Hypertension

ORIGINAL ARTICLE

Comparative Studies of Renin-Null Zebrafish and Mice Provide New Functional Insights

Scott Hoffmann,* Linda Mullins,* Sebastien Rider, Cara Brown, Charlotte B. Buckley, Adrienne Assmus, Ziwen Li, Mariana Sierra Beltran, Neil Henderson, Jorge del Pozo, Alexandre De Goes Martini, Maria Luisa S. Sequeira-Lopez, R. Ariel Gomez, John Mullins

BACKGROUND: The renin-angiotensin system is highly conserved across vertebrates, including zebrafish, which possess orthologous genes coding for renin-angiotensin system proteins, and specialized mural cells of the kidney arterioles, capable of synthesising and secreting renin.

METHODS: We generated zebrafish with CRISPR-Cas9-targeted knockout of renin (*ren*$^{-/-}$) to investigate renin function in a low blood pressure environment. We used single-cell (10×) RNA sequencing analysis to compare the transcriptome profiles of renin lineage cells from mesonephric kidneys of *ren*$^{-/-}$ with *ren*$^{+/+}$ zebrafish and with the metanephric kidneys of *Ren1*$^{c-/-}$ and *Ren1*$^{c+/+}$ mice.

RESULTS: The *ren*$^{-/-}$ larvae exhibited delays in larval growth, glomerular fusion and appearance of a swim bladder, but were viable and withstood low salinity during early larval stages. Optogenetic ablation of renin-expressing cells, located at the anterior mesenteric artery of 3-day-old larvae, caused a loss of tone, due to diminished contractility. The *ren*$^{-/-}$ mesonephric kidney exhibited vacuolated cells in the proximal tubule, which were also observed in *Ren1*$^{c-/-}$ mouse kidney. Fluorescent reporters for renin and smooth muscle actin (*Tg(ren:LifeAct-RFP; acta2:EGFP)*), revealed a dramatic recruitment of renin lineage cells along the renal vasculature of adult *ren*$^{-/-}$ fish, suggesting a continued requirement for renin, in the absence of detectable angiotensin metabolites, as seen in the *Ren1*YFP *Ren1*$^{c-/-}$ mouse. Both phenotypes were rescued by alleles lacking the potential for glycosylation at exon 2, suggesting that glycosylation is not essential for normal physiological function.

CONCLUSIONS: Phenotypic similarities and transcriptional variations between mouse and zebrafish renin knockouts suggests evolution of renin cell function with terrestrial survival. **(*Hypertension*. 2022;79:e56–e66. DOI: 10.1161/HYPERTENSIONAHA.121.18600.)** • **Supplemental Material**

Key Words: actins ■ glycosylation ■ pericytes ■ renin ■ zebrafish

The renin-angiotensin system (RAS) is responsible for blood pressure, sodium homeostasis, and water regulation. The rate limiting enzyme renin is predominantly expressed and synthesized in specialized renal, perivascular, mural cells,[1–3] or renal lineage cells (RLCs), which are involved in renal development[4] and have been implicated in angiogenesis of the renal vasculature.[5–7] Mice lacking renin require neonatal subcutaneous saline injections to survive, show recruitment of RLCs, have no detectable angiotensinogen metabolites (Ang I or Ang II), and show severe defects in renal development.[8–10]

Studies on the RAS have predominantly focused on mammalian systems. However, the optically clear zebrafish larva contains multiple genes orthologous to the human RAS genes—angiotensin-converting enzyme (*ace*, *ace2*), angiotensinogen (*agt*), angiotensin receptors (*agtr1a,*

Correspondence to: Linda Mullins, Cardiovascular Science Centre, Queen's Medical Research Institute, University of Edinburgh, Email linda.mullins@ed.ac.uk
*S. Hoffmann and L. Mullins are joint first authors.
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NOVELTY AND RELEVANCE

What Is New?

Renin knockout delays development of the swim bladder in zebrafish larvae.
Mice and zebrafish show vacuolation of the proximal tubule in the absence of functional renin.
Both mouse metanephric and zebrafish mesonephric kidneys recruit renin lineage cells in the absence of functional renin.

What Is Relevant?

Zebrafish renin lineage cells share multiple functions and comparison reveals novel mechanistic insights relating to variation in homeostatic challenges.

Clinical/Pathophysiological Implications?

