EPIGENETIC MECHANISMS OF RENIN CELL PLASTICITY

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Abstract
Paramount to the survival of animals with a closed circulatory system is the regulation of blood pressure and blood flow to critical tissues\textsuperscript{1,2}. This is accomplished by the renin-angiotensin system (RAS), an enzymatic cascade that culminates in the production of Angiotensin II, a potent vasoconstrictor that controls blood pressure, renal hemodynamics and fluid-electrolyte homeostasis. Across millions of years of development, the RAS has been perfected throughout evolution to enable animals to survive fluctuations in fluid-electrolyte balance and blood pressure\textsuperscript{1,3}. The key event in the RAS and its rate limiting step is the tightly regulated synthesis and secretion of renin, a hormone-enzyme which cleaves angiotensinogen into Angiotensin I which is further converted to Angiotensin II by Angiotensin Converting Enzyme (ACE). Under normal physiologic conditions, renin is secreted by juxtaglomerular (JG) cells located in the walls of the afferent arterioles at the entrance to the glomeruli of the kidney. Although the nominal number of JG cells is small, representing 0.1-0.01\% of all kidney cells\textsuperscript{4}, their production of renin is, under normal conditions, typically sufficient to sustain blood pressure and ensure survival. However, during intense threats to homeostasis by hypotension, dehydration or administration of RAS inhibitors, mesangial cells, smooth muscle cells and pericytes along the afferent arterioles transform into renin expressing cells – a process described as “recruitment”\textsuperscript{5–7}. This process is a result of direct transdifferentiation of the aforementioned cell types in the kidney\textsuperscript{8}. These cells undergo a remarkable switch in cellular identity to express renin and other genes crucial to attain the renin cell program before presumably returning to their original identity once the threat abates and homeostasis is restored. The underlying mechanisms which bestow
this rare and fascinating ability upon these cells remain unknown and are explored in this thesis.
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I. Introduction

Includes writings, data and figures from a first author review article and an internal author research article:


**The Renin-Angiotensin-Aldosterone System (RAS):**

Regulation of blood pressure and fluid electrolyte homeostasis is crucial for survival\(^2,3\). This is primarily accomplished through the Renin-Angiotensin-System (RAS) which has been refined throughout evolution to maintain normal blood pressure and the constancy of extracellular fluid volume and composition\(^3\). The RAS is an enzymatic cascade involving multiple organs and peptides (Fig 1). It begins with Angiotensinogen, a 485-amino acid long peptide, which is released from the liver and circulates in an inactive form. Renin, secreted from juxtaglomerular cells (JG cells) of the kidney, cleaves Angiotensinogen into Angiotensin I which is further processed to Angiotensin II by Angiotensin-Converting-Enzyme produced in the lungs, endothelial cells, and other tissues\(^3\). Angiotensin II, an octapeptide, acts on Angiotensin receptors inducing vasoconstriction and increasing heart rate. In addition, Angiotensin II stimulates NaCl tubular reabsorption by the kidney and aldosterone synthesis and release. These actions result in elevation of blood pressure and maintenance of fluid electrolyte balance\(^3\).

Proper functioning of the RAS results in normal tissue perfusion and delivery of oxygen and nutrients; and its regulation is predominantly controlled by the synthesis and release of renin. Renin is a hormone-enzyme catalyzing the rate-limiting step of the RAS and is produced by juxtaglomerular (JG) cells in a tightly regulated fashion by physiological stimuli. JG cells are sensors that respond to signals conveying the status of the extracellular fluid volume and blood pressure. Thus, a reduction in extracellular volume or blood pressure which occurs due to dehydration, blood loss, heart failure, or treatment with RAS inhibitors, result in increased renin synthesis and release which
ultimately leads to increased Angiotensin II generation and reestablishment of homeostasis.

**Plasticity of renin cells: a strategy for survival:**

In adult mammals, JG cells comprise 0.01% of total kidney cells\(^4\). Under normal circumstances, renin release from those few cells is sufficient to maintain blood pressure homeostasis. However, intense and prolonged threats to homeostasis (such as dehydration, blood loss, hypotension) requiring higher levels of renin, the kidney arterioles adopt an endocrine phenotype: renin cell descendants (smooth muscle cells, pericytes, mesangial cells) are transformed to synthesize renin, resulting in an increase in the number of cells releasing renin to the bloodstream. This process, generally described in the literature as “**recruitment**”\(^9\), occurs by a re-enactment of the embryonic pattern of renin expression\(^7\). The process is believed to be reversible and is evident when there is a chronic threat to homeostasis\(^2,6,7,10–14\). The ability to produce large quantities of renin allows animals to restore their blood pressure and fluid volume and maintain blood supply/oxygen delivery to critical organs thus ensuring survival. Contrary to what has been suggested, the recruitment of renin cells is not thought to be a result of proliferation or migration of cells but rather, occurs by de-differentiation or re-transformation of smooth muscle cells to the renin phenotype. A small percentage of renin-expressing cells may be generated by neogenesis, de novo expression of renin by cells that never expressed it but the predominant mechanism is through transformation of existing vascular cells in the kidney arteriole into renin expressing cells\(^15\). Thus, the smooth muscle cells retain the memory of the renin phenotype which can be activated during intense threats to ensure survival.
At this juncture, it is important to note that the transformation of the neighboring cells does not simply refer to an activation of the renin gene in isolation; rather, they undergo a complete differentiation in terms of their phenotype. They adopt an epithelial morphology, develop lysosome-like granules which store renin and express key molecular programs of the renin cell such as Akrb7; a reliable enzyme marker of renin cells used to detoxify aldehyde by-products of renin synthesis\textsuperscript{16–18}. This plastic ability of vascular renal cells to adopt the renin cell identity and express renin during homeostatic threat is well established\textsuperscript{5,19–21,22}. However, less well described is the reverse process by which the recruited renin cells along the vasculature abandon the renin cell identity and return to their original cell type after physiologic threats have been addressed\textsuperscript{23}. Therefore, we subjected mice to a low sodium diet in addition to the ACE inhibitor captopril in their drinking water, conditions known to stimulate recruitment (transdifferentiation of arteriolar smooth muscle to renin expressing cells) before altering their diet to high sodium chow and saline drinking water to repress the renin cell programme\textsuperscript{8} (Fig 2A). The mice were then harvested at various stages of treatment to demonstrate the activation and subsequent repression of the renin cell program. \textit{In-situ} hybridization for renin revealed a dramatic increase in renin expression both in native renin expressing cells as well as cells along the vascular tree at 7 days of recruitment in contrast to control animals in which renin expression was restricted to the juxtaglomerular area (Fig 2B). However, in animals subjected to the repressive treatment after recruitment, renin expression is only present in the JG area at 2 days and at 5 days even the JG area has sharply diminished expression levels. These results demonstrate the plasticity of vascular renin lineage cells as they both activate and
repress the renin program efficiently depending on the physiological context. qPCR for renin mRNA from the kidney cortices mirrored the *in-situ* results as expression of renin rose rapidly after recruitment and then progressively lessened with longer repression (Fig 2C). These trends were matched by the circulating levels of renin as measured by ELISA from plasma demonstrating that the changes observed in the kidney constituted a functional physiologic response to the pressures applied by the treatments (Fig 2D). These results were also corroborated by qPCR for *AKR1b7*, a bona fide marker of renin cells, where similar trends were observed confirming that the changes undergone by the cells where a complete change in cell identity rather than simply activation of renin expression. Interestingly, staining for renin protein revealed an altogether different pattern from the *in-situ* hybridization results. While renin mRNA rose and fell according to the treatment of the mice, renin protein appeared to persist even after the application of high salt and saline drinking water (Fig 2E). Even after 5 days of repression, renin protein is still seen in vascular cells suggesting that the cells retain the protein and degradation of renin and the acidic granules in which it is housed requires a longer time before being removed. Interestingly, no signs of autophagy were seen in these cells either by staining for the gold standard autophagic marker LC3B (Fig 2F) or electron microscopy (data not shown) hinting at a more complex processing of renin protein as the cells return to their contractile phenotype. The exact mechanisms by which these cells process renin, and its post-translational regulation, remain to be studied and constitutes a fascinating avenue for future research.

**Renin Cells at the Epicenter of Multiple Defense Mechanisms:**
As mentioned above, renin cells have a fascinating plasticity that is uncommon in most mammalian cells\textsuperscript{24}. Furthermore, this plasticity is not only restricted to transdifferentiation to and from renin cells to maintain vascular flow, but it can also occur under other physiological/pathological contexts:

Perhaps the most striking example of this plasticity occurs under condition of hypoxia where renin cells undergo a complete change in cell fate to become erythropoietin secreting cells to increase blood supply to vital tissues \textsuperscript{25}. In mammals, the supply of oxygen (through red blood cell circulation) is key to survival. Renin cells participate in this vital process by constricting blood vessels to increase pressure and maintain blood flow to those tissues, namely the brain, heart and lungs, which are necessary for survival. However, under intense hypoxic conditions (simulated by the deletion of the Von Hippel Lindau tumor suppressor which regulates the hypoxic response) some renin cells abandon their conventional role of blood pressure regulation and instead begin to produce erythropoietin\textsuperscript{26}. Erythropoietin is typically produced by interstitial pericytes in the kidney and acts on bone marrow to elicit greater production of red blood cells and renin cells can contribute to this process\textsuperscript{26}. The ability of renin cells to shift between these two cell fates, both of which control oxygen supply to vital tissues, hints at an ancestral role for renin cells in two endocrine systems — the RAS and erythropoietin— which sustain life by controlling fluid volume, perfusion pressure and oxygen delivery to tissues.

Finally, deletion of different effectors in the RAS pathway is known to cause a chronic induction of renin recruitment by removing the negative feedback exerted by elevated blood pressure\textsuperscript{6,27–29}. One example of such a manipulation is the deletion of aldosterone
synthase (AS), the enzyme responsible for synthesis of aldosterone; a downstream effector of the RAAS cascade. In mice suffering from a knockout of AS, blood pressure is chronically deregulated, they are hypotensive and have pathologically elevated levels of plasma renin. However, unlike in other models of renin recruitment, cells of the renin lineage fail to recruit along the arteriole in the developmental pattern and the JG cells undergo a hypertrophic expansion with increased renin expression instead. This outcome is intriguing especially when one considers that mice harboring AS-KO have an extreme response to stimuli that induce recruitment (low salt and angiotensin-converting-enzyme inhibitors) where recruitment is seen even in tissues outside of the kidney such as the adrenal glands\(^{18}\). Taken together, renin cells have an inherent plasticity which helps sustain life during homeostatic threats and understanding the basis of this plasticity has an important implication for clinical treatment of blood pressure and hypertension.

**Control of Renin Expression and Recruitment:**

Because of their crucial role in maintaining homeostasis and stable blood flow to vital tissues, renin cells have evolved to be exquisitely sensitive to their surroundings. In fact, efforts to decipher the mechanisms by which recruitment occurs have historically been hampered by the absence of a classical, truly comparable JG cell line producing renin and this is thought to be a consequence of losing the signals that are present *in-vivo*\(^{22}\). The stimuli that elicit renin recruitment *in-vivo* have been described in great detail (Fig.3) and it is known that there are various pathways by which renin expression, secretion and renin cell recruitment are controlled\(^{30}\):
One of the most powerful, yet poorly understood, mechanisms by which renin expression is controlled is through pressure-induced calcium entry. Heightened blood pressure in the afferent arteriole exerts a negative feedback loop on renin secretion through a rise in intracellular calcium. Although in most cell types a rise in intracellular levels of calcium elicits exocytosis and release of secreted vesicles, the opposite occurs in renin cells and is termed “the calcium paradox”\(^{31}\). The mechanism by which calcium is thought to play an inhibitory role in renin production is by diminishing the effects of cAMP by activating phosphodi-esterases and inhibiting adenylyl cyclases\(^{32}\). Because cAMP generally enhances renin production/secretion, as described in more detail below, sharply attenuating its levels is thought to be the mode of action for calcium-dependent inhibition. The molecular effectors which lead to a rise in intracellular Ca\(^{2+}\) levels in response to pressure-the so called “renal baro-receptors”- were not well understood but recent studies from our lab have highlighted the role of \(Lmna\) in transducing pressure signals to the nucleus\(^{33}\). This was demonstrated using an aortic co-arctation model where a partial ligation of the aorta between the two kidneys induces a pressure differential between the two kidneys. The renin cells in each kidney respond in an opposite fashion where renin is upregulated in the kidney receiving lower blood pressure/flow while the converse is true for renin cells in the opposite kidney. This response is blunted in animals with conditional deletion of \(Lmna\) in renin lineage cells and renin cells fail to modulate their expression of renin in response to pressure in these animals. However, the exact relationship between \(Lmna\), integrins and calcium in the governance of renin expression remains to be elucidated.
The other main stimulus which elicits renin expression and recruitment, which is highly relevant to this thesis, is the cyclic-adenosine monophosphate (cAMP) signaling pathway. This pathway is activated by noradrenaline binding to β-adrenergic receptors at the surface of renin cells and smooth muscles along afferent arterioles leading to the glomeruli. These are G-protein coupled receptors which transduce the signal into cAMP which ultimately results in CREB binding CRE loci in the renin regulatory regions to enhance transcription \(^{34}\). Although CREB is a putative binding factor for many loci in the genome, Kurtz et al demonstrated that it is a bona fide binding factor at the renin gene through competitive assays, enhancer reporter fusions and deletion studies. Furthermore, it is known that the transcriptional co-activators CREB-binding Protein (CBP) and p300 play a key role as their ablation in renin cells results in both a reduction in renin expression as well as a blunted response to known stressors \(^{35}\). It is suspected that CBP and p300 induce acetylation at the renin gene to facilitate expression of renin mRNA due to their known histone-acetyltransferase activity; but the exact extent to which this contributes to renin expression remains to be investigated\(^{20,30}\). The importance of the cAMP signaling pathway lies in the fact that it is the effector for many of the ligands/extracellular signals that the renin cells receive and plays a vital role in the regulation of renin.

