercially available small molecule libraries hold a vast assortment of biological compounds that are tested to see the previously mentioned interactions. Based on the novel kidney tissue repair program, MD cells are a great model system for identifying new molecules that can augment endogenous kidney tissue regeneration.

The objective of our study is to develop a prototype of an HTS using the recently established, novel immortalized MD cell line aiming to identify MD cell activating compounds to augment kidney tissue repair. Our hypothesis is that cultured MD cells can be used to identify small molecules that activate the kidney tissue repair program. We further hypothesize that changes in MD calcium signaling and ultrastructure are biologically relevant readouts for the identification of lead compounds. Using the MD HTS, we would aim to find a lead compound that would increase calcium levels. This increase would activate MD cells which would in turn facilitate the kidney tissue repair program, leading to a healthy kidney.

MATERIALS

Mouse macula densa immortalized cell line (MD geo) established at the Zilkha Neurogenetic Institute, University of Southern California, MD geo was established from a primary culture of apparently normal mouse kidney fragments, ready-to-use AAV9 particles produced from AAV-CAG-jGCaMP8f-WPRE (Catalog number: 179254), DMEM-F12 cell culture media, Fisherbrand™ Surface Treated Sterile Tissue Culture Flasks, Vented Cap, Fetal Bovine Serum (FBS) Thermofisher Scientific, Penicillin-Streptomycin (5,000 U/mL) Catalog number: 15070063 (Gibco), sterile pipette tips (20 uL, 200 uL, 1000 uL) (Fisher Scientific), pipette (10 mL, 25 mL), single channel pipettes (20 uL, 200 uL, 1000 uL) (Fisher Scientific), DWK Life Sciences Wheaton™ PIPET-PAL Pipet Controller, glass pipettes, 70% ethanol, phosphate buffered saline, TrypLE cell dissociation reagent (Gibco), cell culture microscope, cell culture incubator, cell culture biosafety cabinet, Leica SP8 Dive Confocal Fluorescence Microscope, Leica LAS X image acquisition and analysis software (Leica Microsystems), Excel, Graphpad Prism, Biorender.

METHODS

MD cell culture

MD cells were cultured using aseptic technique as described before (Gyarmati et al, 2021). Briefly, mouse-immortalized MD cells were grown in T75 culture flasks in complete MD cell culture media (DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), 0.001% dexamethasone (DX)) with NGF (0.1ug/mL; N8133, Millipore Sigma) at 33 ºC and 5% CO2. At 80% confluence cells were subcultured (1:5) using TyplLE (Thermofisher Scientific, #12604013) to maximize the number of healthy cells. For differentiation, cells were passaged into 24 or 96 well plates, and incubated at 37°C, 5% CO2 for 14 days in complete MD cell culture media supplemented with NGF (0.1ug/mL; N8133, Millipore Sigma).

AAV transfection

Ready-to-use AAV9 particles produced from AAV-CAG-jGCaMP8f-WPRE (Catalog number: 179254) were used for the expression of the calcium-sensitive green fluorescent dye (GCaMP8f) in MD cells as described before (Vorburger and Hunt, 2002). Briefly, MD cells were cultured as described above. AAV-CAG-jGCaMP8f-WPRE viral particles were resuspended in complete MD cell culture media. MD cells were incubated with AAV-CAG-jGCaMP8f-WPRE viral particles at

multiplicity of infection (MOI): 3 (3 viral particles/1 cell) for 24 hours at 37 ºC. Cell culture media containing the viral particles were then replaced with normal MD culture media. Cells were kept at 37 ºC for differentiation.

Confocal Microscopy

Cells in 24 or 96 well plates were examined with Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) confocal/multiphoton laser scanning microscope systems as described previously (Gyarmati et al, 2021). Briefly, MD cells in glass-bottomed IBIDI plates were imaged in DMEM-F12 basal cell culture media at room temperature and no-flow condition. High temporal and spatial resolution fluorescence imaging was performed to visualize MD cells and changes in MD intracellular calcium signaling in response to test compounds. The images were acquired using a Leica SP8 DIVE multiphoton confocal fluorescence imaging system with a 63× Leica glycerine-immersion objective (numerical aperture (NA) 1.3) powered by a Chameleon Discovery laser at 970 nm (Coherent, Santa Clara, CA) and a DMI8 inverted microscope's external Leica 4Tune spectral hybrid detectors at 510-530 nm (Leica Microsystems, Heidelberg, Germany). Image acquisition (12-bit, 512×512 pixel) consisted of a single time lapse recording for 1-5 minutes (xyt, 526 ms per frame). Changes in CGaMP8f fluorescent intensity was measured after the experiment in the defined ROI using the Quantify package of LAS X software (version 3.6.0.20104; Leica-Microsystems) (Peti-Peterdi, 2006).

