NEW MECHANISMS OF MACULA DENSA CELLS IN RENAL PHYSIOLOGY, DISEASE AND THERAPY

PhD thesis – short version

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1. INTRODUCTION

Chronic kidney disease (CKD) is a condition in which the kidneys are damaged and cannot filter blood as well as healthy kidneys. As a result, excess fluid and waste from the blood remain in the body and may cause other health problems, such as cardiovascular disease and cognitive impairment. Presently, only non-specific therapies are available. Even the newest drugs, SGLT2 inhibitors and GLP-1 agonist can only slow down CKD progression. Clearly, there continues to be an urgent and unmet medical need for specific, mechanism-based, and highly efficient CKD therapies. The unmet medical need and inadequacy of current treatments have led to great interest in regenerative medicine. Improved understanding of endogenous kidney tissue repair and identification of the key cellular and molecular targets are critically important for the development of specific, mechanism-based regenerative therapies that can achieve disease regression.

Physiological adaptation to maintain functional homeostasis is a known driver of physiological regeneration in multiple organisms and mammalian tissues. Physiological signals associated with either loss or gain of organ function, such as fasting for the nervous, endocrine and digestive systems and mechanical force for the skeletal system can trigger potent regenerative responses. Conservation of body fluid and salt was a major evolutionary driver of the highly efficient and complex mammalian nephron.

Macula densa (MD) cells are chief cell type within the kidney and play key sensory and regulatory functions of intrarenal mechanisms that maintain body fluid and electrolyte homeostasis and blood pressure. MD cells are part of the juxtaglomerular (JG) apparatus (JGA), a key anatomical site within the kidney to provide physiological control of basic kidney functions including glomerular filtration rate, renal blood flow and renin release.

The central and peripheral nervous system senses and integrates information about the inner state of the body through the process of interoception, which is essential for maintaining mental and physical health. Interoception involves a variety of neural and non-neural cells and processes in several internal organs by which an organism senses, interprets, integrates, and regulates signals from within itself. There are well-established bidirectional interoceptive communications between the brain and peripheral neural networks in several organs throughout the body including the heart, intestine, and bladder. Despite playing major roles in maintaining body homeostasis, the kidneys have been largely overlooked in the context of interoception. Importantly, no neuronal somata have been described in the renal cortex, although anatomically and functionally far less-complex organs have local neural control and neural networks. However, the MD-specific expression of the neuronal type of nitric oxide synthase (*Nos1*) in the renal cortex has been long established and preliminary transcriptomic analysis of a limited number of single MD cells expressing neuroepithelial features suggest their neuronal differentiation. In addition, recent discoveries in tissue regenerative mechanisms suggest the key role of local neuronal activity in direct control of somatic stem cells. The emerging key role of local neurons in endogenous tissue repair fits well with the new clues on MD cell neuron-like sensing and responding to the environment and with their potential links to physiological tissue regeneration.

Here we aimed to develop an MD specific comprehensive research toolbox to study these overlooked cells in unprecedented detail. Applications of these new research tools addressed the hypothesis that MD cells via their novel sensory neuron-like function control progenitor cell-mediated endogenous kidney regeneration.

2. OBJECTIVES

The overall objective of my work was to explore the importance and new roles of the macula densa (MD) in renal physiology, disease, and therapy.

My central hypothesis is that sensory neuron-like MD cell calcium and NGFR signaling play novel, primary roles in physiological responses to control renal hemodynamics, renin, and tissue remodeling in order to maintain body homeostasis (interoception). Furthermore, I hypothesize that MD cells via secreted paracrine factors act on local progenitor cells to orchestrate endogenous kidney tissue remodeling. In addition, alterations in the neuron-like functions of MD cells represent a novel, neurodegenerative component in the pathogenesis of chronic kidney disease (CKD) and can be therapeutically targeted.

In the present work I aimed to:

1. Develop a next generation MD specific research toolbox, including in vivo animal models, new in vivo multiphoton microscopy approaches to study MD cell function, a newly developed immortalized MD cell line, and bulk and single-cell based MD transcriptome profile.
2. Test the hypothesis that MD cells exhibit sensory neuron-like molecular, structural, and functional features.
3. Test the hypothesis that MD cells regulate endogenous tissue remodeling and repair after injury. We further hypothesized that this program is activated via physiological stimuli and can be therapeutically targeted.