Although the two pathways, described above, are the main regulators of renin expression and relate to molecular signal detection, renin cells also respond to the physical nature of their environment and contact with neighboring cells:

- **Notch Signaling:** Our lab has established that members of the Notch signaling pathway including Notch, Jagged and the transcriptional regulator \textit{recombination}
signal binding protein for immunoglobulin kappa-J (RBP-J) are all expressed in renin cells. Loss of RBP-J specifically in renin cells using a conditional knockout approach highlighted the importance of the Notch signaling pathway due to the mice’s inability to recruit renin-expressing cells. However, deletion of Jagged did not manifest an impairment in renin cells nor their ability to recruit raising the possibility that other Notch ligands could compensate for the loss of Jagged1. Lending further importance to Notch signaling is the fact that deletion of the RBP-J binding site in vivo leads to a reduction of about 60% in renin transcription in As4.1 cells, a tumoral cell line constitutively expressing renin.

- Cellular Junctions: Two examples of the importance of cellular junctions in the regulation of renin recruitment are Connexin-40 and β-integrin. Mice with mutated/knock-out Connexin-40 suffer from a complete mis-localization of renin cells from the afferent arteriole around the glomeruli and to the extraglomerular mesangium associated with the glomerulus. Furthermore, these mice, which develop malignant hypertension and hyperreninemia, are unresponsive to cues from the macula densa as well as classical ACE inhibition. In this condition, the cells react as if they are continuously sensing decrease perfusion pressure, thus their hyperreninemia. Another distinct example of renin cells interacting with their extracellular environment is seen in a conditional deletion model of β-integrin in renin cells. Although the cells seem to be localized properly, they have diminished renin expression and cannot enact an appropriate recruitment response when stimulated. These findings highlight the importance of physical contact as a stimulus for renin cells as well as their ability to recruit.
Whilst there are other molecules and signals which regulate renin expression and recruitment, their mode of action usually relies on influencing one of either cAMP levels or calcium influx. For example, loss of fluid-electrolyte balance by a reduction in circulating NaCl levels is detected by the macula densa, a group of cells located in the juxtaglomerular apparatus adjacent to renin cells and distal nephron tubules. Low NaCl levels is a signal indicating a reduction in fluid volume which the macula densa transduces and activates cyclo-oxygenase 2 (COX-2) to produce prostaglandin-E2 (PGE₂), a paracrine molecule that induces expression of renin and recruitment of smooth muscle cells. This signaling pathway acts by the E4-receptor on the renin cell membrane and stimulates adenylyl cyclase to increase cAMP levels leading to increased renin but the macula densa can also reduce PGE₂ levels when homeostasis is threatened by elevated blood pressure. It is also known that one of the downstream effectors of the RAS pathway, angiotensin II, participates in a prominent negative feedback loop on renin cells by acting on AT1 receptors. AT1 transduces the signal and actsives Gq leading to activation of phospholipase C and release of diacylglycerol and inositol triphosphate which release intracellular stores of calcium leading to a reduction in renin expression. Because angiotensin II is a potent vasoconstrictor, it is not currently known to what degree the suppression of the renin program is due to the physiological inhibition due to high blood pressure or AT1’s activities.

**Epigenetics of Renin Cells and their Descendants:**

As described above, there is a wealth of information on how renin expression is regulated as well as the control of the recruitment process. However, there is a dearth of information on the mechanisms which endows renin cells with the ability to
transdifferentiate and it is likely that the answer lies deeper in the regulation of the epigenome due to the fact that recruited cells are derived from renin-expressing progenitors\textsuperscript{7}. Early in mammalian development, renin expressing cells are widely distributed throughout the renal arterial tree \textsuperscript{44}. As the fetus develops, those renin-expressing cells differentiate into smooth muscle cells and expression of renin is progressively restricted to the classical JG position found in the adult animal\textsuperscript{7}. Under physiologic conditions of fluid/electrolyte balance, expression of renin from the juxtaglomerular cells is sufficient to cope with the requirements of everyday life. However, when homeostasis is threatened, smooth muscle cells along the renal arterioles reacquire the renin cell program in a pattern resembling the one found in the embryo\textsuperscript{5}. This process can occur in both natural contexts such as in dehydration, sodium depletion and hemorrhaging or through artificial manipulations using renin-angiotensin blockers.

We have previously shown that those vascular cells which have the ability to develop renin expression are descended from renin expressing cells and thus are described as “cells of the renin lineage”\textsuperscript{7,19,45}. We have also demonstrated that renin expressing cells have a unique set of epigenomic features including enrichment of activating marks and accessible chromatin at renin identity genes known as super-enhancers\textsuperscript{38}. Furthermore, perturbation of chromatin organizers such as CTCF have a drastic effect on renin lineage cells and diminish their ability to express renin highlighting the importance of the epigenetic landscape\textsuperscript{46}. Nevertheless, the resting/basal epigenetic state of renin lineage cells has not been described and what changes occur in these cells at the level of the epigenome and transcriptome remains undiscovered. Because the ability to induce and
activate the renin cell program is dependent upon the developmental history of the cells and the identity of their progenitors, it is logical to infer that these cells retain a molecular memory of the renin program which is activated under physiological distress. One of the most important ways in which cells pass on information to their descendants is via their epigenetic landscape/chromatin configuration\textsuperscript{47,48}. Therefore, we hypothesize that renin cell descendants possess a unique configuration of chromatin marks and enhancers which endow them with the molecular memory of the renin cell identity. Said memory may be imprinted upon their epigenome with poised histone marks and accessible chromatin to enable rapid remodeling and activation of the renin cell identity and this hypothesis constitutes the core of this thesis where we explore in Chapters III and IV the epigenetic and transcriptional landscape of renin cells and their descendants.

Overview:
The governance of cell fate is a critical factor in the survival of all living organisms. To ensure stability of cell identity and cell fate, organisms have evolved sophisticated epigenetic processes to encode a regulatory network which silences genes associated with other cell types while promoting expression of genes critical to their own cell type. Thus, a cell which can malleably change its identity and fate would serve as a fascinating model for studying the mechanisms of epigenetics and cell fate. Given that the renin cell displays these characteristics, my thesis examines the epigenetic basis of the recruitment process and the various molecular factors involved in bestowing this remarkable ability on renin cells.
In **Chapter II**, we answer a long-standing debate on the contribution of proliferation to the recruitment process. We demonstrate, using multiple techniques and under various models of recruitment including acute and chronic conditions, that there is a minimal amount of proliferation in recruitment. We also show that the cell cycle (as analyzed through the transcriptome of recruited renin cells) is heavily downregulated in these cells indicating the lack of proliferation in this phenomenon.

In **Chapter III**, we isolate vascular cells from the renin lineage to assay their transcriptome and epigenetic profile before and after they are subjected to recruitment. We find that vascular renin lineage cells share a surprising level of similarity with renin cells with differences being concentrated at endocrine and contractile loci which undergo remodeling to allow the cells to vacillate between these two cell states.

In **Chapter IV**, we delve deeper into the epigenetic data we have collected from renin cells to elucidate the mechanisms by which the epigenetic landscape facilitates expression of the renin program. By comparing our renin cell ATAC data to ATAC-seq from many cell types, we identify *Mef2c* as a critical transcription factor whose perturbation leads to an attenuation of the renin cell program. Similarly, our previous work\textsuperscript{[38]} defining the repertoire of super-enhancers in renin cells led to the identification of *Klf2*, the highest expressed transcription factor with a super-enhancer in its regulatory region. Taken together, we demonstrate the utility of epigenetic data, elevating beyond being simply a descriptive pursuit.

In **Chapter V**, I end with a discussion of my work as well as future directions for investigation.
Fig.1 The-Renin Angiotensin-System: The rate-limiting step of this cascade involves the secretion of renin from juxtaglomerular cells in response to sympathetic stimulation, changes in perfusion pressure and salt levels in the urinary system. Renin, a hormone enzyme, cleaves angiotensinogen into angiotensin I, an inactive deca-peptide. Angiotensin I is further processed into angiotensin II by angiotensin-converting-enzyme (ACE) to produce angiotensin II. Angiotensin has a critical role in restoring homeostasis namely by inducing vasoconstriction to increase blood pressure and stimulating the adrenal gland to produce aldosterone which promotes sodium re-uptake in the kidney. Together, these actions regulate blood pressure and fluid-electrolyte balance, thus restoring homeostasis.
Figure 2:

(a) Harvest Controls

(b) Untreated Recruited Repression 2 Days Repression 5 Days

Renin ELISA

(c) mRNA Expression

(d) Relative Renin Expression

(e) Recruited Recruited Repression 2 Days Recruited Repression 5 Days

Recruited Recruited Repression 2 Days Recruited Repression 5 Days

Untreated Recruited Recruited Repression 2 Days Recruited Repression 5 Days

Recruited Recruited Repression 2 Days Recruited Repression 5 Days

Recruited Recruited Repression 2 Days Recruited Repression 5 Days

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Recruited Recruited Repression 2 Days Recruited Repression 5 Days

Recruited Recruited Repression 2 Days Recruited Repression 5 Days
**Fig.2 Forward + Reverse Plasticity of Vascular/Renin Expressing Cells:** The recruitment process involves transformation of vascular cells into renin expressing cells before returning to their original cell fate. A) Schematic depicting the treatment mice were given to stimulate recruitment (through 7 days of homeostatic threat with low sodium and ACE inhibition) before inducing a reversal through high salt and saline drinking water. B) *In-situ* hybridization for renin mRNA confirmed recruitment after 7 days with a gradual loss of renin expression in the recruited cells with progressively higher salt loading. C) Relative renin mRNA expression as measured by qPCR from kidney cortices. D) Circulating levels of renin protein as measured by ELISA for renin in blood plasma. E) Immunostaining for renin protein revealed an interesting contrast to the *in-situ* results where renin protein persisted even after treatment with high salt. F) Immunostaining for autophagy gold standard marker LC3B showed no co-localization.
with renin protein indicating that the protein was not being processed by canonical autophagy pathways.
Fig. 3 Pathways Controlling Renin Expression and Recruitment: Schematic of the various ligands, receptors and signaling pathways that elicit/repress renin expression, secretion and renin cell recruitment. Note the importance of calcium and especially cAMP which is involved in transducing the signal from multiple elements and forms the core regulatory factor for renin in the cell.
II. PROLIFERATION DOES NOT CONTRIBUTE TO MURINE MODELS OF RENIN CELL RECRUITMENT

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_Proliferation does not contribute to murine models of renin cell recruitment._ Guessoum O, Zainab M, Sequeira-Lopez MLS, Gomez RA. Acta Physiol. (2020);00:e13532.
Abstract

Aim:

Renin cells are essential for regulation of blood pressure and fluid-electrolyte homeostasis. During homeostatic threat, the number of renin cells in the kidney increases, a process termed as recruitment. It has been proposed that recruitment occurs by proliferation, yet no systematic studies have been performed. We sought to determine the extent to which proliferation contributes to the recruitment process.

Methods:

Mice were subjected to recruitment before analysing the renin cells’ cell cycle. For acute threats, we subjected SV129 and C57Bl6 mice to a low sodium diet plus captopril. Tissue sections from treated mice were co-stained for proliferation markers (Ki67, PCNA, pH3 and BrdU) and renin. Chronic recruitment was studied in deletion models of aldosterone synthase and angiotensinogen through co-immunostaining and counting mitotic figures in periodic acid-Schiff-stained sections. Finally, RNA-seq of renin cells isolated from recruited mice was performed to study mitotic signature.

Results:

Mice subjected to low salt and captopril displayed increases in renin cell number (312 ± 40 in controls to 692 ± 85 in recruited animals, P<.0001), 10-fold increases in renin mRNA and fourfold increases in circulating renin. Co-staining these kidney sections for proliferation markers revealed negligible proliferation of renin cells (<2%), indistinguishable from control animals. Similarly, chronic models of recruitment—aldosterone synthase KO and angiotensinogen KO—had negligible proliferation.
Additionally, the transcriptome of recruited renin cells revealed overall downregulation of mitotic pathways when compared to proliferative cell lines.

**Conclusion:**

Acute and chronic physiological threats to homeostasis produced a distinct increase in renin-synthesizing cells, but we found no evidence to suggest the involvement of proliferation.
Introduction:

Regulation of blood pressure and fluid-electrolyte homeostasis is key to the survival of animals with a closed circulatory system\(^4^9\). The Renin-Angiotensin-System (RAS) is responsible for such regulation and the tightly regulated production and secretion of renin from juxtaglomerular cells (JG) of the kidney constitute the system's rate-limiting step. Renin catalyses the formation of angiotensin I from angiotensinogen in a cascade leading to angiotensin II generation which elicits vasoconstriction and sodium reabsorption ultimately resulting in increased blood pressure. A fascinating phenomenon involving JG cells is that, under conditions in which homeostasis is threatened and blood pressure falls, the number of renin cells in the kidney increases\(^5,3^1\). This process has been termed 'recruitment' to indicate that cells distant from the glomerulus, along the afferent arterioles, and sometimes in the glomerular mesangium and peritubular interstitium express renin\(^7\). However, the extent to which hyperplasia of renin cells contributes to the increase in renin cell number as opposed to transformation of existing cells to the renin phenotype is not fully understood.