High-throughput screen

HTS was performed using mouse MD-GT cells plated in 96 well IBIDI plates. First, tool compounds, such as arginine-vasopressin (peptide CYFQNCPRG, a V1a receptor agonist(Aoyagi et al, 2008)) and gastrin (CCKBR agonist) were used to establish 5-point dose-response curves in the 0.1-10 μM range and to validate the assay. Then focused collections of commercially available small molecule libraries were selected based on their match with the MD cell gene profile and neuronal differentiation, such as the NINDS' 1,040 compound library, MicroSource Discovery Systems, LOPAC, Toscriscreen libraries(Lo and Hughes 2011, Li et al, 2015). MD-GT cells in replicates were incubated with compounds for 5 minutes and Agilent BioTek Synergy H1 Microplate Fluorescence Reader (Agilent, Santa Clara, CA) was used to read fluorescence intensities at 510-530 nm.

Data analysis

Data are expressed as average ± SEM and were analyzed using Student's t-tests (between two groups), or ANOVA (for multiple groups) with post-hoc comparison by Bonferroni test. $P<0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism 9.0c (GraphPad Software, Inc.).

RESULTS

Generation of the MDgeo-G mouse MD reporter cell line

Previous studies revealed that intracellular calcium signaling is a biologically relevant readout of MD cell activation. To generate an HTS that is able to detect an MD cell activation signal, MD cells expressing the calcium sensitive fluorescence reporter were generated. To express the green calcium sensitive fluorescent reporter protein GCaMP8f in MDgeo cells, cells were first cultured to semi-confluence (80%) at 33 °C and transfected using AAV9/GCaMP8f viral particles as shown in Fig. 1A. Transfected MDgeo-G cells preserved their epithelial characteristics as indicated by their cobblestone pattern similar to MDgeo cells (Fig. 1B). Confocal fluorescence microscopy of MDgeo-G cells showed intense green fluorescence of GCaMP8f in the cytoplasm and nuclei of MD cells (Fig. 1C) compared to non-transfected cells (Fig. 1D).

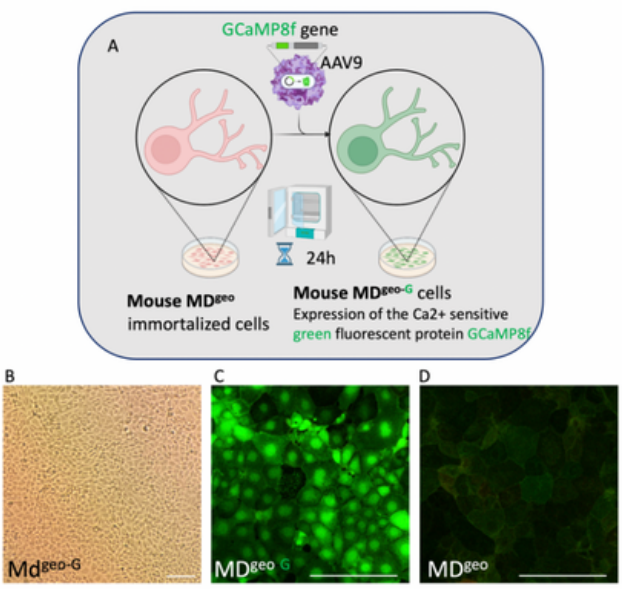

Figure 1 Generation of the MDgeo-G mouse MD cell line
A: Schematic illustration of the workflow of viral transfection of the mouse MDgeo immortalized cell line using ready-to-use AAV9 particles.
B: Representative light microscopy image of cultured MDgeo-G cells at confluence. Note the cobblestone pattern indicating healthy epithelial morphological features.
C-D: Representative confocal fluorescence microscopy image of cultured transfected MDgeo-G cells expressing the green calcium sensitive fluorescent reporter GCaMP8f (C) and non-transfected cells (D). Bars are 50μm.