The zebrafish provides important information about the evolution of renin lineage cell function. Our results suggest a continued, but noncritical requirement for renin in adult mouse and zebrafish

Nonstandard Abbreviations and Acronyms

AMA	anterior mesenteric artery
CW	conditioned water
EGFP	enhanced green fluorescent protein
JG	juxtaglomerular
RAS	renin-angiotensin system
RFP	red fluorescent protein
RLC	renal lineage cell

agtr1b, agtr2), renin receptor (*atp6ap2*), mineralocorticoid receptor (*nr3c2*), and renin (*ren*)—lacking only an orthologue to the Mas receptor.[11] Renin has been linked to teleost survival in water of fluctuating osmolarity.[12] Placing zebrafish larvae in dilute (1/20) conditioned water (CW; 60 mg/L sea salts), increases renin expression and circulating Ang II levels,[13–15] analogous to low-salt diet administration in mammals. Despite a functional RAS, zebrafish larvae are unable to survive reduced salinity in the presence of the Ace inhibitor, Captopril.[16] As in mammals, RLCs in the adult zebrafish mesonephros are present along afferent arterioles in a preglomerular position, express smooth muscle and pericyte markers, *acta2* and *pdgfrb*, respectively and are recruited under low salinity or Captopril challenge.[17–19]

Electron microscopy of perivascular RLCs or labeling of acidic organelles with fluorescent dyes have revealed that the zebrafish RLCs contain dense core, acidic granules, suggestive of renin synthesis, storage, and processing.[17] In mammals, it is thought that glycosylation sites are involved in the processing of renin and sequences encoding these highly conserved glycosylation sites are found in exons 2 and 4 of the zebrafish renin gene. However, the functional role of glycosylation in zebrafish renin remains to be elucidated.

The zebrafish *ren* transcript is first expressed in larvae at 24 hours post fertilization and has been implicated in the development of the pronephros, which is an active filtration organ from 3 dpf (days post fertilization).[16,20] At the larval stage, renin expression is largely limited to the anterior mesenteric artery (AMA), which supplies the swim bladder. This buoyancy aid typically becomes visible from 4 to 5 dpf, when the zebrafish larvae reach 3.4 to 3.7 mm in length,[21] unless they are exposed to low salinity.[13]

Zebrafish kidney development terminates with the mesonephros and despite lacking the structural complexity of a metanephric kidney, nephron structure, and tubular segmentation is highly conserved and easily accessible.[22] Furthermore, the zebrafish mesonephric kidney retains the ability to regenerate and restore damaged nephrons, making it an ideal model for studying renal injury and repair.[23,24]

In this study, we generated an allelic series of *ren* mutations using CRISPR/Cas9-targeting in zebrafish. Crossing of the *ren*$^{-/-}$ zebrafish to a double fluorescent reporter strain (*Tg(ren:LifeAct-RFP; acta2:EGFP)*), enabled high resolution imaging of the mesonephric renal vasculature, and the generation of informative scRNAseq 10× data sets. The transcriptional profiles of RLCs from *ren*$^{-/-}$ and *ren*$^{+/+}$ mesonephros were compared with those of equivalent mouse metanephric RLCs.

METHODS

The authors declare that all supporting data are available within the article (and its Supplemental Material). Zebrafish scRNAseq libraries have been submitted to ArrayExpress (E-MTAB-11079). Mouse scRNAseq data have been submitted to GEO public repository (GSE180873); (NCBI tracking system No. 22225156).

Detailed methods are available in the Supplemental Material. All zebrafish (*Danio rerio*)[25] experiments were approved by the local ethics committee and conducted in accordance with the Animals (Scientific Procedures) Act 1986 in a United Kingdom Home Office approved establishment.

Statistical Analysis

Statistical analyses were performed (GraphPad Prism version 8.4.3 for Mac; GraphPad Software, San Diego, CA), by ANOVA and post hoc Sidak multiple comparisons test. Values are

reported as means±SD, and $P<0.05$ was considered statistically significant.

RESULTS

Viability of Ren$^{-/-}$ Larvae and the Pronephric Kidney

CRISPR-Cas9 targeting of G0 embryos produced chimeric founders, with a range of indels at the target site. These were resolved by backcrossing to the wild-type strain, WIK, and subsequent genotyping of F1 fish. Indels were verified by sequencing of polymerase chain reaction products[26] spanning the target site.

In one fish (*ren*$^{-/-}$), removal of 8 bp near the glycosylation site in exon 2 was predicted to cause a nonsense mutation, bringing a stop codon into frame in the third exon, and thus truncating the protein product (Figure S1A). F2 fish showed a Mendelian ratio of 20:38:15 (wild type: heterozygous: homozygous) indicating that homozygous knockout fish were viable in water of normal salinity (CW) and suggesting that an active RAS is not vital during early stages of development (Figure S2F).

A second knockout line, with a 9 bp deletion spanning the glycosylation site was also generated (*ren*$^{\Delta 9/\Delta 9}$), together with a knock-in line (*ren*$^{KI/KI}$), generated using an antisense ss-oligo (Figure S1B and S1C). One out of 40 fish screened carried the knock-in allele, which replaced amino acids encoding the glycosylation site, rendering it disabled. Fish homozygous for both the 9 bp deletion (*ren*$^{\Delta 9/\Delta 9}$) and the 9 bp substitution (*ren*$^{KI/KI}$) were viable.