The initial description of the increased number of renin cells along the arterioles in response to decreased perfusion and hypotension found no evidence of DNA synthesis as assessed by tritiated thymidine uptake\(^9\). This led to the conclusion that the increased number of renin-synthesizing cells was caused by 'metaplasia' of existing vascular cells rather than cell proliferation. This finding is supported by our previous work indicating that cells descended from a renin-expressing progenitor are capable of re-expressing renin\(^7\). In addition, it has been reported that recruitment involves cells exhibiting de novo
renin expression as determined by dual colour lineage tracing in a process termed neogenesis \textsuperscript{15}. However, and in contrast to these findings, other groups proposed that proliferation of JG cells plays a key role during recruitment and use the term hyperplasia to describe the process \textsuperscript{50–53}(Figure 1). Despite these conflicting findings, a systematic assessment of renin cell proliferation with various proliferation markers and transcriptomic analysis has, to date, not been conducted to conclusively resolve this question.

The prevailing hypothesis in the study of renin recruitment involves phenotypic switching/transdifferentiation of existing vascular cells into renin-expressing cells (Figure 1). In this study, we use several models in which blood pressure and fluid homeostasis are threatened to stimulate the increase in renin cell number to test the hypothesis that proliferation plays a role in the recruitment process (Figures 1 and 2). We performed co-immunostaining for renin and various proliferation markers to determine the number of renin cells undergoing cell division during basal physiological conditions and under homeostatic threats. Additionally, we utilized Fluorescence-Activated Cell Sorting (FACS) to purify recruited renin cells. These cells were subjected to RNA-seq to examine their transcriptome and compare them with proliferative cell lines to determine if they had a mitotic signature. Taken together, these assays allowed us to measure the contribution of proliferation to renin cell recruitment.
Results:
To determine the extent to which proliferation occurs during acute homeostatic threats, we treated mice with a diet low in sodium and captopril in their drinking water for a week to stimulate the recruitment of renin-expressing cells. We performed these experiments on both the C57Bl6 and SV129 strains, two strains historically used for studying renin cells. Furthermore, we also treated mice with a diet high in salt and saline in the drinking water. Staining for renin protein confirmed the effects of the treatments as there was an increase in vascular, renin-expressing cells in the low salt + captopril condition, whereas there was a clear reduction in renin-expressing cells in the high salt condition (Figure 3A). Expansion of renin cell number largely occurred in afferent arterioles (aa), whereas in the control group, renin cells remained localized to the juxtaglomerular area. Conversely, a diet high in sodium results in diminished renin expression and fewer renin-expressing cells. This was also confirmed by the juxtaglomerular index (JGI) for each of these conditions (JG Index: controls = 52.7%, low salt = 78% and high salt = 44%, \( P < .01 \)) (Figure 3B). Furthermore, qPCR for renin mRNA on kidney cortices from each of these conditions paralleled the staining results as there was an increase of 10-fold in renin mRNA in the low salt plus captopril group (\( P < .0001 \)) and a tendency towards reduction with a threefold decrease in the high salt diet group (\( P = .059 \)) (Figure 3C). Finally, to confirm that increased expression of renin constituted a functional, physiological response, we measured circulating renin levels by ELISA and found a highly significant increase in circulating renin in mice subjected to the low salt diet plus captopril (\( P < .001 \)) which confirmed the above findings (Figure 3D). Circulating renin measurements for mice treated with high salt and saline revealed a significant increase in the SV129 strain but not for Bl6 animals although the trend tended towards reduction
in renin levels (from 63641 to 41943 pg/mL, \(P = .36\)). Finally, we collected urine from the treated mice and quantified the sodium content which was reduced from \(~250\) to 20 mmol/L in mice subjected to low sodium + captopril and significantly elevated to up to 400 mmol/L in the high salt + saline treatment (Figure S1, \(P < .01\)). These results are indicative of the efficacy of the treatments as mice treated with low salt and captopril attempt to retain sodium to expand extracellular fluid volume and raise blood pressure while mice exposed to high salt attempt to excrete it to lower blood pressure. Taken together, these results indicate that homeostasis was perturbed, resulting in recruitment of renin cells in mice treated with low salt and captopril and repression of renin in mice treated with high salt and saline.

Having demonstrated that the mice responded to the treatments appropriately, we proceeded to perform co-immunofluorescence for renin and Ki67 to identify dividing renin cells in each condition (Figure 4). All proliferation events in the kidney were counted and assigned to one of several groups: tubulointerstitial (Figure 4Ai), JG cells positive for renin expression (Figure 4Aii), JG cells negative for renin expression (Figure 4Aiii) and glomerular cells (Figure 4Aiv). Figure 4B illustrates the results of counting all the events from two non-consecutive kidney sections for each treatment and reveals that \(>90\)% of the proliferation occurs in the tubular compartment while other cell types (including renin-expressing cells) rarely undergo cell division. Because the different conditions result in vastly different numbers of renin-expressing cells, we sought to normalize the number of proliferating renin-positive cells. This was accomplished by dividing the proliferating renin cell number by the total number of renin cells for mice in each treatment. Control mice with more than 300 renin cells were found to have six
dividing renin cells on average while recruited mice had fewer than 10 dividing renin cells out of 692 (Figure 4C&D). Therefore, the proportion of dividing renin cells under basal conditions was low (<2%) and was not significantly increased, that is remained unchanged during recruitment ($P = .44$). Considering that recruitment generates an increase from 312 to 692 renin cells between the control and low salt + captopril condition, this degree of proliferation cannot account for the increased number of renin cells observed. In addition, we performed co-staining for renin and other proliferation markers, such as pH3 and PCNA, and observed identical results, further emphasizing the lack of proliferation during stress (Figure S2). Therefore, these findings suggest that proliferation plays a negligible role in the increase in renin cell number observed after a whole week of a sustained homeostatic threat.

To identify renin cells undergoing DNA synthesis and actively entering S-phase, we injected BrdU into the control and recruitment groups and performed double immunostaining for renin and the BrdU analog. Figure 5A&B depict examples of proliferating renin cells identified in the mesangium and JGA respectively. All such events were counted and divided by the total number of renin cells per section and the results are reported in Figure 5C. Although the presence of BrdU + renin cells was detected in the recruited samples, which was notably absent in the controls, the prevalence of such events was exceedingly rare and constituted less than 0.2% of all renin cells. Therefore, the DNA synthesis assay strongly suggests that proliferation does not contribute significantly to the increase in renin cell number during acute recruitment.
Having established that renin cells do not proliferate to restore homeostasis in response to a sustained physiological threat of an entire week, we sought to determine whether proliferation plays a role in more prolonged/chronic models of renin recruitment. Specifically, we measured proliferation in mice homozygous for aldosterone synthase deletion and mice homozygous for angiotensinogen deletion\textsuperscript{14,28,56}. Each of these models exhibits increased renin expression and heightened renin cell number as a result of chronic homeostatic imbalance (Figure 6A). We performed co-immunostaining for the proliferation markers described above and renin to quantify proliferating renin cells in aldosterone synthase KO tissues (Figure 6Bi), and found comparable results with very low/negligible levels of proliferation (less than 1\%) in this model (Figure 6Bii). In addition, we performed PAS staining in angiotensinogen KO tissues across different ages throughout development to visualize nuclei undergoing mitosis (Figure 6Ci). These were quantified and normalized to glomerular number before comparison to wild-type Bl6 controls which revealed no significant increases in proliferation. Therefore, in models of subacute and chronic homeostatic threats accompanied by massive increases in the number of renin cells, we found no evidence of increased proliferation. We must conclude, therefore, that renin cells do not undergo proliferation to restore homeostasis.

Finally, we analyzed the transcriptome of recruited renin cells to determine whether they expressed genes associated with progression through the cell cycle. We challenged C57Bl6 mice bearing a Ren1C-YFP transgene which labels all renin-expressing cells with YFP using the conditions described above to stimulate recruitment\textsuperscript{38} (Figure 7A). YFP+/renin-expressing cells were then isolated by FACS and processed for RNA-seq to
measure the expression of cell cycle-associated genes such as Ki67. Expression of Ki67 in both recruited and untreated YFP + cells was exceedingly low (<5 transcripts per million). Furthermore, data on cell types known to have a high proliferative capacity were extracted from the ENCODE database and used to compare to the expression levels in our cells (Figure 7B). Expression of Ki67 was about fivefold higher in the HeLa and MCF-7 breast cancer tumoural lines than in the recruited renin cells. Additionally, BTG2, a known tumour suppressor\textsuperscript{57,58} was highly enriched in the recruited cells but sharply diminished in the proliferative cell lines indicating the anti-mitotic state of these cells.

Our initial analyses focused on cell cycle genes such as tumour suppressors but, to expand our study and increase its value, we decided to perform a whole transcriptome evaluation to determine differentially regulated pathways. For this analysis, we used RNA-seq data from As4.1 cells\textsuperscript{38}, a tumoural cell line which expresses renin and may serve as a better basis for comparison when looking at changes occurring at the level of the transcriptome (Figure 7C). We used the DeSeq2 package to find differentially regulated genes between the recruited renin cells and the As4.1 cells, which were then examined to determine up/downregulated pathways using the DAVID-KEGG Annotation. About 2830 genes were found to be differentially regulated (Figure 7D), but the most downregulated pathway in the recruited renin cells relative to the As4.1s was the cell cycle, confirming our previous data (Figure 7Eii). Upregulated pathways, by comparison, include pathways known to play an important role in renin cells such as the PPAR signalling pathway, metabolism of xenobiotics involving genes such as AKR1B7 etc (Figure 7Ei). Therefore, our results mirror both our findings regarding proliferation as
well as previous literature involving renin cells. These results improve upon and support our initial findings of proliferation playing an inconsequential role in the increase in renin cell number seen during recruitment.

Discussion:

The question of renin cell proliferation in response to homeostatic threat has been subject to contradictory findings in the field of renin research. This work finds very little to no contribution of proliferation to the increase in renin cell number seen during stress. To our knowledge, ours is the first work to systematically assess proliferation using various assays including various proliferation markers co-stained with renin, DNA synthesis assays, mitotic figure observation and transcriptomic profiling. We used different strains of mice alongside both acute and chronic models of homeostatic threat and failed to detect a significant degree of proliferation in any of these models. Because none of these methods or models support proliferation having anything beyond a minute effect on the increase in renin cell number, we conclude that the increase in renin cell number is likely due to phenotypic switching of neighbouring, vascular smooth muscle, mesangial and interstitial cells to adopt a renin-expressing cell identity. Furthermore, we also treated mice with a diet high in salt and saline in the drinking water, conditions known to stimulate an increase in proliferation throughout the kidney as the bioenergetic burden on tubules is increased. As expected, we observed a large increase in the proliferation of the tubular compartment and interstitial cells, yet renin cell proliferation remained low in response to a broad range of physiological challenges indicating that other mechanisms may be responsible for the increase in renin cells in response to stress. Considering that a single mouse kidney section contains between 50,000 and
100,000 cells, the exceedingly low proportion of renin cells undergoing proliferation serves to highlight that mechanisms other than proliferation govern the recruitment process. In addition, the high expression of BTG2, the downregulation of cell cycle pathways and the exceedingly rare occurrence of reninomas suggest that the mitotic programme of these cells is highly repressed. The reasons for this repression are unclear but one may speculate that a cell critical for maintaining homeostasis and survival would be under constant and precise regulation to prevent their tumourigenesis. Although the historical difficulties in isolating renin cells (low cell number, etc) have limited studies of the cell cycle and its components in the renin cell, emerging technologies and techniques may help overcome such limitations.

During early embryonic life, renin expression is widespread throughout the kidney and in the vasculature in particular where it is thought to aid in the formation of vessels. However, as ontogeny proceeds, expression of renin gradually recedes until it is spatially restricted to the juxtaglomerular area. However, when blood pressure falls or during dehydration, renin is expressed by cells in the vasculature in a pattern mimicking the foetal pattern. It can be assumed that numerous cells re-developing renin expression is an evolutionarily advantageous trait as it allows a rapid response to loss of homeostasis. Considering that the RAS plays a crucial role in survival by ensuring constant perfusion and oxygen delivery to critical organs, the ability to rapidly address loss of homeostasis would dramatically increase the chances of survival. This line of thought is supported by the fact that the RAS and the ability of renin cells to be recruited are conserved across various species ranging from fish to mammals. Increasing the number of cells engaging in the production of renin exponentially increases the quantity
of renin released allowing acute physiological threats to be dealt with in a timely manner ensuring survival.

From an evolutionary standpoint, one may speculate that proliferation of renin cells, on the other hand, is a costly and ineffective strategy to cope with homeostatic threats. After threats have been addressed, renin expression must quickly fall to avoid adverse cardiovascular events and/or due to excessively high blood pressure. If proliferation and migration of JG cells truly occurred, those cells would have to undergo rapid apoptosis and be phagocytosed by nearby cells to maintain the structural integrity of the kidney. These events have not been observed. From a bio-energetic standpoint, this process is unlikely as it requires large amounts of energy and resources to synthesize the new cells to be ultimately destroyed. Furthermore, the temporal requirements for proliferation are inappropriate to serve as a mechanism for addressing acute homeostatic threats. The shortest time required for mammalian cells to undergo cell division is estimated to be between 24 and 48 hours. Acute threats, such as dehydration, can be lethal within a much shorter time frame making proliferation an ineffective mechanism for coping with such threats. These arguments, in addition to all the supporting evidence demonstrating a lack of proliferation, strongly refute proliferation as a mechanism to restore homeostasis neither for acute or chronic threats.