3. METHODS

Male and female, 6-12 weeks old C57BL6/J or BalbC mice were used in all experiments. Transgenic mouse models with the expression of various fluorescent reporter proteins and gene knock-out strategies were generated by intercrossing Cre or Cre-ER^{T2} mice with floxed mice. Mice underwent dietary (physiologica) and pharmaceutical interventions to stimulate MD cell function. Adriamycin (ADR) induced nephropathy was used as a pre-clinical CKD model. At the 2 weeks peak of disease, 4-weeks treatment of pre-existing pathology was initiated using MD cell biologicals. At the end of 4 weeks treatment, mice were euthanized, and tissues harvested for histological analysis.

Serial intravital imaging using multiphoton microscopy (MPM) with or without abdominal imaging window (AIW) implantation was used to acquire visual clues of MD cell function and changes in kidney tissue microenvironment. The images were acquired using a Leica SP8 DIVE multiphoton confocal fluorescence imaging system. Serial MPM imaging of the same glomerulus was performed for up 14 days.

Glomerular filtration rate (GFR) measurements were performed using the MediBeacon Transdermal Mini GFR Measurement System (MediBeacon).

Immunofluorescence detection of proteins on kidney tissue sections and whole-mount cleared kidney tissue was performed for qualitative analysis. Samples were examined with Leica TCS SP8 confocal system. Image analysis software Image J and Leica LAS X were used for image analysis.

Bulk and single-cells RNA sequencing and bioinformatics was used to study the molecular fingerprint of MD cells. RNA-seq data was analyzed using Partek Flow, GraphPad Prism 9.0.1, and QIAGEN Ingenuity Pathway Analysis (IPA).

A new immortalized mouse macula densa cell (MD^{geo}) line was developed for cell culture studies. For functional characterization, changes in MD^{geo} cell NO synthesis and PGE2 production was measured in response to low salt stimulation using either a NO sensitive fluorescent dye or the PGE2 biosensor cell assay, respectively.

Conditioned MD^{geo} cell culture media was generated after full differentiation of MD^{geo} cells via applying physiological activation by temporary exposure to low-salt conditions for 6 hours every other day for a total of 3 times. Mass spectrometry analysis was performed to study the composition of MD^{geo} cell culture media.

Immunoblotting of mouse cortical homogenates or MD^{geo} cell homogenates were used for the quantitative measurement of protein expression.

Data are expressed as means+/- SEM, were analyzed using Student's t-tests or ANOVA with post-hoc comparison by Bonferroni test. $P<0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism.

4. RESULTS

4.1 Development and characterization of a next generation MD cell research toolbox

4.1.1 The generation of MD targeting in vivo mouse models.

Although macula densa (MD) cells are chief regulatory cells in the nephron, they have been difficult to study in full detail due to their inaccessibility and limited research tools available to specifically target them. To overcome these limitations, we first developed several MD cell targeting in vivo genetic mouse models using the classic MD cell-specific marker nNOS in a Cre/lox-based genetic strategy. The newly developed transgenic MD-GFP and MD-GT mouse models combined with the altered level of Cre induction strategies enabled the visualization and functional assessment of single MD cells, the MD plaque as a functional unit, as well as the isolation of MD cells for in vitro cell culture studies and RNA seq and transcriptome analysis.

4.1.2 The development of new in vivo multiphoton microscopy approaches to study MD cell function

To study MD function in vivo we developed several unbiased intravital imaging approaches using multiphoton microscopy (MPM). Calcium signaling, changes in renal hemodynamics, and the local kidney tissue microenvironment using serial MPM imaging was studied to search for direct visual clues concerning renal physiology and disease. MPM imaging also provides qualitative and quantitative in vivo visual clues regarding alterations in renal hemodynamics, kidney structure and function and the potential major molecular and cellular mechanistic drivers at play during disease pathogenesis.