Validation of the MDgeo-G in vitro calcium assay

To validate that MDgeo-G cells are able to respond to physiologically relevant stimuli, tool compounds such as arginine-vasopressin (AVP) and isoproterenol were used. Time-lapse confocal fluorescence microscopy imaging of MDgeo-G cells was used to confirm MD cell viability, activity, and responsiveness. As demonstrated in representative images of MDgeo-G cells at baseline (Fig. 2A) and after 5 minutes incubation (Fig. 2B) and representative recordings (Fig. 2C, E) and statistical summary (Fig. 2D,F), MD cell calcium increased about 2-fold in response to tool compounds (AVP: F/F0=1.5 +/- 0.02, p<0.0001; Isoproterenol: F/F0=1.7+/-0.08, p<0.0001, n=6 each). MD cell activation (calcium signal) in response to tool compounds was steady and sustained for over 5 minutes as shown in the representative recordings. Therefore, future application of HTS used the 5 minutes reading time-point.

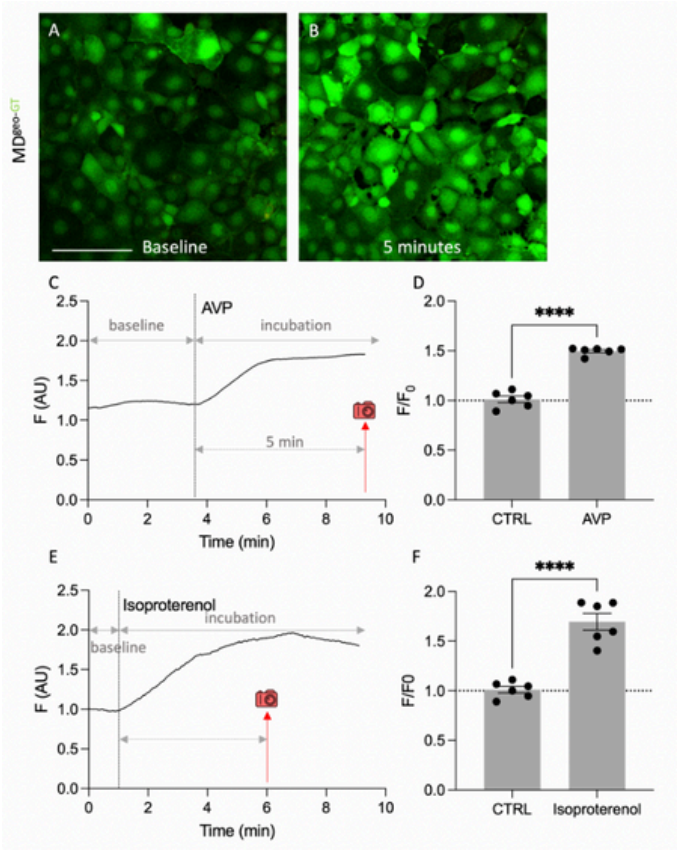

Figure 2 Validation of the MDgeo-G in vitro calcium assay.
A-B: Representative images of cultured Mdgeo-G cells at baseline (A), and after 5 minutes incubation with tool compound (B). Note the increased GCaMP8f fluorescence intensity (green) after 5 minutes incubation period as compared to baseline. Bar is 50 m.
C-F: Representative recordings (C,E) and statistical summaries (D,F) of changes in MD cell calcium signaling in response to AVP (C-D) and Isoproterenol (E-F). Data are means ± SEM, ****P<0.0001 using Student's t-test, n=6.

Development of MDgeo-G based HTS to identify new drugs for CKD

Since an efficient HTS requires high sensitivity, quantifiability, reproducibility, and fast data acquisition and analysis, the earlier used image-based analysis was converted to a

fluorescence microplate reader-based system. First, to calibrate intracellular calcium changes in MD cells to changes in GCaMP8f fluorescence intensity, ionomycin was used to permeabilize MD cell membrane to calcium. Step-by-step increases in calcium concentrations in MD cell culture media by added CaCl2 resulted in significant increases in the 1-5-fold range in GCaMP8f fluorescence intensity as illustrated by the heatmap (Fig. 3B). In addition, different seeding density of MD cells/well was tested to understand if different cell number/well has an effect on the measured GCaMP8f fluorescence intensity. As it is shown on the heatmap, there was no significant effect of increased cell number/well on the measured values (Fig. 3B). The addition of tool compounds, isoproterenol, angiotensin II, AVP, furosemide, and gastrin caused elevated HTS signals to variable degrees. Among all tool compounds, gastrin had the most potent effect (Fig. 3C, Table 1).