Though viability was unaffected by loss of renin expression, a significant delay in somatic growth (length) and appearance of the swim bladder in *ren*$^{-/-}$ zebrafish, was observed using the Vertebrate Automated Screening Technology system (Figure S2A). Zebrafish length was significantly reduced at 4 dpf (*ren*$^{-/-}$, 3.58±0.03 mm [n=45]; *ren*$^{+/+}$, 3.79±0.02 mm [n=29]; $P<0.0001$), and 5dpf (*ren*$^{-/-}$, 3.76±0.03 mm [n=37]; *ren*$^{+/+}$, 3.89±0.03 mm [n=29]; $P=0.004$; Figure S2B). At 5 dpf, *ren*$^{-/-}$ zebrafish had a dramatically reduced swim bladder size (0.055±0.006 mm^2, n=80) and in 42.5% cases lacked a swim bladder completely, compared with *ren*$^{+/+}$ zebrafish, (0.109±0.002 mm^2; n=61) which all had a swim bladder. By 8 dpf, there was still a significant difference in swim bladder area but not length of *ren*$^{-/-}$ larvae compared with controls. Despite this, no obvious behavioural difference was noted.

We investigated pronephric development in *ren*$^{+/+}$ and *ren*$^{-/-}$ zebrafish crossed to the transgenic reporter line *tg(wt1b:EGFP)*, where Wt1b is localized to the proximal tubule and the glomerulus.[27] The pronephros of *ren*$^{+/+}$ and *ren*$^{-/-}$ *Tg(wt1b:EGFP)* zebrafish were imaged at 3-,4-, and 5 dpf, and the anterior glomerular distance and posterior glomerular distance were measured (Figure S2C). Anterior glomerular distance and posterior glomerular distance were significantly increased in *ren*$^{-/-}$ *Tg(wt1b:EGFP)* at all 3 stages of development suggesting a delay in glomerular fusion in *ren*$^{-/-}$ larvae (Figure S2D and S2E). No significant difference in viability was observed when exposing *ren*$^{-/-}$ zebrafish to either 1/20 CW (3 mg/L sea salt), or Captopril in CW compared with *ren*$^{+/+}$ treated zebrafish. However, no *ren*$^{-/-}$ zebrafish survived in 1/20 CW combined with Captopril, compared with 12.5% survival of *ren*$^{+/+}$ zebrafish (Figure S2F).

Larval RLC Ablation

In larvae, renin expression is limited to the AMA, which supplies the swim bladder.[16] Despite the delay in development, no significant difference was observed in the area of AMA fluorescence in the *ren*$^{-/-}$; *Tg*(*ren:LifeAct-RFP;acta2:EGFP*) line compared with *ren*$^{+/+}$;*Tg*(*ren:LifeAct-RFP;acta2:EGFP*)[16,28] at 5 dpf, (Figure 1A through 1C; n=5 per group), suggesting that renin expression is not essential in CW.

To determine whether or not RLCs in the AMA exhibit contractile pericyte functions, we specifically ablated the RLCs in 3 dpf *Tg*(*ren:KillerRed*) larvae, using a Bessel beam on the SPIM microscope as previously described.[29] We recorded time-lapse images and measured the maximum luminal width of the AMA blood vessel, accounting for its pulsatility with each heartbeat. We demonstrated that the width significantly increased (from 19.208±1.307 µm to 21.678±0.615 µm; n=5; $P=0.01$) following ablation of the RLCs (Figure 1D and 1F and Video S1[30]).

The Ren$^{-/-}$ Mesonephric Kidney: Renin Assay

Ang I and Ang II metabolites were predicted by comparison of zebrafish angiotensinogen protein sequence with that of higher mammals (Figure S1E), synthesized and used as standards for the indirect kidney renin assay. Ang I and Ang II were measured in pooled mesonephric kidney samples isolated from *ren*$^{+/+}$, *ren*$^{-/-}$, *ren*$^{\Delta 9/\Delta 9}$ and *ren*$^{KI/KI}$ fish. Results are given in Table 1. Almost no angiotensin metabolites were detected in the *ren*$^{-/-}$ kidneys, confirming that renin activity is absent in these fish. Neither disabling (*ren*$^{KI/KI}$) nor removal (*ren*$^{\Delta 9/\Delta 9}$) of the glycosylation site in exon 2 affected the levels of Ang I and Ang II in their respective mesonephric kidneys.