4.1.3 The development of the immortalized mouse MD cell line

A new immortalized mouse MD cell line that we named mMD^{geo} was generated for cell biology studies *in vitro.* The MD phenotype of mMD^{geo} cells was validated by the high expression of classic MD cell markers including

Cox2, Nkcc2, and Romk. As validation of their intact physiological function, mMD^{geo} cells exhibited the classic phenotype of increased pERK1/2, Cox2, and pp38 expression in response to low salt culture conditions. Another classic hallmark of MD cells, Nos1-mediated NO release, and Cox2-mediated PGE_2 production was confirmed intact in mMD^{geo} cells.

4.1.4 Profiling of the MD transcriptome

Next, we aimed to establish the molecular signature of MD cells and their function in control and activated (low salt - LS) condition. A total of 28,000 MD cells and 50,000 control cells from adjacent tubule segments were isolated from the cortex of freshly digested MD-GFP kidneys (n=2 mice for MD and n=4 mice for control cells from each condition) for bulk RNA isolation and transcriptome analysis. For single-cell RNA sequencing and transcriptome analysis, 894 and 1296 MD cells were analyzed from control and LS-induced conditions, respectively, each from a single MD-GFP mouse. High-level expression of known MD cell markers validated our RNA sequencing approach.

4.2 Neuron-like features of MD cell structure and function

4.2.1 A new view of MD cell microanatomy

The combination of membrane-targeted fluorescence reporter expression in the MD-GFP mouse model with the sporadic labeling of single MD cells enabled the visualization of the microanatomical details of MD cells and depicted an elaborate network of major and minor processes at the cell base, projecting toward the glomerulus, the extraglomerular mesangium, the AA and EA, and other MD cells, that we named maculapodia,. The density and length of maculapodia was dependent on gender and physiological activation or inactivation of MD cell function. These findings were confirmed in vivo using MPM imaging of MD-GFP mouse kidneys, and ex vivo, using freshly isolated MD cells.

4.2.2 Interoceptive neuron-like functions of MD cells in vivo

Mice with ubiquitous calcium reporter expression and combined with in vivo MPM imaging were used to study calcium signaling in all renal cells and to identify potential chief neuron-like cell types within the renal cortex. Time-lapse intravital MPM imaging of Sox2-GT mice found robust, spontaneous Ca^{2+} transients in MD cells. Among all cells MD cells showed by far the highest cumulative elevations in Ca^{2+}. To study the spatial and dynamic details of MD cell Ca^{2+} responses with truly single-cell resolution, MD-GT mice were used. Single-cell Ca^{2+} imaging revealed the presence regular pacemaker-like Ca^{2+} transients. MD cells also responded to several diverse stimuli with altered steady-state Ca^{2+} and/or firing frequency, including most importantly altered tubular fluid composition (low salt diet) and mechanical strain (tubule flow), but also local autacoids (angiotensin II), systemic neurohormone administration (arginine-vasopressin (AVP), gastrin), and metabolic states (diabetic hyperglycemia).

Imaging of cleared and immunolabeled MD-GFP mouse kidney tissue showed close anatomical association between MD cell basal processes (GFP+) and the sympathetic (tyrosin-hydroxylase+) and sensory (calcitonin gene-related peptide+) nerve endings. The local expression of synaptophysin, a major synaptic vesicle protein specifically in MD cells and in nearby nerve endings suggest that MD cells synapse with each other and with the sympathetic and sensory nerves forming a peripheral renal nervous system.

4.2.3 Neuron-specific molecular signature of MD cells

The analysis of the top 50 MD enriched genes of bulk RNAseq using tissue-specific gene enrichment analytic tools assigned a highly significant brain tissue identity to MD cells. In addition, canonical pathway analysis identified several functional neuronal pathways, including axon guidance as the top and most significant pathway in MD cells. Single-cell RNA sequencing

and transcriptome analysis further emphasized the neuronal characteristics of MD cell molecular signature. The high MD expression of neurohormone and metabolic receptors such as the AVP (Avpr1a), gastrin and cholecystokinin (Cckbr) and glucagon receptor (Gcgr) further highlight the systemic sensory function of MD cells to maintain whole body homeostasis and their role in inter-organ crosstalk.