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
CTRL vs. Iso	-0.5522	-1.371 to 0.2662	No	ns	0.2602
CTRL vs. AngII	-0.09162	-0.9100 to 0.7267	No	ns	0.9978
CTRL vs. AVP	-0.4373	-1.256 to 0.3810	No	ns	0.4616
CTRL vs. Furo	-1.304	-2.122 to -0.4854	Yes	**	0.0015
CTRL vs. Gastrin	-3.816	-4.634 to -2.997	Yes	****	<0.0001

Table 1 Results of statistical analysis of MD cell calcium changes in response to tool compounds. Statistical analysis was performed by using One way ANOVA and Dunnett's multiple comparison test and GraphPad Prism software.

First applications of the MDgeo-G based HTS

For a preliminary, real-world testing of the newly established HTS a small library of small molecules consisting of 28 compounds was screened. The controls and compounds were tested in triplicates as illustrated in the matrix in Figure 4A. Among the 28 small molecule compounds tested, one compound, C-10 produced the greatest HTS signal, and was considered as a promising lead compound (Fig. 4B). Subsequent data mining confirmed that C-10 is a potent agonist of the calcium sensing receptor (Casr). The molecular structure (Chemical name: R568) of C-10 is illustrated in Figure 4C. In addition, MD cell transcriptomic data from earlier published database(Gyarmati et al, 2021) revealed the high and MD specific expression of the Casr compared to control cells in the kidney (Fig. 4D).

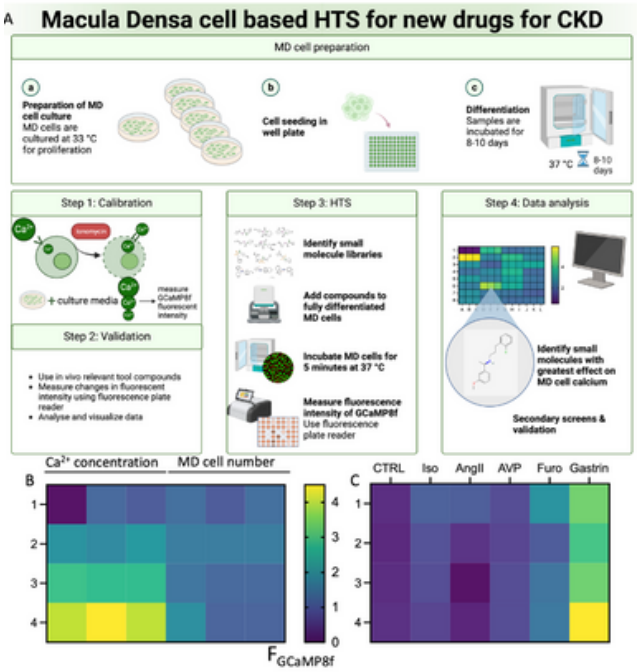

Figure 3 Development of MDgeo-G based HTS.
A: Schematic illustration of the step-by-step development of the MD cell based HTS for new drugs for CKD, including MD cell preparation, calibration and validation steps of the assay, steps of the screening, and data analysis and visualization.
B: Heatmap of assay calibration using step-by-step increases of Ca2+ concentrations in cell culture media and permeabilized MD cells; or different numbers of seeded MD cells.
C: Heatmap of assay validation using tool compounds.
Blue-yellow-colored squares represent the values of GCaMP8f fluorescence intensity/well in a 24 well cell culture plate measured by microplate fluorescence reader. Pseudo-color intensity range of GCaMP8f fluorescence is shown in the middle.