Histology

Periodic Acid Schiff stain[31] was used to visualize renal structures in adult zebrafish mesonephros (Figure S3A and S3B). Kidneys of *ren*$^{-/-}$ zebrafish exhibited widespread, severe vacuolation of the proximal tubular epithelial cells compared with very rare, mild vacuolation in *ren*$^{+/+}$ controls (n=3 per group). Absence of Periodic Acid Schiff staining suggests that the vacuoles do not contain polysaccharides or glycoproteins. No

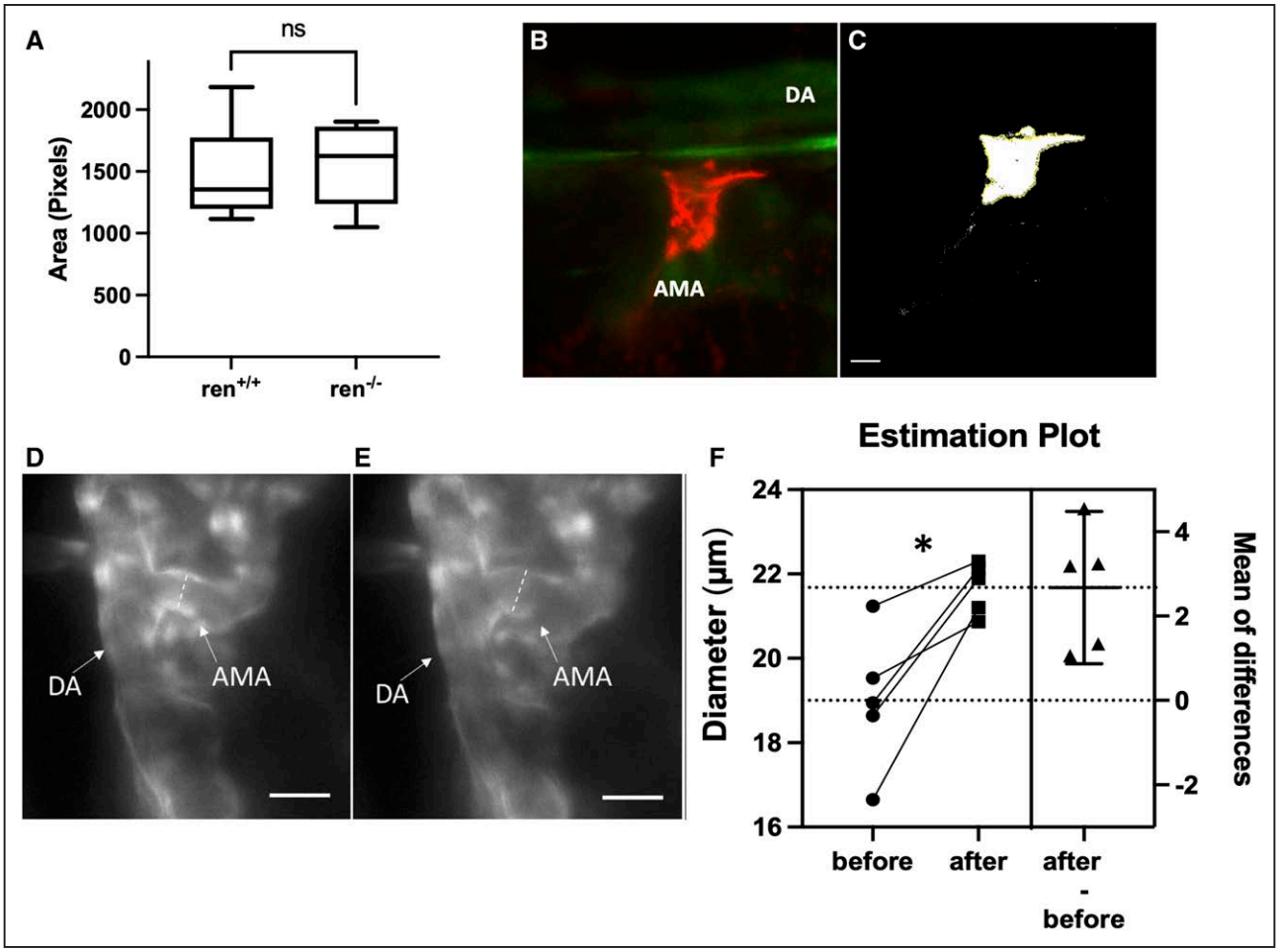


Figure 1. Anterior mesenteric artery (AMA) analysis.
A, Quantification of the LifeActRFP fluorescent signal at the AMA in *Tg*(*ren:LifeAct-RFP;acta2:EGFP*) 5 dpf larvae on a $ren^{+/+}$ or $ren^{-/-}$ background (n=5 per group); (**B**) SPIM microscopy of pronephric AMA showing LifeActRFP (Ren-expressing cells) and EGFP (Acta2-expressing cells) fluorescence and (**C**) representative gray scale image for mean area analysis. Laser ablation of renin-expressing cells in the AMA of Tg(*ren*:mem-KillerRed) 3 dpf larvae showing *kdrl*:*GFP* signals at (**D**) t=0 min and (**E**) t=60 min of the ablation protocol. The dorsal aorta (DA) can be seen with the AMA budding off it. Images represent single axial planes. Scale bars represent 30 µm; AMA diameter was measured at the same two locations for each fish (dotted lines); (**F**) an estimation plot and mean of differences are shown; (paired *t* test*: *P*=0.0147).

differences were observed in the distal tubule, which lacks a brush border. Vacuolation of proximal tubular epithelial cells was also observed in the $Ren1^{cYFP}$ $Ren1^{c-/-}$ mouse kidney, compared with the $Ren1^{c+/+}$ control (Figure S3C and S3D).