Having established that proliferation is a minimal contributor to the recruitment process, future studies should focus on elucidating the molecular mechanisms governing the ability of cells in the vasculature to adopt the renin cell identity. Our group has previously demonstrated, using ATAC and ChIP-seq, that native JG cells as well as recruited renin cells have activating marks at various loci which regulate the expression
of core genes of the renin cell identity\textsuperscript{38}. Additionally, we have also found a set of transcription factors that are expressed in recruited renin cells and it is likely that these are involved in changing the epigenetic landscape and activating the necessary genes for recruitment. It remains to be determined what is the native state of the recruited cells before recruitment and while they are still smooth muscle, mesangial and pericyte in nature. Whether they are prepared to adopt the renin cell identity by having poised epigenetic marks at particular loci or if they must undergo a complete remodelling of their epigenetic landscape remains unknown but represents an exciting avenue for future research.

In summary, we used multiple methods in an attempt to detect proliferation of renin cells during homeostatic threats and found that the cells do not undergo significant proliferation to produce the dramatic expansion of renin cells seen during stress. Thus, based on available experimental data, we must conclude that phenotypic switching of renin lineage cells into renin cells is the main contributor to elevating renin levels to cope with physiological threats.

**Acknowledgments:**

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**Conflicts of Interest:** None declared
Figure 1: Proliferation vs plasticity. Hypotheses put forward to explain the increase in renin cell number seen during stress. One possibility is proliferation and migration of existing renin cells to bring about an overall increase in renin cell number. An alternative hypothesis is phenotypic switching of neighbouring cells in the kidney. A systematic evaluation of proliferation in the recruited kidney has not been performed to date.
**Figure 2:** CONSORT diagram of mouse subjects. Consort diagram illustrating the genotype and number of mice utilized for each experiment throughout this paper.
Figure 3:

a) Control Low Salt + Captopril High Salt + Saline

b) JGI B16 JGI SV

b) JGI B16 JGI SV

B16 Renin Expression

SV Renin Expression

Renin ELISA B16 Renin ELISA SV

Test group Test group Test group Test group

% Captopril % Captopril

Test group Test group Test group Test group

Relative renin expression Relative renin expression

Test group Test group Test group Test group

Concentration (pg/mL) Concentration (pg/mL)

Test group Test group Test group Test group

p = 0.059 p = 0.062

**** = p < 0.0001

ns

*** = p < 0.001

* = p < 0.05

ns
**Figure 3:** Changes in renin expression constitute a physiological response against homeostatic threats. A, Kidney sections of mice stained for renin protein, marker of renin cells, show a notable increase in renin cell number in the captopril-treated group. aa, afferent arteriole. *Glomeruli. B, Juxtaglomerular Index (JGI) quantification for each condition is consistent with staining results. JGI, number of JGAs positive for renin staining/total number of glomeruli × 100. C, Relative renin expression normalized to GAPDH using kidney cortices from study animals. D, Changes in renin mRNA expression are reflected in circulating/active renin protein levels in the circulation to respond to loss of homeostasis. n ≥ 4 and all points on graphs represent individual mouse test subjects.
Figure 4:

a) i) Tubulo-Interstitial Cells
   ![Image of Renin, Hoescht, and Ki67 staining for Tubulo-Interstitial Cells]
   ii) JG+
   ![Image of Renin, Hoescht, and Ki67 staining for JG+]
   iii) JG-
   ![Image of Renin, Hoescht, and Ki67 staining for JG-]
   iv) Intra-Glomerular
   ![Image of Renin, Hoescht, and Ki67 staining for Intra-Glomerular]

b) Control Ki67 Proliferation
   ![Graph showing Ki67 proliferation in Control group]
   Captopril-Ki67 Proliferation
   ![Graph showing Ki67 proliferation in Captopril group]
   HighMA-Ki67 Proliferation
   ![Graph showing Ki67 proliferation in HighMA group]

c) Renin Cell # B6
   ![Graph showing Renin Cell # B6 proliferation by diet group]
   Renin Cell # SV
   ![Graph showing Renin Cell # SV proliferation by diet group]

d) % Proliferating Cells B6 Ki67
   ![Graph showing percentage of proliferating cells B6 Ki67 by diet group]
   % Proliferating Cells SV Ki67
   ![Graph showing percentage of proliferating cells SV Ki67 by diet group]
Figure 4: Quantification of proliferating renin cells reveals proliferation is infrequent and not significantly increased during acute homeostatic threats. A, Kidney sections of test groups co-stained for renin protein and Ki67. Proliferating cells were identified in: (i) Tubular cells, (ii) JG cells positive for renin, (iii) JG cells negative for renin and (iv) Intra-glomerular cells. Dotted lines indicate glomeruli. B, Quantification of all proliferating events in kidney sections organized by cell type across control, recruited and high salt + saline conditions. C, Quantification of total number of renin cells per section in each condition. D, Normalization of proliferating Ki67 + renin-positive cells obtained by dividing the number of dually positive (Ki67+, renin+) cells by the total number of renin + cells per section × 100. n ≥ 5 and all points on graphs represent individual mouse test subjects.
Figure 5: Co-staining Renin and BrdU Reveals Negligible Proliferation of Renin Cells During Recruitment: (A) A proliferating recruited mesangial cell expressing renin. B, Proliferating renin cells in the juxtaglomerular area. C, Quantification of all renin-expressing cells displayed in a pie chart with BrdU ± cells illustrated. n = 3 representing mouse subjects.
Figure 6: Proliferation of renin cells under chronic conditions: (A) Immunofluorescence for renin protein in two conditions of chronic stress: aldosterone synthase KO and angiotensinogen KO. B, (i) Quantification of all proliferating events in aldosterone synthase KO tissues co-stained for renin and Ki67 as in Figure 3. (ii) Normalization of proliferating renin cells by total renin cells by: # of JG + cells/Renin Cell # per section \( \times \) 100. C, (i) PAS staining in angiotensinogen KO tissues to identify mitotic figures in vessels as shown by the arrow. (ii) Quantification of mitotic figures across several post-natal ages and compared to wild-type Bl6 controls. \( n \geq 2 \) and points on graphs represent individual mouse test subjects.
Figure 7:

a) [Images of cell cultures labeled Control and Recruited]

b) [Graphs showing K67 and BTG2 expression levels across different cell types]

c) [Image of tissue staining for As4.1]

d) [MA Plot indicating expression levels]

e) i) [Bar chart showing upregulated KEGG pathways]

ii) [Bar chart showing downregulated KEGG pathways]
**Figure 7:** Transcriptome analysis of isolated cells; expression of proliferation-associated genes does not increase during physiological threats: (A) Frozen tissue sections of mice bearing a transgenic YFP which labels renin cells and reports the activity of the renin promoter. Conditions observed were basal physiological conditions and after subjection to homeostatic threats. A clear expansion of the number of YFP + renin cells is seen under stress B, Transcriptome profiling of renin compared to cell lines known to have a high degree of proliferation. (i) Expression of Ki67 proliferation marker. (ii) Expression of BTG2/Anti-Proliferation Factor 2. C, Tumoural cell line As4.1s, which constitutively express renin, stained with neutral red. Adapted from reference 16. D, Using the R package DeSeq2, 2830 genes were identified as differentially expressed between these two cell types. The manta-ray (MA) plot depicts the expression level and significance of the genes used. E, (i) Depiction of up/downregulated pathways in recruited renin cells when compared to the renin-expressing, cancerous As4.1 cell line. (ii) The most downregulated pathway in this comparison is the cell cycle.
Supplementary Figure 1: Urine Sodium Measurements: Urine was collected from mice and Na+ was measured for each condition. Consistent with the results in Figure 1, the recruited mice had lower sodium in their urine and the high-salt + saline group had higher levels. A) Bl6 strain. B) SV strain mice.

n ≥ 3 and all points on graphs represent individual mouse test subjects.
Supplementary Figure 2: Quantification of Proliferation using Other Proliferation Markers: A) Small intestine positive control staining for each proliferation marker. Kidney sections of test groups co-stained for renin protein and B) PCNA or C) pH3. Proliferating cells were identified as one of: tubular-epithelium (TE), JG cells + for renin expression, JG cells - for renin expression and Intra-glomerular cells (G).

\( n \geq 5 \) and all points on graphs represent individual mouse test subjects.
Supplementary Figure 3: Validating BrdU staining: A) Staining for BrdU in small intestine of all animals was performed to confirm BrdU entry and circulation alongside no primary antibody negative staining control. B) Co-immunostaining for renin and BrdU alongside singly stained consecutive sections to confirm specificity of signals.
III. TRANSCRIPTOME AND EPIGENETIC LANDSCAPE OF RENIN LINEAGE CELLS
**Introduction:**

Paramount to the survival of animals with a closed circulatory system is the regulation of blood pressure and blood flow to critical tissues\(^1,2\). This is accomplished by the renin-angiotensin system (RAS), an enzymatic cascade that culminates in the production of Angiotensin II, a potent vasoconstrictor that controls blood pressure, renal hemodynamics and fluid-electrolyte homeostasis. Across millions of years of development, the RAS has been perfected throughout evolution to enable animals to survive fluctuations in fluid-electrolyte balance and blood pressure\(^1,3\). The key event in the RAS and its rate limiting step is the tightly regulated synthesis and secretion of renin, a hormone-enzyme which cleaves angiotensinogen into Angiotensin I which is further converted to Angiotensin II by Angiotensin Converting Enzyme (ACE). Under normal physiologic conditions, renin is secreted by juxtaglomerular (JG) cells located in the walls of the afferent arterioles at the entrance to the glomeruli of the kidney. Although the nominal number of JG cells is small, representing \((0.1-0.01% \text{ of all kidney cells})\)^4, their production of renin is, under normal conditions, typically sufficient to sustain blood pressure and ensure survival. However, during intense threats to homeostasis by hypotension, dehydration or administration of RAS inhibitors, mesangial cells, smooth muscle cells and pericytes along the afferent arterioles transform into renin expressing cells – a process described as “recruitment”\(^5-7\).

We have previously demonstrated that this process of recruitment does not involve proliferation and is a result of direct transdifferentiation of the aforementioned cell types in the kidney\(^8\). These cells undergo a remarkable switch in cellular identity to express renin and other genes crucial to attain the renin cell program before presumably returning to their original identity once the threat abates and homeostasis is restored. The underlying mechanisms which bestow this rare and fascinating ability upon these cells remain unknown.
We have previously shown that those vascular cells which have the ability to develop renin expression are descended from renin expressing cells and thus are described as “cells of the renin lineage”\(^7,19,45\). We have also demonstrated that renin expressing cells have a unique set of epigenomic features including enrichment of activating marks and accessible chromatin at renin identity genes known as super-enhancers\(^38\). Furthermore, perturbation of chromatin organizers such as \(CTCF\) have a drastic effect on renin lineage cells and diminish their ability to express renin highlighting the importance of the epigenetic landscape\(^46\). Nevertheless, the resting/basal epigenetic state of renin lineage cells has not been described and what changes occur in these cells at the level of the epigenome and transcriptome remains undiscovered. Because these cells have previously expressed renin, we propose that they retain the memory of the renin cell identity and such memory resides in their epigenetic landscape. We hypothesize that this memory is imprinted upon their epigenome with poised histone marks and accessible chromatin to enable rapid remodeling and activation of the renin cell identity. In this study, we demonstrate the plastic ability of vascular renin lineage cells to both activate the renin cell identity under stress as well as return to their original identity once homeostasis has been restored. Furthermore, we profile the transcriptome and epigenome of these cells and highlight their poised state through accessible chromatin before undergoing a shift from contractile to endocrine state. Finally, we demonstrate the importance of transcription factors in conjunction with the epigenetic landscape in the regulation and activation of the renin cell identity.
Results:

Chromatin Accessibility of Vascular Renin Lineage Cells:

To study the epigenetic landscape of vascular renin lineage cells and how it is altered during recruitment, we bred mice bearing a transgenic yellow fluorescent protein under the control of the renin regulatory region to mice harboring Cre-Recombinase under the control of the Myosin Heavy Chain 11 promoter and Rosa26-TdTomato (Fig 1A). The resultant offspring (Ren1c-YFP; Myh11-CreERT2; R26RTdTomato) have two fluorescently labelled populations in the kidney: dual TdTomato and YFP fluorescence in JG cells while vascular cells (which derive from the renin lineage) are labelled with TdTomato alone (Fig 1A). This allows separation of the two cell types by Fluorescently Activated Cell Sorting FACS by selecting for Td+/YFP- vascular cells under their basal state (Supplementary Figure 2). After subjecting these mice to recruitment, the vascular cells develop YFP expression as they begin to express renin and thus can be sorted to compare to the Td+/YFP- cells to capture and study their epigenome and transcriptomes. We utilized this model to perform ATAC-seq on both populations to study the accessibilome of the vascular cells before and after they transform into renin expressing cells. We initially compared our recruited (YFP+) ATAC-seq data to existing whole kidney ATAC-seq datasets in order to confirm that we successfully isolated renin expressing cells during the recruitment process. Using the Diffbind package in R studio, we ran a Pearson Correlation analysis on the called peaks from our recruited renin cell ATAC alongside the whole kidney ATAC dataset (Supplementary Figure 1A). We observed that our recruited renin cell ATAC replicates shared a majority of their peaks with a correlation of 90% while only being correlated ≈55% with the whole kidney ATAC datasets. We hypothesized that the shared peaks represented peaks associated
with housekeeping/generic cellular functions and that the differential peaks harbored the renin identity specific regions. To confirm this, we applied the differentially called peaks to GREAT (Genomic Regions Enrichment of Annotations Tool)\textsuperscript{66} in order to obtain a functionally annotation of the enriched regions. This analysis yielded a number of Gene Ontology (GO) terms keenly associated with the renin cell identity such as “regulation of heart contraction” and “regulation of blood circulation” (Supplementary Figure 2B). Furthermore, the genes associated with the differential regions that were used to perform the annotation included known and essential genes involved in the renin programme such as renin itself but also including genes such as *Acta2, Myh11, Pln* and *Hopx*\textsuperscript{16}. Thus, we confirmed the specificity of our recruited renin ATAC.