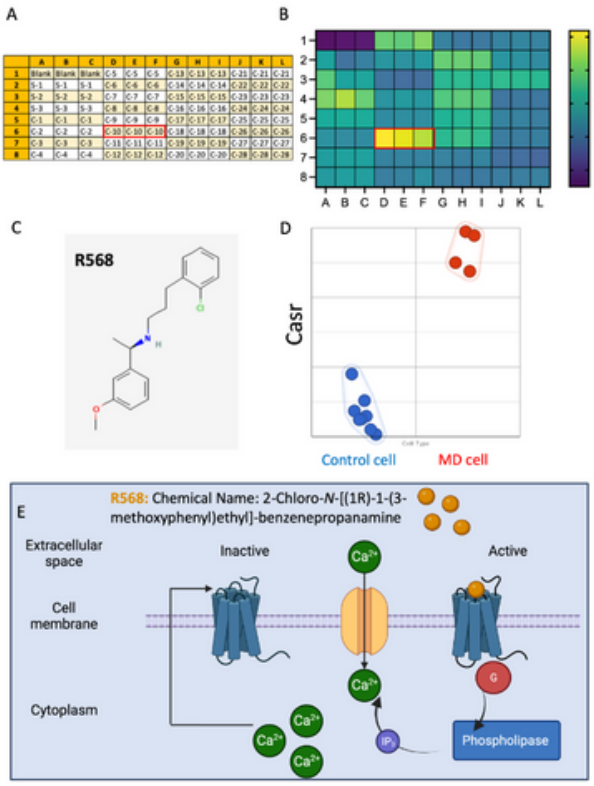

Figure 4 First applications of the MDgeo-G based HTS.
A: Experimental design of a 96 well plate with controls (S) and test compounds (C). S and C were tested in triplicates.
B: Heatmap of HTS signal intensity in response to the first 28 test compounds. Red square highlights the compound with the greatest effect. Blue-yellow-colored squares represent the values of GCaMP8f fluorescence intensity/well in a 96 well cell culture plate measured by microplate fluorescence reader.
C: Molecular structure of the hit compound C-10: the calcium sensing receptor (Casr) agonist R568.
D: The expression of Casr in freshly isolated mouse MD cells compared to control kidney tubule cells based on bulk RNA sequencing and transcriptome analysis. Dot plot is adapted from Gyarmati et al, 2021 with permission.
E: Schematic illustration of the effect of R568 on MD cell calcium.

DISCUSSION

The present study successfully developed a new, live cell-based HTS prototype using gene technology, and new scientific knowledge regarding the novel functions of a key kidney cell type, the macula densa that is linked to kidney regeneration. The presently developed HTS is addressing an important problem, an unmet medical need to develop highly effective treatment for common and devastating diseases that affect the kidneys. One important novelty of our approach is the targeting of the newly identified kidney regenerative mechanisms orchestrated by the chief kidney cell type, the macula densa (Gyarmati et al, 2021). Additional innovation in our work is the use of the unique living MD cell line that was developed and made available by our host research laboratory at USC (Gyarmati et al, 2021). State-of-the-art gene editing technology using viral gene delivery enabled the successful development of the MD-HTS that uses physiologically relevant calcium signaling as a readout. The other technical aspects of our work used standard HTS signal detecting methods, such as fluorescence intensity plate reader (FLIPR) assay that are widely used in drug development (Schroeder and Neagle, 1996).

The first real-world application of the new HTS identified a new potential lead compound that is a promising small molecule drug candidate for CKD. C-10 (R568), a potent Casr agonist, had the greatest stimulatory effect on MD cell activation (calcium signaling) (Fig. 4B), which suggests that this compound or its future derivatives maybe potent inducers of kidney regeneration in vivo. The C-10 induced high HTS signal is consistent with the high MD specific expression of Casr (Fig. 4D) that was established previously (Gyarmati et al, 2021). The potential mode of action of R568 in MD cells is depicted in Figure 4E, and likely involves binding to the Casr (Sundararaman and van der Vorst, 2021) localized in the MD cell membrane, and triggering intracellular calcium signaling that in turn activates kidney regeneration.

Potential limitations of our study include the small scale of the used small molecule library, since a typical application by the pharmaceutical industry routinely uses commercially available libraries that consists of hundreds of thousands of screened compounds. However, the presently developed

MD-HTS can be used on a much larger scale in future work, using current technology and fully automated platforms. Another potential limitation is that MD molecular targets identified via the HTS may have side effects due to the expression of the same receptors in other cell types, and organs. For example, Casr is expressed throughout the cardiovascular system and the parathyroid gland (Sundararaman and van der Vorst, 2021). Secondary screens and preclinical testing using whole animal systems are important and usual steps in the drug development process.

In conclusion, our study provided useful information and developed new research tools that can be used in future phases of drug development for CKD. Since MD-like chief cells are localized in other organs (Gyarmati et al, 2021), our new assay may be used in future studies to develop potential therapeutics for other failed organs and chronic diseases, including diabetes, heart diseases, and aging-related degenerative diseases.

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