Spinning disc confocal microscopy[32] of excised mesonephric kidneys isolated from $ren^{+/+}$ and $ren^{-/-}$ *Tg(ren:LifeAct-RFP, acta2:EGFP)* fish revealed that RLCs are located intermittently along the afferent arterioles in $ren^{+/+}$ fish giving a banded or striped pattern (Figure 2A). However, RFP (red fluorescent protein) labeling in $ren^{-/-}$ mesonephric kidneys showed extensive continuous expression and the virtual absence of striped/banded patterning, whilst EGFP (enhanced green fluorescent protein) fluorescence, which marked smooth muscle cells, was significantly reduced (Figure 2B). This mirrors the RLC recruitment seen in mice.[33]

Table 1. Angiotensin Metabolites in Mesonephric Kidneys Isolated From Zebrafish Homozygous for Mutated Renin Alleles

Genotype	Ang 1–8, pg/g	Ang 1–10, pg/g
$ren^{+/+}$	1159.0	6790.1
$ren^{-/-}$	<5	<5
$ren^{\Delta 9/\Delta 9}$	966.1	5496.0
$ren^{KI/KI}$	1626.9	8844.9

Renin Complementation

To determine whether the $ren^{-/-}$ knockout phenotype could be rescued, either by the deletion mutant $ren^{\Delta 9/\Delta 9}$ or the knock-in mutant, $ren^{KI/KI}$, each was crossed with $ren^{-/-}$*Tg*(*ren:LifeAct-RFP*; *acta2:EGFP*) to generate obligate heterozygotes: $ren^{-/\Delta 9}$; *Tg*(*ren:LifeAct-RFP*$^{+/-}$; *acta2:EGFP*$^{+/-}$) and $ren^{-/KI}$;*Tg*(*ren:LifeAct-RFP*$^{+/-}$; *acta2:EGFP*$^{+/-}$), respectively. Kidney squashes from these were assessed for complementation of the