4.3 The role of MD cells in endogenous tissue remodeling and regeneration after injury

4.3.1 The molecular profile of the MD cell program for tissue regeneration

In response to loss of body fluid and salt as the strongest physiological stimuli of MD cells, bulk and single-cell transcriptome analysis of activated MD cells identified growth and transcription factors and chemokines (Fabp3, Egf, Ccn1, Foxq1, Cxcl12) and angiogenic factors (Vash2, Pamr1, Vegfa, Ccn3) highly expressed in MD cells, suggesting tissue remodeling regulatory functions. The newly identified MD angiogenic factors include the Ccn family of matricellular proteins, Ccn1 and Ccn3 (ranked #38 highest enriched MD gene). Mass spectrometry analysis of the low-salt conditioned newly established immortalized MD^{geo} cell line secretome (MD culture media) identified the angiogenic matricellular protein CCN1 as one of the top enriched MD-secreted proteins confirming the transcriptome data.

4.3.2 Live tracking and MD-centric pattern of endogenous tissue remodeling

To establish the dynamics and pattern of endogenous kidney tissue remodeling with single-cell resolution in the intact living mouse kidney, unbiased tracking of the same tissue volume of kidney cortex over several days and weeks was performed using a combination of serial intravital MPM and genetic cell fate tracking. The loss of body fluid and salt as a MD-activating physiological stimulus caused substantial recruitment of mesenchymal and

endothelial precursor cells. Importantly, the geometrical epicenter (highest cell density) of both mesenchymal and endothelial cell recruitment was the base of MD cells at the glomerular vascular pole in each nephron. Newly recruited $Ng2^+$ mesenchymal progenitor cells differentiated to multiple cell fates including vascular smooth muscle, renin, mesangial, parietal epithelial and proximal tubule cells and podocytes. Both $Cdh5^+$ and Ren^+ cells produced clonal remodeling of the vasculature, interstitium and glomerulus closest to the MD, further suggesting the presence of mesenchymal and endothelial progenitor cells at the glomerular vascular pole. This effect was blocked by pharmacological inhibition of the MD-function, suggesting the key role of MD cells in this tissue remodeling process.

4.3.3 Ngfr signaling is key in MD neuron-like and tissue remodeling functions

The nerve growth factor receptor (*Ngfr*) was the highest expressed growth factor receptor in MD cells (ranked #64 most enriched MD gene) suggesting the primary role of Ngf signaling in the function of MD cells. Ngfr expression was significantly increased by low salt culturing condition in MD^{geo} cells and supplementation of the MD^{geo} culture media with NGF significantly increased cell proliferation and was absolutely essential for the long-term survival of MD^{geo} cells in culture.

In vivo MPM imaging of the newly developed MD-GT-NGFRKO mice revealed that compared to WT mice, the frequency of MD cell Ca^{2+} transients showed a 4-fold increase in MD-NGFR KO mice. Importantly, instead of the regular periodic oscillations that were observed in WT mice, MD-NGFR KO mice featured irregular chaotic-like oscillations in whole-MD Ca^{2+}. Cell-to-cell connectivity of MD cells was greatly reduced in MD-NGFR KO mice compared to WT. Short-term treatment with NGF (10 µg/kg sc daily for one week) caused significant increases in the number of renin-producing

juxtaglomerular (JG) cells and in GFR in WT mice, while renin cell number and GFR were significantly lower in MD-NGFR KO mice. MD-NGFR KO mice featured kidney tissue fibrosis and showed increased levels of the classic neurodegeneration marker p-tau (S199) in MD cells.