Having confirmed that our ATAC-seq of recruited renin cells captured the essential features of the transdifferentiation of the renin lineage cells, we then proceeded to compare that dataset to the Td+/YFP- vascular renin lineage cells prior to their recruitment and transformation into renin expressing cells. We initially examined the accessibility signals in the next generation sequencing (NGS) plots with a focus on renin and other genes that are central to the renin programme. Interestingly, we noted that recruited renin cells have a highly accessible peak in the previously characterized “classical enhancer” region roughly 2.7Kb upstream of the renin promoter while the vascular renin lineage cells had a much weaker peak yet it was not completely closed (Fig 1B). We observed similar patterns at *AKR1b7* (Supplementary Figure 2B) while there was a fascinating reverse pattern for genes associated with the contractile/smooth muscle identity such as *Acta2* and *Myh11* where the recruited renin cells had much less accessibility at those genes compared to the vascular renin lineage cells.
(Supplementary Figure 2C). While renin cells are known to have contractile features such as expression of smooth muscle genes in addition to their endocrine phenotype, these results suggest that the vascular renin lineage cells exist in a partially poised state with some measure of accessibility at renin identity genes. Once the stimuli that trigger recruitment are received, it appears that the chromatin is remodeled to diminish the contractile nature of the renin lineage cells and, instead, activate the endocrine, renin cell identity. This interpretation is supported by a Pearson Correlation analysis of the called peaks between these two populations which are extremely similar with a correlation of up to 89% (Fig 1C). Further evidence supporting this interpretation is the GREAT annotation of the differential peaks from the vascular renin lineage cells which is enriched with typically vascular/contractile cell functions such as “actin cytoskeleton organization” and “vascular development” (Fig 1D). Taken together, the ATAC-seq results indicate that the vascular renin lineage cells, while nominally acting as contractile agents to regulate blood flow, exist in a primed state with most of the epigenome partially accessible and ready to activate the renin cell identity.

**Transcriptome of Renin Lineage Cells:**

Having profiled the accessibility of the epigenome of vascular renin lineage cells before and after recruitment, we turned our attention to the expression profile of these cells. We isolated vascular cells as outlined in Fig 2A and used the FACS sorted cells for RNA-seq in order to profile their transcriptome. For recruited vascular cells, we used data from our previous work where we had isolated renin cells from mice subjected to recruitment and compared them to the vascular, untreated cells. We performed a principal components analysis (PCA) to study how the samples corresponding to each
population would segregate and found, as expected, that samples belonging to the same treatment (i.e. untreated vascular cells vs recruited cells) clustered together (Fig 2A). Interestingly, the vascular cells appeared to be less homogeneous than the recruited samples as the latter population samples clustered more tightly than the former. This is because vascular renin lineage cells encompass several cell types including mesangial cells, pericytes and vascular smooth muscle which converge towards the renin cell identity under stress possibly reducing the differences between them. We then performed a differential expression analysis using DeSeq2 to identify important differences between the two populations. We noted that renin, Acta2 and Myh11 were all significantly different in the two populations in patterns similar to our observations with ATAC seq where renin was upregulated during recruitment while the latter two contractile genes were diminished as the endocrine identity was enacted (Fig 2B). Given that we had previously analyzed the recruited dataset and confirmed its enrichment for the renin cell identity, we then focused on the differentially identified genes that were upregulated in the vascular smooth muscle cells. We performed a functional annotation of those genes using Metascape to gain a global overview into how these vascular cells differ from their endocrine state during recruitment (Fig 2C). We noticed a clear enrichment of terms associated with calcium regulation (a crucial ion in the contraction and relaxation of contractile cells) as well as annotations consistent with observations at the level of the accessibilome. Overall, the ATAC and RNA-seq both support the finding that the cells transition from a contractile to endocrine identity by downregulating key features of the other before adopting the cell state required to ensure survival.
Discussion:

Our current studies are, to our knowledge, the first to describe the epigenome and transcriptome of vascular renin lineage cells. We show, by ATAC-seq, that the cells are highly similar even after they transform into renin expressing cells and that those differences manifest in subtle ways such as reduced accessibility at endocrine genes whilst retaining contractile genes in a more open state; a pattern which is quickly reversed as they rapidly adopt the renin cell identity. The vast similarities between these two cell states is likely a key factor in the ability of vascular renin lineage cells to transform into renin cells under stress; a sound strategy for survival given that threats to fluid electrolyte homeostasis can quickly become lethal if blood pressure and flow are not swiftly restored. Furthermore, our results assaying the transcriptome are consistent with this model of a spectrum of renin-like cells where the vascular cells are poised on the precipice of becoming renin cells but have some highly specific contractile features characteristic of vascular cells. In addition, the presence of H3K4me3 and H3K27Ac at the renin locus alongside the silencing mark H3K27me3 renders the renin promoter (and other genes with a similar profile) bivalent and capable of activating once the proper molecular signal has been transduced from the cytosolic periphery into the nucleus. However, it is not known to what extent the epigenome drives these changes in cell fate or is a result of molecular writers aiming to aid in the enhancement / diminishing of expression depending on the need. We, and others (11,32,33,) have shown that direct application of epigenetic marks is sufficient to modulate gene expression to some degree but it has proven difficult, at least for renin cells, to capture the full measure of renin gene activation simply by placing marks such as H3K27Ac.
This is likely due to the fact that the epigenome encompasses a whole host of marks likely acting in concert to promote gene expression as well as the fact that multiple other factors such as topological conformation, and binding of relevant transcription factors etc are not taken into account by such experiments. Nevertheless, these experiments prove the functional nature of the epigenome and that describing the epigenetic state provides a significant insight into the mechanism of gene expression.

Another benefit of describing the epigenome is that it can be used to make inferences regarding the activity of epigenetic writers and transcription factors which may play a central role in the governance of cell identity. Our work on MEF2C (described below) originated from a deeper analysis of our ATAC-seq data beyond simply describing the accessibility of various elements of the genome. By applying stringent cutoffs, comparisons to a multitude of other cell types and the appropriate normalizations, we managed to isolate / capture those critical regions which denote the renin cell identity. While such a dataset is inherently valuable and can be used in multiple ways, our search for enriched motifs brought us to MEF2C which we thoroughly validated through staining and in-situ hybridization before employing a classical genetics approach to demonstrate its functional / important role in regulating the renin cell identity. It can be considered a stroke of serendipity that we had previously identified MEF2c as a potential regulator of the renin cell programme but its recurrent appearances in different assays and experiments highlights its importance in the renin cell and proves the value of such approaches to making use of next generation sequencing data. It is possible to move to an even higher level of analysis through digital footprinting where binding of transcription factors is inferred by the absence of accessibility due to their inhibition of
the Tn5 transposase. By leveraging the power of the transposase to produce high resolution maps of accessibility, there are a multitude of uses for epigenetic datasets and it is our hope that the field continues to move beyond simply descriptive assays into more nuanced and deeper insights into cell fate and function.

In summary, we demonstrated the fascinating ability of vascular renin lineage cells to move between the renin cell programme and their original cell fate. We produced the first descriptions of the epigenome and transcriptome of these cells to highlight their dual / poised nature and how that facilitates their ability to transdifferentiate. Finally, we provided an example of how to probe ATAC-seq (and other types of epigenetic datasets) to produce new insights and identify novel targets in the regulation of cell identity.
Figures:

Figure 1: Chromatin Accessibility of Vascular Renin Lineage Cells:

A)

B)

C)

D)
Figure 2: Transcriptomics of Renin Lineage Cells:

A)

B)

C)
Supplementary Figure 1:

A)

B)
IV: TRANSCRIPTION FACTORS REGULATING THE RENIN CELL IDENTITY AND RECRUITMENT:

Includes writings, data and figures from a internal author research article and a first author research article in preparation:

Introduction:
The renin-angiotensin-aldosterone system (RAAS) is an enzymatic cascade which controls blood pressure and fluid electrolyte balance in the body\textsuperscript{68}. Initially, angiotensinogen is secreted from the liver and is proteolytically cleaved into Angiotensin I through removal of 4 amino acids by the aspartyl protease renin\textsuperscript{19,22}. Angiotensin I is further processed into Angiotensin II by Angiotensin-converting enzyme which is secreted from the lungs. Angiotensin II is the key effector of this cascade as it is a potent vasoconstrictor which raises blood pressure as well as stimulating the adrenal glands to produce aldosterone which promotes sodium reuptake to increase fluid volume and restore osmolality. The rate-limiting step in this cascade is the quantity of circulating renin which, in adult mammals, is produced by a few JG cells which constitute only 0.01\% of the total kidney cells mass\textsuperscript{4}. Under normal conditions of everyday life, secretion of renin by those few JG cells is sufficient to maintain homeostasis. However, under a serious threat to homeostasis, smooth muscle cells along the kidney arterioles are “recruited” into renin-expressing cells and secrete renin until the crisis is overcome\textsuperscript{69}. An interesting consequence of this phenomenon is that, unlike many endocrine tissues in the body, the method used to achieve greater levels of circulating renin rely on increasing renin cell number rather than renin output per cell\textsuperscript{20}. It is also notable that those cells capable of being recruited descend from renin expressing progenitors which indicates that they somehow retain the memory of the renin phenotype\textsuperscript{7}. The mechanisms that underlie the recruitment process and why cells descended from renin-expressing progenitors have the ability to transdifferentiate back into renin cells are currently unknown.
Because the ability to induce and activate the renin cell program is dependent upon the developmental history of the cells and the identity of their progenitors, it is logical to infer that these cells retain a molecular memory of the renin program which is activated under physiological distress. One of the most important ways in which cells pass on information to their descendants is via their epigenetic landscape/chromatin configuration. We had previously explored this hypothesis by profiling the accessibilome of renin expressing cells by ATAC-seq\textsuperscript{38}. We found that cells exhibiting the renin cell program (including JG cells at rest and recruited vascular cells in both acute and chronic models of stress) share a unique repertoire of accessibility genome including the renin gene itself as well as other regions important for the renin program. Similarly, ChIP-seq for H3K27Ac in a model of chronic recruitment (\textit{Ren1c-KO} mice in which the renin gene is ablated and in which vascular cells are driven to constantly express the renin identity\textsuperscript{11,70}) revealed a set of “super-enhancers” which are unique to renin cells and enriched for functions critical to renin cell function. These findings brought new insight into the regulation of the renin cell identity and the role of epigenetics which had not previously been described.

An interesting note on epigenetic data is that it can be extended beyond simply describing the status of genes and the combination of marks / accessible chromatin present at different loci. Because histone marks and accessible chromatin co-operate to promote transcription factor binding to effect changes in gene expression, we sought to explore our previous ATAC-seq and super enhancer data to identify candidate transcription factors which regulate the renin cell identity. Through this approach, we
found two molecular players which have a key role in regulating the renin cell identity: *Mef2c* and *Klf2*. 
Results:

**Identifying Key Transcription Factors Regulating the JG Program through ATAC-seq – MEF2C:**

ATAC-seq data can be probed at individual loci to determine epigenetic changes that happen at the level of individual genes; but it is also possible to perform deeper analysis at the global scale which permits identification of the transcription factors / motifs playing a key role in driving and maintaining cell identity. To that end, we performed an in-depth analysis of our ATAC-seq data from native JG cells and compared it to multiple ATAC-seq datasets from other cell types (cell types collected from the ENCODE database and summarized in Supplementary Table 1) in order to define those regions/peaks which characterize the JG program (Fig. 1A). After quantile normalization and capping at the 99th percentile, we intersected significant peaks with our base called peaks from JG cells to generate a matrix. The matrix was further refined by restricting only to peaks which were at least 2-fold higher in JG cells than in the other samples and we selected those peaks above 0.9 normalized intensity (Fig. 1B). This process produced 2495 peaks which identify the most salient/critical peaks distinguishing JG cells from all other cell types.

To verify that the identified regions/peaks demonstrated specificity for the renin cell program, we applied our peaks to GREAT and observed an enrichment for terms keenly associated with the JG cell identity including “Notch signalling” “regulation of blood pressure” and “response to decreased oxygen” thus validating our analysis (Fig. 2A).

Having confirmed the specificity of our peak set, we then performed Motif analysis using the MEME suite (Multiple Expectation maximizations for Motif Elicitation) to search for relevant transcription factors. We identified MEF2C (Myocyte Enhancer Factor 2C), a
member of the MEF family of transcription factors well-known for their roles in development and differentiation particularly in muscular cells\textsuperscript{72,73}, whose motif was significantly enriched in these “renin-specific” regions (Fig. 2B). To confirm expression of \textit{MEF2C} in renin cells and validate it as a target for classical genetics, we stained tissues from wild-type mice, mice treated with ACE inhibitors for 6 months and mice lacking the renin gene (Ren1c-KO animals; a chronically recruited model of renin expression)\textsuperscript{11,29}. \textit{MEF2C} is seen clearly in the JG area in wild-type animals and in the afferent arterioles and mesangium in the recruited animals in the same pattern as renin confirming its expression in renin expressing cells (Fig 2C). We also performed RNA-scope for \textit{MEF2C}, an extremely sensitive and specific \textit{in-situ} hybridization technique capable of detecting single molecules of RNA\textsuperscript{74}. RNA-scope with dual probes for renin and \textit{MEF2C} in the aforementioned models revealed a strong co-localization of both transcripts further validating the expression of \textit{MEF2C} in renin cells (Fig 2D).