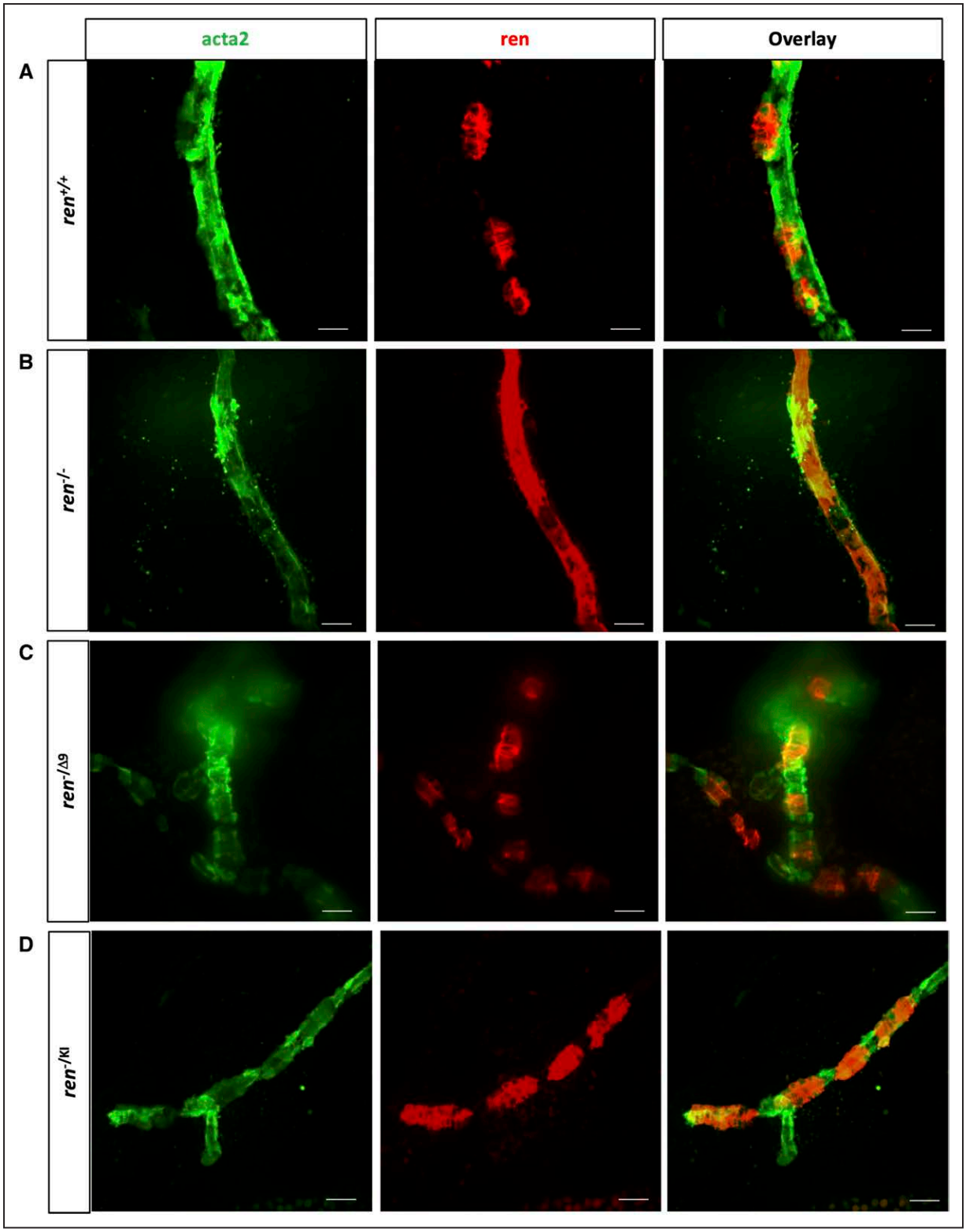


Figure 2. Spinning disc microscopy showing the expression of fluorescent reporters *ren:LifeAct-RFP* and *acta2:EGFP*. Mesonephric kidney squashes from (**A**) *ren*$^{+/+}$, (**B**) *ren*$^{-/-}$ or the obligate heterozygous fish (**C**) *ren*$^{-/\Delta 9}$, and (**D**) *ren*$^{-/KI}$. Parts show separate red, green and merged channels. Scale bar = 50µm.

banding pattern in afferent arterioles (Figure 2C and 2D). Both substitution and removal of the glycosylation site rescued the knockout phenotype.

scRNAseq Analysis

ScRNAseq 10× libraries were made from. *ren*:LifeAct-RFP^{+}- and *acta2*:EGFP^{+}-expressing cells,

fluorescence-activated cell sorted from *ren*$^{+/+}$ and *ren*$^{-/-}$ double-transgenic fish. Dimensionality reduction (t-distributed Stochastic Neighbour Embedding [tSNE])[34] was performed (using resolution 0.1 to set the granularity of the clustering) on the merged libraries following principal component analysis (Figure S4), and similar cells were grouped into 5 clusters (Figure S4A). Violin plots and feature plots were used to explore the transcription of key genes of interest (Figure S4B and S4C). Cluster 2 contained the majority of renin- and LifeActRFP- expressing cells while clusters 0, 1, and 2 contained acta2- and EGFP-expressing cells. RLCs from the control library (ZF1) represented 11.9% of the fluorescent-activated cell sorted cells, while 36.7% of cells from the RenKO library (ZF2) resided in this cluster. This probably reflects the proportion of juxtaglomerular cells from ZF1 versus the recruited RLCs from ZF2. Lists of genes differentially expressed in each cluster were generated, and several cluster 2-specific transcription factors were identified (Figure S4C) including *cnot4b*, *twist1a*, *nr2f5*, *nkx3.2*, *cited4a,* and *sox6*. Other transcripts enriched in RLCs included signaling proteins such as *angptl3* and *rgs5b*, and also *hmox1a* and *tfpia*. Pseudotime analysis[35] was projected onto the seurat-generated clusters and confirmed the relationship between smooth muscle cells and RLCs (Figure S4D).

Subsequently, cluster 2 was separated into subclusters, to identify genes that distinguish juxtaglomerular (JG) cells from recruited cells (Figure S5). Using a resolution of 0.5, 3 clusters were resolved (Figure S5A). The distribution of cells from the 2 libraries indicated that cluster C2 contains cells from the control library (ZF1) and, therefore, represents JG cells, while cells from the *ren*$^{-/-}$ library (ZF2), representing recruited RLCs, could be divided into clusters C0 and C1. Genes differentiating the sub-clusters were interrogated further (Table 2). Transcription of *ren* was 4-fold higher in JG cells (C2) than RLCs, while transcription factors *cnot4b* and *twist1a* were upregulated in cluster 1 and *rbp4* was more highly expressed in C0 of the recruited cells (Figure S5B and S5C). The top genes listed for each cluster were assessed by gene ontology analysis (http://geneontology.org). C0 showed a 16-fold enrichment for transcripts related to actomyosin structural organization (including *tagln, acta2, lmod1b, csrp1a*), C1 showed enrichment for the vegf pathway (*vegfaa, pgfb,* and *gng2*) and lysosomal and lytic vacuoles, but no significant enrichment was seen in C2 (data not shown). Pseudotime mapping suggested that cluster 0 may represent a transition stage between JG cells and RLCs.