4.3.4 Therapeutic targeting of the MD program for kidney repair

Human translational studies using transcriptomic data from the European Renal cDNA Biobank (ERCB) as well as protein expression data of CKD and control kidney biopsies uncovered that CCN1 is among the top 1% under expressed genes in CKD patients independent of the etiology of the disease. To study whether targeting of the presently identified MD cell program for kidney tissue regeneration provides therapeutic benefit, the effects of treatment with MD-derived biologicals were tested in the robust CKD model of Adriamycin. Treatment of the pre-existing GS was initiated using one of the following five biologicals: saline (PBS), recombinant CCN1 in low or high-dose, control DMEMF12 culture medium or low salt conditioned cell culture medium of the MD^{geo} MD cell line. Animals were followed-up for 4 weeks of treatment. In contrast to control PBS or DMEMF12 medium which had no significant effect, treatment with CCN1 or low salt conditioned MD medium caused strong improvements in albuminuria. In contrast to all other groups, treatment with low salt conditioned MD medium dramatically improved GFR, which returned to normal baseline levels indicating functional regression of FSGS pathology. Subsequent kidney histological analysis showed severe GS and tissue fibrosis in control PBS and DMEMF12-treated groups, while CCN1 or low salt conditioned MD medium treatments greatly improved FSGS histopathology, $p57^{+}$ podocyte number and tubulointerstitial fibrosis suggesting structural regression of FSGS.

5. CONCLUSIONS

1. The newly developed next generation MD research toolbox and its applications painted a paradigm shifting new view of the macula densa.
2. Genetic labeling and MPM imaging of single MD cells found physiologically regulated dense network of major and minor processes at the cell base, called maculapodia. These dynamic and secretory microanatomical features serve as structural basis for cell-to-cell communication within the JGA, between the MD itself and other target cells.
3. MD cells are the nephron central command. Calcium imaging of MD cells and other kidney cell types complemented with transcriptome analysis uncovered new cell physiology features, including the autonomous rhythmic calcium firing and the coordination and clustering of single MD cell calcium transients into cumulative whole-MD calcium output to drive whole organ function.
4. Physiological activation of the nephron central command (MD) drives endogenous nephron remodeling and repair including the glomerular filtration barrier (GFB), endothelium and podocytes. MD cells synthetize and secrete numerous angiogenic, cell migration and patterning, growth and extracellular matrix signaling and remodeling factors that act on local progenitor cells in a paracrine fashion to orchestrate tissue remodeling.
5. The loss and conservation of body fluid and salt, the known major driver of the evolution and the function of the mammalian nephron is the strongest stimulus to activate the MD regenerative program. Hence it drives endogenous kidney tissue remodeling and has clinical implications in the long-known protective effects of low dietary salt intake and ACEi to slow down CKD progression.

6. Therapeutic targeting of the MD specific tissue regenerative program may serve as a novel specific and mechanism-based regenerative therapy that can achieve disease regression. Human translational studies revealed that CCN1, one of the top MD specific secreted angiogenic factors in the kidney, are among the top 1% under expressed genes in CKD regardless of disease etiology. Targeting of the novel MD tissue remodeling program using MD biologicals resulted in the regression of CKD in a pre-clinical mouse model. The identification and characterization of this new MD mechanism provides key initial steps to develop a new class of drugs, MD cell mimetics, targeting the MD cell neuronal and tissue regenerative program.

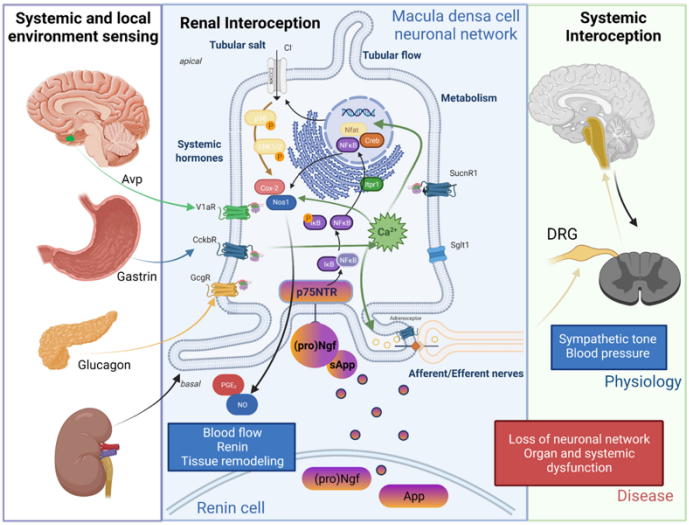

Figure 1. Schematic illustration of the novel MD cell function as sensory neuron-like chief cells in the kidney. MD cells sense and respond to local and systemic stimuli and serve as the nephron central command, as well as paly a major role in whole-body homeostasis and disease development.

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