Having identified \textit{MEF2C} through our ATAC-seq analysis and verified its expression in renin cells under various physiological conditions, we proceeded to conditionally delete \textit{MEF2C} in renin lineage cells using our \textit{Ren1d-Cre} mice (Fig 3A). We confirmed successful deletion of \textit{Mef2c} in renin expressing cells by co-immunofluorescence for renin and \textit{MEF2C} (Supplementary Fig 1A+B). Wild-type animals showed a clear co-localization of the two proteins in the JG area which was conspicuously absent in mutant animals confirming that the \textit{Cre-Recombinase} had successfully ablated the floxed \textit{MEF2C} gene. At 4 months of age, these mice presented with lower renin expression as evidenced by immunostaining for renin protein (Fig 3B). The intensity of renin staining was markedly reduced (indicating less renin protein) and the number of
JG areas with renin staining was reduced indicating also a reduction in the number of renin expressing cells as seen by the Juxtaglomerular Index (JGI) (Fig 3C). Measurements of renin mRNA by qPCR and by renin ELISA showed similar trends with lower renin levels in both despite not reaching significance (Fig 3D). Overall, these results demonstrate the utility of ATAC-seq as it allows description of the regulatory state of individual genes but can also be leveraged to unravel key regulators and determinants of cell identity including the renin cell.

While the accessibility patterns are an important aspect of the epigenome, other cis-acting factors such as histone marks play a critical role in facilitating transcription factor binding and gene regulation. Therefore, we probed our previous H3K27Ac data for the transcription factors bearing a super-enhancer in their own regulatory region (Fig 4A)\(^{38}\). To maximize the possibility of identifying a transcription factor which had a key role in regulating the renin cell identity, we restricted our search to only those TFs with a putative binding site / motif in the renin enhancer region. Finally, we then selected the highest expression and accumulation of H3K27Ac (i.e. with the strongest super-enhancer). These criteria led us to the *Kruppel-like Factor 2 (Klf2)* gene. To validate *KLF2* as a target for classical genetics and ablation in renin lineage cells, we first sought to confirm its presence in renin cells. We performed *in-situ* hybridization in recruited animal tissues and found that in acutely recruited mice, clear expression of *Klf2* can be seen in the JG area overlapping the typical location of renin expressing cells. In addition, chronically recruited mice lacking the renin gene (*Ren1c-KO*) had clear expression of *Klf2* not only in the JG area, but also in the mesangium, pericytes and
afferent arterioles where recruitment takes place in this model. We these results, we thereby confirmed our *in-silico* findings regarding *Klf2*'s presence in renin cells.

Having validated the expression of *Klf2* in renin cells, we could move to more functional studies involving classical genetics and ablation. We bred mice bearing floxed *Klf2* alleles to our *Ren1d-Cre Recombinase* to induce conditional deletion in cells of the renin lineage. At 4 months of age, there were clear differences in renin expression when staining for renin protein was performed (Fig 5A). The intensity of renin staining was lower and this was also matched by a reduction in the number of renin expressing cells as seen by the Juxtaglomerular Index (JGI) (Fig 3B). Similarly, expression at the level of the mRNA and circulating renin protein were also attenuated (Fig 3C&D). Taken together, these results highlight the role of *Klf2* in the regulation of the renin cell program and, at a broader level, underlie the importance of epigenetic and histone modifications in driving the expression of genes central to cell fate.
Discussion:
A key benefit of describing the epigenome is that it can be used to make inferences regarding the activity of epigenetic writers and transcription factors which may play a central role in the governance of cell identity. Our work on *MEF2C* originated from a deeper analysis of our ATAC-seq data beyond simply describing the accessibility of various elements of the genome. By applying stringent cutoffs, comparisons to a multitude of other cell types and the appropriate normalizations, we managed to isolate / capture those critical regions which denote the renin cell identity. While such a dataset is inherently valuable and can be used in multiple ways, our search for enriched motifs brought us to *MEF2C* which we thoroughly validated through staining and *in-situ hybridization* before employing a classical genetics approach to demonstrate its functional / important role in regulating the renin cell identity. It can be considered a stroke of serendipity that we had previously identified *MEF2c* as a potential regulator of the renin cell programme but its recurrent appearances in different assays and experiments highlights its importance in the renin cell and proves the value of such approaches to making use of next generation sequencing data. It is possible to move to an even higher level of analysis through digital footprinting where binding of transcription factors is inferred by the absence of accessibility due to their inhibition of the Tn5 transposase. By leveraging the power of the transposase to produce high resolution maps of accessibility, it becomes possible to move beyond simple descriptive assays and develop more nuanced and deeper insights into cell fate and function.
Figure 1. Identifying Unique/Core Renin Cell Elements: a) Conceptual strategy used to identify the most salient / critical renin identity regions. By comparing our renin cell ATAC-seq data from wild-type mice to over 40 other cell types collected from the ENCODE database, we could define the regions most specific to the renin cell. b) Scatterplot depicting individual peak regions after quantile normalization, capping at the 99th percentile and selecting for peaks which were at least 2 fold higher in JG cells than any other cell type. The top right box represents the resulting 2495 peaks which were extracted for further analysis.
Figure 2: Identification and Validation of Mef2c

A) GO Biological Process

B) Optimal Alignment

C) Wild-type  Recruited  Ren1c-KO

D) Mef2c  Renin
Figure 2. Identification and Validation of Mef2c: a) The 2495 peaks selected for their specificity in renin cells were plugged into the GREAT program for functional annotation to confirm that they encompassed the genes and functions critical to the renin cell. b) The MEME suite identified Mef2c’s motif as one which was enriched in those highly specific renin identity regions. c) Immunostaining for Mef2c revealed that it is expressed in JG cells under wild-type conditions and its expression is increased in the vasculature during homeostatic threat in a pattern resembling renin recruitment indicating that is expressed in renin cells. d) RNA-scope for Mef2c and renin revealed that Mef2c is co-expressed with renin in JG cells (red arrows) and also in the arterioles of the kidney (white arrows).
Figure 3: Classical Genetics; Mef2c Deletion in the Renin Lineage

A) 

\[ \text{Ren1}\(^d\) \quad \text{Cre} \]

\[ \text{loxP} \text{Mef2c}^{fl} \text{loxP} \]

\[ \text{loxP} \text{Mef2c}^{fl} \text{loxP} \]

B) 

Control (Mef2c\(^{fl/fl}\))

Mef2c cKO (Mef2c\(^{fl/fl}\); Ren1\(^d\) \(\text{cre/}\)+)

C) 

Mef2c JGI

\[
\begin{align*}
\text{Control} & \quad n = 6 \\
\text{Mef2c cKO} & \quad n = 6 \\
** & \quad p < 0.01
\end{align*}
\]

D) 

Mef2c qPCR

E) 

Plasma Renin: Control vs cKO
Figure 3. Classical Genetics; Mef2c Deletion in the Renin Lineage: a) Schematic depicting the breeding strategy to conditionally delete Mef2c in the renin lineage. b) Immunostaining for renin protein in control/wild type mice as compared to mice with conditional deletion of Mef2c in the renin lineage. There is a clear reduction in the quantity of renin protein being expressed in the mutants as well as the number of cells expressing it. c) Juxtaglomerular index of wild-type and Mef2c-cKO animals. d) Relative renin expression in kidney cortices from wild-type and Mef2c-cKO animals. e) Circulating plasma renin levels in wild-type and Mef2c-cKO animals as measured by ELISA.
Figure 4: Identification and Validation of \textit{Klf2}; a super-enhanced Transcription Factor in Renin Cells

A)

B)

Acute Dehydration (Low Salt + Captopril)  Deletion of the Renin Gene (Ren1c-KO)
Figure 4. Identification and Validation of *Klf2*; a super-enhanced Transcription Factor in Renin Cells: a) Rank ordering of Super-Enhancers (ROSE) in renin cells using H3K27Ac signal. *Klf2* was identified as the transcription factor with the strongest K27Ac signal and highest expression in renin cells. b) *In-situ hybridization* for *Klf2* in acute and chronic models of renin recruitment. As seen under low salt and captopril, *Klf2* is clearly expressed in the JG area where renin cells are located. Under the chronically recruited *Ren1c-KO* model, expression is seen in the mesangium and afferent arterioles where recruitment of renin cells takes place indicating that *Klf2* is expressed by recruited renin cells.
Figure 5: Deletion of Klf2 in Renin Lineage Cells

A) Control: Ren1d+/+  Klf2<sup>flox/flox</sup>  
Mutant: Ren1d<sup>Cre/+</sup>  Klf2<sup>flox/flox</sup>

B) Klf2 JGI

C) Klf2 qPCR

D) Plasma Renin Klf2
**Figure 5. Deletion of Klf2 in Renin Lineage Cells:** a) Immunostaining for renin protein in control/wild type mice as compared to mice with conditional deletion of *Klf2* in the renin lineage. b) Juxtaglomerular index of wild-type and *Klf2-cKO* animals. c) Relative renin expression in kidney cortices from wild-type and *Klf2-cKO* animals. d) Circulating plasma renin levels in wild-type and *Klf2-cKO* animals as measured by ELISA.
Chapter V: DISCUSSION

In this thesis, we have described the recruitment process and were the first to clearly demonstrate the reverse plasticity of vascular renin lineage cells after homeostatic threats have been addressed. Furthermore, we answered a long-standing question in the field of renin recruitment regarding the contribution of proliferation to recruitment in the negative. We described the epigenetic landscape of vascular renin lineage cells and showed that, prior to their transformation into renin expressing cells, they have a highly similar accessibility profile and transcriptome which could prime them to activate the renin program when required. Finally, we demonstrate the utility of epigenetic data such as ATAC and ChIP-seq which we leverage to identify Mef2c and Klf2, important transcription factors in the regulation of the renin cell identity. Here, I speculate on some unanswered questions / interesting avenues for further investigation:

The uncoupling of the transcriptome and proteome during the reversal of recruitment:

As described in Chapter I, vascular smooth muscle cells have the fascinating ability to transform into renin expressing cells during homeostatic threats, but we have shown that they also malleably attenuate the renin program (as seen at the level of the mRNA) when the threats have been addressed. Curiously, the finding that renin protein lagged temporally relative to the expression patterns of renin and other markers such as AKR1b7, suggested that the RAS may have evolved a backup option where, in case another homeostatic threat emerges, the cells are able to release renin protein quickly without needing to transform and resynthesize it. The persistence of renin protein in the cells would seem to support this hypothesis as the levels of circulating renin have
returned to normal indicating that the cells have ceased releasing the protein into the circulation. This hypothesis could be tested by subjecting the mice to another round of homeostatic threat and assessing the strength of the recruitment response (this can be done by measuring circulating renin levels and mRNA levels in the kidney). Furthermore, how long renin protein is retained in the recruited cells remains unknown and should be assayed by taking longer timepoints after application of the repressive treatment. Additionally, it was highly peculiar that no signs of autophagy were observed by electron microscopy or staining for LC3B suggesting that another, hitherto unknown, mechanism is used to degrade and remove renin protein. Historically, it has been difficult to study the processing and trafficking of renin protein due to the absence of physiologically relevant cell models\textsuperscript{19,22} as well as the fact that tagging the renin protein with large, folded fluorophores creates artifacts which result in the protein becoming non-functional (personal communication, Dr. Mullins). It is possible that further technological advancements will allow labelling and observation of the protein in its more native state to further our understanding of how recruited renin cells return to their original phenotype.

**Proliferation and chronic threats to homeostasis:**

As described in Chapter II, we see no evidence of proliferation in acute or chronic models of recruitment. Using various techniques including immunostaining for proliferation markers, mitotic figure counting, transcriptome pathway analysis, we instead find that renin cells appear to have a highly suppressed cell cycle program. This is evident from the downregulation of cell cycle genes, the high expression of tumor suppressors such as BTG2, etc. These results are consistent with clinical observations
on the rarity of reninomas with less than 100 confirmed cases reported since 1967. It is evident that evolution has placed a high emphasis on the prevention of oncogenesis in renin cells. The reasons behind this can be inferred from the critical importance of renin cells in maintaining homeostasis wherein their tumorigenesis would have catastrophic effects on the survival of the animal, thus rendering their genomic and mitotic stability a crucial factor to be maintained. In cases where this balance is perturbed (as seen in patients presenting with reninomas), the body typically suffers from severe hypertension, hypokalemia and an increased risk of life-threatening events such as strokes and heart attacks if left untreated. Therefore, the importance of suppressing the cell cycle in renin cells is paramount in ensuring such events do not occur. The exact mechanisms by which the cell cycle is continuously suppressed in JG cells remain unknown due to the difficulties in isolating renin cells in sufficient quantities to study the molecular effectors (e.g., cyclins and ubiquitin ligases) which control the cell cycle. As technology advances and techniques with higher sensitivity and lower sample requirements than Western blots emerge, these exciting and important questions should be investigated.