The control and knockout libraries were further analyzed using canonical correlation analysis (Figure S6; resolution 0.2 gave 8 clusters, cluster 1 containing most RLCs). This confirmed multiple genes with altered transcription in response to renin knockout, including transcription factors *cnot4b*, and *nr2f5*, and signaling factors such as *angptl3, rgl1*, *nedd9*, and *npr1b.* Gene ontology analysis suggested an 8.6-fold enrichment in markers of lysosomes or lytic vacuoles in response to renin knockout.

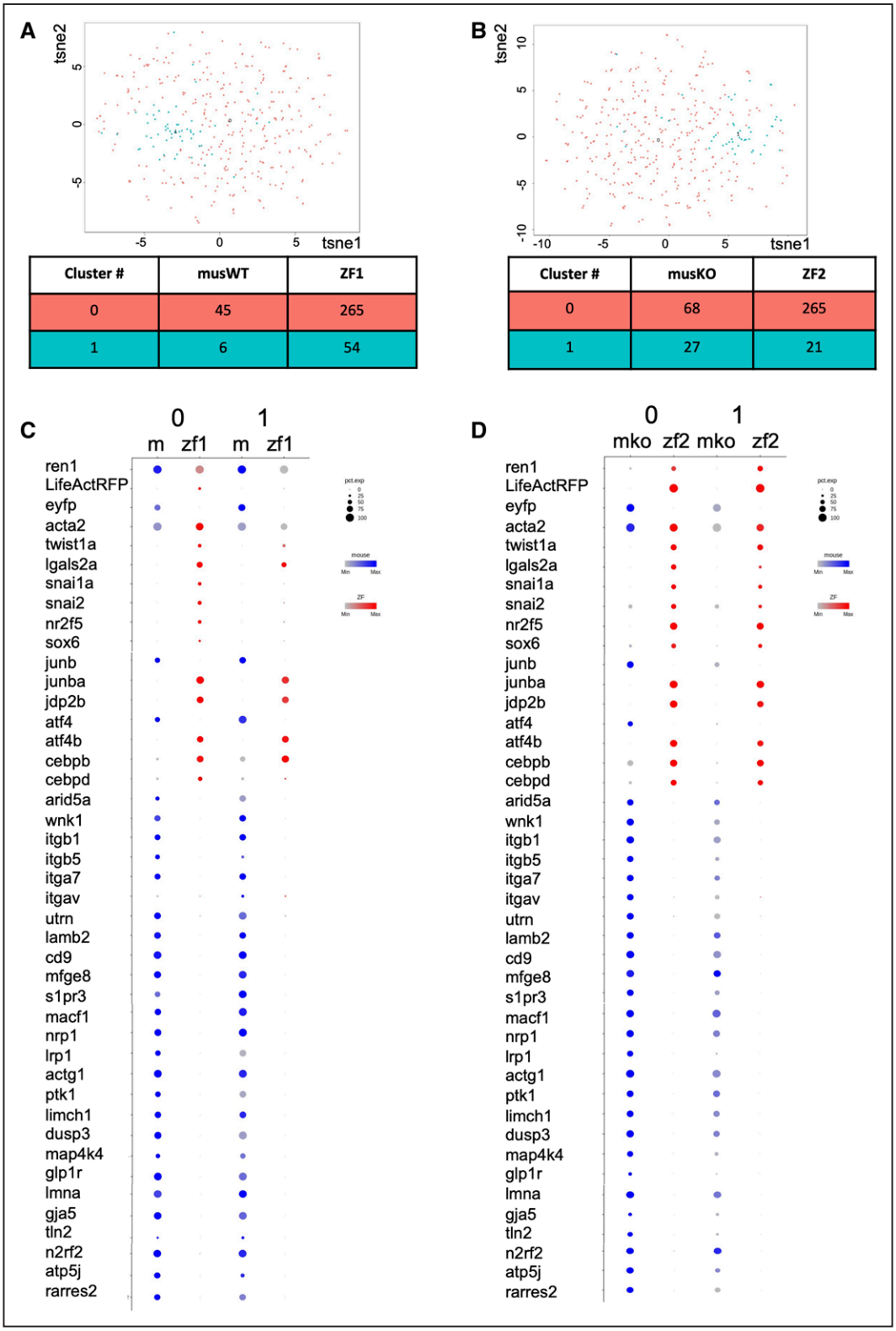
Table 2. Factors Involved in Transcription Control, Signaling Pathways, Actin Filament Organization and Ion Transport, Which Are Differentially Expressed Between JG cells and RLCs (Separated by Subcluster Analysis)