An important aspect of the renin cell cycle can be seen in chronic models of recruitment such as the Ren1c-KO, aldosterone-synthase knockout (AS-KO) and the angiotensinogen knockout (Agt-KO) murine models. Each of these models have an excessively activated renin cell program and display varying levels of recruitment. Furthermore, mice subjected to these deletions which have a diminished chronic positive feedback loop on renin expression which leads them to develop vascular lesions and present with concentric vascular hypertrophy wherein the arterial walls
become enlarged and heavily disorganized\textsuperscript{29,70}. Interestingly, and as described in depth in \textbf{Chapter II}, the levels of proliferation in these models of recruitment remains very low. Thus, a question asks itself: where do these cells come from? One possible answer is the neogenesis hypothesis. As described by Todorov and Hugo\textsuperscript{15,45}, renin cells may be produced by \textit{de novo} expression from cells not derived from the renin lineage. By using a \textit{Ren1d-Cre Recombinase} with a \textit{Rosa26-TdTomato/stop/GFP} reporter mouse, there is a brief interval of time in which cells newly expressing renin contain both the \textit{TdTomato} fluorophore and the \textit{GFP} fluorophore and this can be used to detect cells with \textit{de novo} expression of renin. This is a rare event (<10%) which has been observed during recruitment and under basal physiology but may serve as a source of renewal for renin cells without incurring the risks involved with mitotic activity which can introduce the risk of mutations leading to tumorigenesis. In the case of chronic models of recruitment with concentric vascular hypertrophy, the neogenesis phenomenon could produce more renin cells which persist due to the constant and unceasing need for more renin due to the dysregulated homeostasis induced by these genetic models. This hypothesis remains to be tested and raises another question of how the excessive accumulation of renin cells in the arterial wall is maintained. Presumably, these cells must be resistant to autophagic/apoptotic signals and the “normal” cues which maintain the structural integrity of tissues, but this remains an area without much clarity. The underlying mechanisms which allow persistence of these cells poses a question of high clinical relevance as their presence is accompanied by secretion of a host of extracellular matrix proteins which may exacerbate the pathology of these vascular lesions\textsuperscript{29}. Given that such lesions also arise with the conventional treatment for
hypertension such as ACE inhibitors or angiotensin receptor blockers, understanding the cell cycle pathways and mechanisms implicated in these models could provide targets for treating this pathology. Therefore, further studies on the cell cycle and proliferation (or lack thereof) of renin cells could have enormous implications for patient health and prognoses making it a critical area for further study.

What makes vascular renin lineage cells but not other cell types from the renin lineage “recruitable”?

In Chapter III, we described the poised nature of vascular cells of the kidney (all of which derive from the renin lineage) and how their accessibilome and transcriptome exist in a primed state to activate the renin program. An intriguing question which we did not explore is the nature of non-vascular renin lineage cells and their epigenetic profile. As mentioned in Chapter III, we turned away from the Ren1d-Cre Recombinase as it labelled renin lineage cells from the tubular compartment as well which are generally not regarded as being recruitable. It would be particularly interesting to have an active smooth muscle reporter mouse bred to our Ren1d-Cre; TdTomato mice. This combination of reporters renders renal smooth muscle cells labelled in green and red while other renin lineage cells in TdTomato alone. Isolating each of these populations independently and performing CUT&Tag as well as ATAC and RNA-seq could provide some very interesting insights into the mechanics of recruitment and what exact combination of markers / transcription factors are required to enable smooth muscle cells to undergo recruitment. We predict that, in contrast to the vascular smooth muscle cells, the collecting ducts and proximal tubules of the renin lineage would have silencing marks and closed chromatin which precludes them from being in a poised state which
can activate the renin cell identity (Fig 1). Another approach to isolate those salient elements which permit a cell to undergo recruitment could be to compare renal vascular smooth muscle cells to vascular smooth muscle cells from other organs. While the ability of vascular cells, particularly those in the coronary arteries, to undergo phenotypic switching in pathological models such as atherosclerosis has been extensively described\textsuperscript{77}, to date, there have been no reports of extra renal smooth muscle transformation into renin expressing cells. Therefore, using the same mouse described above to isolate these two vascular populations would allow this comparison. This comparison would prove very useful as the differences by and large between these two subpopulations should mainly be restricted to those that allow renal smooth muscle cells to be recruited as opposed to their counterparts in other tissues. It is possible that the chromatin is closed for extra renal SMCs in those renin cell identity genes which are partially accessible in the renal SMCs or that a different configuration of histone marks leads to a more repressed gene status which precludes their transformation under homeostatic threat. The possibilities are many and this would be a particularly interesting experiment to pursue for future researchers in the field of renin recruitment.

A small subplot in the story of recruitment involves tubules of the renin lineage. Despite the fact that recruitment is largely a phenomenon restricted to the vascular cells of the renin lineage, in murine models of Angiotensinogen knockout, renin can be detected in tubules even after nephrogenesis is complete at P45. We have previously shown that deletion of renin from the tubular compartment has no effect on the physiology of renin expression both under basal conditions and during recruitment\textsuperscript{78}. The mice respond to homeostatic threat appropriately by elevating circulating levels of renin to levels
comparable to those seen in wild-type animals. Conversely, deletion of renin from the vascular compartment recapitulates the *Ren1c-KO* with global deletion of renin and a complete inability to raise renin levels during homeostatic threat. The fact that tubules can express renin, an admittedly rare event which has only been observed in the model of angiotensinogen deletion, raises a question on the nature of their epigenetic profile at the renin gene in particular. It would be curious to discover whether they harbor silencing marks as depicted in Fig 1 or whether they exist somewhere along the spectrum with smooth muscle cells but simply farther away from renin cells. These questions are intriguing and should be pursued by those with an interest in exploring recruitment in the tubular compartment.

**The epigenome; beyond description and into function and mechanism:**

In this thesis, mainly demonstrated in Chapter III, we utilized various “omics” techniques to profile the vascular renin lineage cells before and after they underwent recruitment and transformation into renin expressing cells. Our current studies are, to our knowledge, the first to describe the epigenome and transcriptome of vascular renin lineage cells. We show, by ATAC-seq, that the cells are highly similar even after they transform into renin expressing cells and that those differences manifest in subtle ways such as reduced accessibility at endocrine genes whilst retaining contractile genes in a more open state; a pattern which is quickly reversed as they rapidly adopt the renin cell identity. The vast similarities between these two cell states is likely a key factor in the ability of vascular renin lineage cells to transform into renin cells under stress; a sound strategy for survival given that threats to fluid electrolyte homeostasis can quickly become lethal if blood pressure and flow are not swiftly restored. Furthermore, our
experiments profiling the transcriptome are consistent with this model of a spectrum of renin-like cells where the vascular cells are poised on the precipice of becoming renin cells but have some highly specific contractile features characteristic of vascular cells. In addition, the presence of H3K4me3 and H3K27Ac at the renin locus alongside the silencing mark H3K27me3 renders the renin promoter (and other genes with a similar profile) bivalent and capable of activating once the proper molecular signal has been transduced from the cytosolic periphery into the nucleus. However, it is not known to what extent the epigenome drives these changes in cell fate or is a result of molecular writers aiming to aid in the enhancement / diminishing of expression depending on the need. We, and others\textsuperscript{38,79,80}, have shown that direct application of epigenetic marks is sufficient to modulate gene expression to some degree but it has proven difficult, at least for renin cells, to capture the full measure of renin gene activation simply by placing marks such as H3K27Ac. This is likely due to the fact that the epigenome encompasses a whole host of marks likely acting in concert to promote gene expression as well as the fact that multiple other factors such as topological conformation, and binding of relevant transcription factors are not taken into account by such experiments. Nevertheless, these experiments highlight the capacity of NGS data to explore function and that describing the epigenetic state provides a significant insight into the mechanism of gene expression.

**Advances in Next Generation Sequencing Technologies and the future of renin cell omics**

At this point, it is important to mention that this thesis, as a whole, has relied on bulk NGS assays such as bulk RNA-seq, bulk ATAC-seq and CUT&Tag\textsuperscript{81,82}. However, in
recent years, these technologies have witnessed an explosion in new capacities and single-cell capabilities in an attempt to decipher the heterogeneity of cell populations\textsuperscript{83–85}. These new tools open the possibility to probe variation at the cellular level and could be a great boon in improving our understanding of renin cell biology. For example, it is well believed that, during recruitment, the first cells to transform into renin expressing cells are those nearest to the juxtaglomerular apparatus while those further away in the vascular tree are recruited in response to persistent and stronger threats to homeostasis\textsuperscript{9,70,86}. Is this variation in the capacity to be recruited a consequence of cellular communication with those closest to the JG area receiving the strongest input or is there an inherent molecular signature which explains this heterogeneity? The advent of single cell technologies promises to answer these fascinating questions and shed light upon mysteries decades in the making.

However, the limitations of said technologies in their current state should not be understated. For example, single cell RNA-seq techniques such as 10x Genomics typically collect only 10-15% of the total mRNA transcripts present in a particular cell\textsuperscript{87}. This leads to the phenomenon known as “dropout” events where, even for cells of the same cell type, a gene can be detected as lowly-moderately expressed in one cell while having a complete zero count in others as a false negative due to the inefficiency and stochasticity of mRNA capture. To avoid this issue, the typical procedure is to reduce the dimensionality of the data by UMAP or other processes\textsuperscript{88} before aggregating the cells to form clusters which can allow deeper insight and use the signal from many cells of the same cell type to “fill in the blanks” generated by dropouts. Whilst this approach does negate the dropout issue and is immensely useful in separating sub-populations of
in scRNA-seq samples generated from heterogeneous tissues, it falls short of the desired goal of investigating heterogeneity at the single-cell level. The resultant data becomes no different than using FACS to purify a subpopulation before performing bulk RNA-seq which falls short of the promise of these technologies. Furthermore, another limitation of these technologies is that they are "biochemical" wherein cells are extracted from their natural environment and their components removed and studied. By bursting the cells to reach its internal components, much of the spatial information which is key to understanding the variability in form and function of cells is lost, particularly for cells such as the JG cell for whom the environment and surroundings are essential for their function. Additionally, the isolation procedure to extract the RNA or nuclei (depending on the goal of the experiment) can perturb the metabolism of cells and alter their transcriptional profile\textsuperscript{89}. Fortunately, the development of in-situ technologies where RNA-seq and other omics technologies which is performed on fixed tissues can alleviate these problems as well as maintain the critical spatial information used to identify cells and their relationships/interactions with one another\textsuperscript{90,91}. Although these technologies are currently limited in terms of their resolution and sensitivity regarding lowly expressed transcripts, my belief is that spatial omics will be transformative not only in the academic field, but also in the clinic once we are able to perform them affordably and rapidly on patient samples. Suffice to say that we live in an exciting time of discovery, and I look forward to the coming breakthroughs we will be able to make through these new and exciting tools.
Discussion Figures:

Figure 1: Model of Epigenetic Landscape of Renin Lineage Cells

(A) JG cells (SE-bearing gene regulatory region)

Open chromatin

H3K27Ac

H3K9Ac

H3K9Me3

Significant Ren1 mRNA transcription

(B) VSMCs (poised chromatin)

Presence of poised marks: low/no TF binding

Sporadic/Low Ren1 mRNA transcription

(C) Tubular cells/collection ducts (silenced chromatin)

Closed chromatin: presence of silencing marks, with no binding of TF

Absent Ren1 mRNA transcription

Figure 1: Model of Epigenetic Landscape of Renin Lineage Cells A) Cells actively expressing renin have a distinct epigenetic signature at the renin gene (and others) characterized by the presence of open chromatin, activating histone marks such as H3K27Ac and binding of key transcription factors (TF) such as CREB and Med1. Together, these features constitute a super-enhancer which drives strong expression of renin and other key genes. B) Vascular smooth muscle cells which activate the renin program under stress may have poised epigenetic features such as H3K4me1 and low
transcription of the renin gene. The absence of open chromatin and activating marks prevents these cells from ectopically expressing renin until the body is challenged by physiological threats requiring increased renin output. C) Tubular cells (and other cell types) may possess silencing epigenetic features including closed chromatin, repressive epigenetic features such as H3K27me3 and restricted transcription factor binding. As a result, these cells lack the ability to activate the renin cell programme.
Chapter VI: MATERIALS AND METHODS

Mice:

I. Proliferation Studies: To study proliferation of renin cells, we studied mice with manipulations known to affect renin expression. Mice aged 2-3 months were studied and included wild-type C57BL6 mice (Jackson Laboratories) and several genetically modified mouse strains, including mice with deletion of aldosterone synthase (AS), which display a prominent increase in the number of renin cells along the arterioles\textsuperscript{14}, mice with deletion of the angiotensinogen gene, which show a dramatic expansion of renin cells in vessels and pericytes\textsuperscript{6,28}, and mice with expression of YFP driven by the Ren1c regulatory region (Ren1c-YFP mice), which reports activity of the renin promoter\textsuperscript{21}. A minimum of five animals per group were examined with a mixture of males and females. Physiological challenge to induce increased renin expression was performed using a 7-day treatment with low sodium diet (0.1%, 7034, ENVIGO) plus captopril added to the drinking water (0.5 g/L) (20). We also challenged a group of mice with a diet high in sodium (3.2% Na, TD.92012) and saline drinking water. Mice were anaesthetized intra-peritoneally with tribromoethanol (300 mg/kg) prior to harvest and terminated by cervical dislocation subsequent to tissue harvesting.

II. Epigenetic / Transcriptomic Studies: To label and study vascular smooth muscle cells of the kidney, we bred animals bearing our transgenic Ren1c-YFP (described above) to mice harboring Cre-Recombinase under the control of the Myosin Heavy Chain 11 promoter and Rosa26-TdTomato\textsuperscript{63,64}. The resultant offspring (Ren1c-YFP; Myh11-CreERT2; R26RTdTomato can be used to sort vascular cells under their basal state as well as after they have been recruited.
III. Transcription Factor Deletion Studies: Klf2 and Mef2c

Floxed Mef2c mice were obtained from Jackson labs (Vong et al) and floxed Klf2 mice were a kind gift from Dr. Stephen Jameson (University of Minnesota).

All animals were handled in accordance with the National Institutes of Health guidelines for the care and use of experimental animals, and the study was approved by the Institutional Animal Care and Use Committee of the University of Virginia.

Genotyping was done using DNA isolated from tails, and sent to Transnetyx (Cordova, TN)

Blood collection:

After anaesthetizing, a 23-gauge needle was inserted into the cardiac stalk and used to draw blood which was collected into BD microtainer tubes containing EDTA (BD 365974-1). Blood was then centrifuged at 1000 g for 10 minutes to separate blood components.

Urine collection:

Mice were scruffed and gentle, manual pressure was applied to the transabdominal area to elicit urination. The urine was collected into 1 mL Eppendorf tubes and submitted to testing for sodium content.

Immunohistochemistry for renin:

Subsequent to treatment, kidneys were harvested and fixed in Bouin's solution and prepared for immunostaining, as previously described. 27 Kidney sections were deparaffinized, hydrated and blocked in 3% BSA and normal goat serum before
exposure to our antibody against renin [rabbit polyclonal 1:500 dilution]. 4 Visualization was performed using the appropriate Vectastain Elite ABC kit (Vector Laboratories), and sections were counterstained with haematoxylin. Kidney sections were mounted and examined under a microscope (Leica DFC 480) and imaged with a digital camera (Leica DFC310 FX).

**Periodic acid-Schiff's staining:**

Bouin's fixed kidney sections were deparaffinized and hydrated before immersion in 0.5% periodic acid solution (Sigma # P-7875) for 10 minutes. Sections were washed three times with ddH2O following oxidation and subsequently immersed in Schiff's reagent (Sigma # S-5133) for 20 minutes. Sections were then washed to remove excess Schiff's reagent and nuclei were counterstained with haematoxylin for 2 minutes followed by running tap water. Sections were then incubated in lithium carbonate to enhance staining contrast for 5 seconds followed by washing. Finally, sections were dehydrated and mounted before imaging.

**DNA synthesis studies:**

To detect actively dividing cells which were replicating their DNA, 5-Bromo-2-deoxyuridine (BrdU) was dissolved in PBS at 10 mg/mL and injected into mice intra-peritoneally. To detect both rapidly dividing cells and their slower counterparts, injections were performed twice; 2 hours prior to harvest for the former and 24 hours prior to harvest for the latter.

**Double Immunostaining for Renin and BrdU:**
Kidneys and small intestines (used as a positive control to confirm BrdU incorporation) were fixed in formalin, embedded and sectioned. Staining was performed using diaminobenzidine for BrdU and VIP-purple for renin as described previously in 27 and using the anti-renin [rabbit polyclonal 1:500 dilution] 4 and the rat anti-BrdU antibody (Abcam ab6326).

**Morphometric measurements:**

The juxtaglomerular apparatus (JGA) index was calculated as the number of renin-positive JGA/total number of glomeruli and expressed as a percentage. To determine the number of renin-expressing cells per section, we counted the number of renin-positive cells in each JGA plus the number of renin-positive cells along the arterioles with visible glomeruli attached to them. To determine the number of proliferating cells in angiotensinogen KO tissues, we stained samples from ages E18 (n = 2), P5 (n = 2), P21 (n = 3), P45 (n = 4) and P70 (n = 4) with the periodic acid-Schiff stain and counted proliferation events in afferent arterioles leading to glomeruli. We then divided this value by the total number of glomeruli and expressed the result as a percentage.

**Immunofluorescence for proliferation markers and Mef2c:**

To detect proliferating renin cells in formalin-fixed tissue sections, we used co-immunofluorescence with an anti-rat renin antibody 1:200 28 alongside proliferation marker antibodies against pH3 (Cell Signalling; #9701, 1:200), Ki67 (Novus NBP-54791, 1:200) or PCNA (Cell Signalling; 13110S, 1:1000). Sections were deparaffinized and rehydrated using xylene and a succession of alcohols. Antigen retrieval was conducted
by boiling for 10 minutes in 10 mmol/L sodium citrate and pH 6.0. Kidney sections were incubated overnight at 4°C with primary antibodies. Sections were then exposed to Alexa Fluor-488 donkey anti-rabbit and Alexa Fluor-568 donkey anti-goat (1:500 dilution) secondary antibodies and counterstained with Hoechst (Invitrogen). Kidney sections were mounted and examined under a microscope (Leica DFC 480) and imaged with a digital camera (Leica DFC360 FX). An average of two kidney sections separated by 70-100 µmol/L was counted for proliferative events within the entire section for each biological replicate. Additionally, the number of co-localizations of renin and proliferation markers was counted and used to compute the % of proliferating renin cells for each condition.

**Plasma renin measurements:**

Plasma renin concentration was determined using ELISA following the manufacturer's instructions (RayBiotech) as we previously described. 29

**Quantitative PCR**

To measure mRNA for renin and GAPDH in RNA from isolated kidney cortices, we conducted quantitative PCR (qPCR), as described previously (20), by reverse transcribing mRNA isolated from cells and amplified using the following primers: GAPDH [5– AAC TTT GGCATT GTG GAA GGG CTC-3=(forward) and 5– ACC AGT GGATGCG AGG GAT GAT GTT–3=(reverse)], and Renin [5– ACA GTA TCC CAA CAG ACA GAG AGA ACA AG –3=(forward) and 5– GCA CCC AGG ACC CAG ACA–3=(reverse)].

**Isolation of renin cells**
Ren1c-YFP mice at 2 months of age, alongside physiological untreated controls, were treated with a low sodium diet (0.1%, 7034, ENVIGO) plus captopril added to the drinking water (0.5 g/L) for 7 days. At the end of the treatment period, YFP + cells were isolated by FACS and cells were used to perform RNA-seq.

**RNA-seq**

For renin cells isolated from Ren1c-YFP mice, the Clontech SMARTer-Seq Lysis, RT and PCR solutions (Mountain View, CA; Catalog No. 634833) were used to generate bulk cDNA samples (~400 cells from the resuspended cells post-FACS). The cDNA was diluted with Fluidigm dilution buffer the following day and stored at −20°C. The samples were quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Samples underwent next-generation sequencing library preparation as described in the Fluidigm C1 manual using an Illumina Nextera XT library prep and index kits (Catalog Nos. FC-131-1096 and FC-131-1002). Briefly, cDNA samples were diluted, tagmented and indexed with unique barcodes for downstream analyses. After the addition of indices and amplification, samples were multiplexed and cleaned using AMPure beads. All samples were sequenced on a HiSeq2500/4000 platform.

We used FastQC to assess the quality of FASTQ file reads. Prior to alignment, we removed low quality reads and adapter sequences using Trimmomatic 0.36. We aligned FASTQ reads to the GRCm38/ENSEMBL mouse genome using Salmon 0.7.2 and transcript-level estimates of expression were scaled up to gene-level estimates using the Tximport 1.2.0 R package, with the ‘lengthScaledTPM’ argument for abundance estimation.
KEGG pathway analysis

The DeSeq2 package was used to identify differentially regulated genes between As4.1 cells and recruited renin cells. Those genes were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to identify biologically relevant pathways. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to integrate and annotate functional genomic information.

Statistical analysis

qPCR experiments were performed in triplicate, and qPCR measurements were conducted three times per replicate. All results are presented as means ± SD. Statistical significance between two groups was determined by unpaired Student's t-test. A P ≤ .05 was considered significant.
Appendix I: Factors to Consider when Using Transgenes

As mentioned in Chapters II and III, our lab has extensively used a transgenic fluorescent murine model \((Ren1c-YFP)\) to isolate and study renin expressing cells\(^8,21,29,33,38\). An interesting and cautionary note we made was when we attempted to perform ChIP-seq using cells isolated from mice bearing this transgene. During ChIP-seq, a small fraction of the biological sample being immunoprecipitated is set aside as an “input”. This “naked” DNA is not immunoprecipitated and serves as a negative control after sequencing to allow calling of enriched peaks in the immunoprecipitated sample. In an ideal case, the input should have uniform absence of signal across all genes as no regions have been enriched by immunoprecipitation. However, this is not typically the case as multiple factors may lead to unexpected signals in specific regions. For example, PCR amplification bias where certain regions in the genome are more amenable to being amplified during library generation, repetitive regions in the genome may be mapped to the same place due to their similarity etc\(^92,93\). In our next-generation sequencing experiments using cells isolated from mice bearing the \(Ren1c-YFP\) transgene, we observed a large amount of signal at the regulatory region of the renin gene in input samples which we attributed to the possibility that the transgene had inserted itself many times in the murine genome. This would lead to an abundance of renin regulatory regions which would be mapped to the native renin gene. Therefore, such signals were false positives and could cloud our interpretation and analysis of experiments such as ATAC and ChIP-seq. This represented a concern for us due to our interest in mapping the epigenetic landscape of the renin regulatory region and defining its state during basal and recruited states. Therefore, we sought to validate our previous findings\(^38\) by using a \(Ren1d-GFP\) knock-in at the renin gene\(^55\) and perform ATAC-seq to
compare with our datasets isolated using the *Ren1c-YFP* transgenic mice. As can be seen in panel b, the signals at the classical enhancer of the renin gene are practically identical in the YFP replicates when compared to the GFP sample which lacked the multiple copies present in the former. This verified our previous findings and confirmed that the classical enhancer region is indeed accessible and permissive for transcription factor binding which is a key aspect in the regulation of the renin gene. Additionally, while next-generation sequencing data can be probed at individual loci to ascertain their status, the true value of such data is the ability to view the genome as a whole. In this particular case, the *Ren1c-YFP* transgene has no effect on the majority of our ChIP and ATAC-seq analyses. Therefore, we stand by our previous results but offer these results as a cautionary tale when using transgenes to study gene regulation. If they must be used and a “true control” such as our *Ren1d-GFP* is not available, it is prudent to know the exact number of copies present as well as their insertion site and this information can be used to subtract this “noise” bioinformatically from the data to draw true conclusions. Suffice to say that one should be wary and cautious of how the tools used to study a phenomenon may change it or influence their observations and steps should be taken to minimize such artifacts to avoid downstream issues with data analysis and conclusions.
Appendix Figure I. Factors to Consider when Using Transgenes:

a) NGS plot of an input sample from cells isolated using the *Ren1c-YFP* transgenic mice. Note the excessive signal at the 5Kb upstream region which was cloned alongside the YFP gene in the generation of the mice. 

b) NGS plot depicting ATAC-seq signal from isolated renin cells from recruited *Ren1c-YFP* mice (top two tracks) and from recruited *Ren1d-GFP* mice which, crucially, **have no extra copies of the renin regulatory region**. As can be seen, the signals at the classical enhancer are virtually identical, thus validating our previous findings.
Appendix II: Optimizing ATAC-seq Protocols for Isolated Cells

Our initial experiments isolating vascular smooth muscle cells and performing ATAC-seq met with challenges in obtaining data with high signal-noise ratio (Fig 1A). The Transcriptional Start Site enrichment (a measure of how much signal was concentrated at the gene promoter which is a hallmark of successful ATAC-seq experiments) was quite low (≈2-3) and the number of regions that could be called as “peaks” was fewer than 10,000. These readings are suboptimal and not conducive to more in-depth studies such as differential region analysis and digital footprinting. To obtain higher quality ATAC-seq datasets, certain conditions should be optimized such as incubation time of the transposase with the nuclei, the quantity of transposase relative to nuclei etc. Whilst these parameters have been tested and optimized in Buenrostro et al (2013), it is possible that certain cell types (such as our vascular renin lineage cells) require different conditions and this is likely exacerbated by the fact that these are in-vivo, FACS sorted cells of which some may be damaged or lower in viability. Therefore, we sought to optimize our ATAC-seq procedure by testing various concentrations of transposase, varying incubation time, adding PBS to the transposition mixture (a change suggested in Corces et al in their Omni-ATAC-seq paper) to find suitable conditions which would allow generation of high-quality datasets (Fig 1B). Quality control was tested using qPCR for predicted open regions (GAPDH and β-actin) alongside closed regions (exon bodies of NeuN, a classical neuronal marker and Vmn2r19, a vomeronasal receptor not expressed in renin cells or the kidney). We found that reducing the concentration of the Tn5 transposase by half and adding PBS to the transposition mixture dramatically increases the enrichment of open regions as evidenced locally at the housekeeping genes and also globally after sequencing in the
TSS score (Fig 1C&D). Given the stress placed on the cells during FACS sorting and isolation (see Chapter VI: Methods for details on isolation procedure), it is possible that the viability of some of the cells is compromised and using a lower transposase enzyme prevents over-transposition of the nuclei which can lead to lower signal to noise ratio. Furthermore, PBS is a chaotropic buffer which can facilitate access of the transposase to the open regions between nucleosomes and increase the signal at the desired, accessible regions (personal correspondence with Dr. Ryan Corces). Overall, our optimization process was successful, and this work charts a path to optimizing ATAC-seq for “difficult” cells which can be used as framework for obtaining high quality datasets.
Appendix II Figures:

a) Initial TSS scores from ATAC-seq datasets before optimization were low indicating poor quality.

b) Conceptual figure demonstrating the process used to optimize the ATAC procedure to obtain high quality datasets. Various conditions were tested using sorted cells and quality was assessed by qPCR for open and closed regions before computing the delta Ct to infer the signal to noise ratio.

c) Bar graphs

d) TSS Score 6.9

Appendix Figure II: Optimizing ATAC-seq Procedure for In-vivo FACS sorted Cells: a) Initial TSS scores from ATAC-seq datasets before optimization were low indicating poor quality. b) Conceptual figure demonstrating the process used to optimize the ATAC procedure to obtain high quality datasets. Various conditions were tested using sorted cells and quality was assessed by qPCR for open and closed regions before computing the delta Ct to infer the signal to noise ratio. c) Bar graphs
demonstrating the increase in quality after applying PBS and reducing transposase quality. The first 4 samples were smooth muscle replicates after optimization compared to a positive control As4.1 cell line sample with high quality and the last 2 bars represented old samples before optimization with low quality. d) The optimized samples in panel c were subjected to Next-generation sequencing and, after alignment and processing, a TSS score was computed showing a greatly improved signal to noise ratio after applying the changes described.
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