Cluster 0 (RLC)	Cluster 1 (RLC)	Cluster 2 (JG cell)
rbp4	*twist1a*	*cited4a*
hmgxb4a	*cnot4b*	*nkx3.2*
	nr2f5	*id1*
acta2	*cremb*	
EGFP	*fosl2*	*ren*
myl9a	*vegfaa*	*dio1*
tagln	*angptl3*	*tnmd*
tpm1	*rgs5b*	*tnfaip8l3*
pfn2	*rgl1*	*tnfsf12*
tfpia	*pgfb*	*rergla*
mmp2	*gng2*	*rasl12*
lmod1b	*npr1b*	*nrarpa*
csrp1a	*gpr137ba*	*kctd12.2*
tpm4b	*alkal1*	
	sgk1	
	igfbp5b	
	cd164	
	socs3b	
	ackr4b	
	adora2aa	
	cnpy1	
	psap	
	ptn	
	nocta	
	agtrap	
	kcne4	
	clcn5b	
	ednraa	

JG indicates juxtaglomerular cells; and RLC, renal lineage cell.

Comparison of RLCs From Zebrafish and Mouse

ScRNAseq expression matrices from RLCs of the *ren*$^{+/+}$ and *ren*$^{-/-}$ zebrafish libraries were compared with expression matrices generated using C1 Fluidigm methodology[36–39] on Ren1^{c}YFP fluorescence-activated cell sorted cells isolated from *Ren1*$^{c+/+}$ and *Ren1*$^{c-/-}$ mice respectively,[40,41] using canonical correlation analyses.[34] Cluster analysis of the 2 wild-type libraries gave 2 clusters at a resolution of 0.5 (Figure 3A). Since cluster 0 included the majority of mouse and zebrafish cells, these were designated as JG like. By analogy, at a resolution of 0.6 following canonical correlation analysis of the mouse and zebrafish renin knockout libraries, cluster 0 was designated as



Cluster #	musWT	ZF1
0	45	265
1	6	54

Cluster #	musKO	ZF2
0	68	265
1	27	21

Figure 3. Merged expression matrices following canonical correlation analysis.
(**A**) Wild-type mouse and zebrafish juxtaglomerular cells (resolution 0.5) and (**B**) renin knockout mouse and zebrafish renin lineage cells (resolution 0.6). Associated tables give contributing cell numbers from respective matrices; (**C** and **D**) genes differentially expressed between respective mouse and zebrafish libraries, irrespective of genotype, are shown in dot plots.

recruited RLCs (Figure 3B). Dot plots show a number of transcripts, irrespective of genotype, which were mouse RLC- specific, including a number of integrins. The mouse *Ren1*$^{c+/+}$ and *Ren1*$^{c-/-}$ libraries both showed significant enrichment of gene transcripts related to thyroid receptor binding (5.7-fold), regulation of focal adhesion assembly

(5.7-fold), and integrin binding (4.0-fold), as assessed by gene ontology analysis, compared with the respective zebrafish libraries. There was a 4.3-fold enrichment of gene transcripts associated with positive regulation of blood pressure in the mouse libraries, while both zebrafish libraries showed a 4-fold enrichment of gene transcripts associated with sprouting angiogenesis (Table 3).

DISCUSSION

Using the CRISPR/Cas9 system we targeted zebrafish *ren* exon 2 and generated multiple allelic variants. The survival of *ren*$^{-/-}$ fish might reflect their aquatic environment, since homozygous *Ren1*$^{c-/-}$ mice require daily administration of saline solution to prevent neonatal death.[9] This suggests that transition to land created additional homeostatic challenges, possibly leading to the development of the macula densa in the metanephric kidney and baroreceptors.

Although ren$^{-/-}$ larvae were completely viable, the delay in growth, late appearance of the swim bladder, and delayed glomerular fusion most likely resulted from an adverse effect on salt handling. Low salt in combination with Captopril resulted in complete loss of viability of *ren*$^{-/-}$ larvae (compared with reduced survival of wild-type larvae in low salt and 0.1 mmol/L Captopril[16]). Renin is increased in ion-poor fresh water,[13] implicating renin functionality in salt absorption, but zebrafish larvae rely predominantly on the 5 types of ionocytes in their gills and skin, which provide alternative routes for establishing salt homeostasis, until the kidneys are fully developed.[42] The Na^+Cl^- co-transporter-rich NCC ionocytes are thought to be responsive to Ang II,[43] though this does not explain the loss of viability of *ren*$^{-/-}$ larvae (which presumably lack Ang II), in low salinity plus Captopril.

In larvae, renin expression is limited to the AMA region, which supplies the swim bladder. We saw no increase in renin reporter expression in the AMA of *ren*$^{-/-}$ larvae grown in CW. However, optogenetic ablation of the RLCs, caused an increase in arterial diameter, suggesting loss of tone. The observed increase in lumen diameter would correspond to ≈22% increase in lumen cross-sectional area. To our knowledge, this is the first direct demonstration of contractile functionality in RLCs, in vivo, though it does not suggest that renin or Ang II are involved per se, rather that the cells have dual functionality. This should be explored in the mesonephric and metanephric kidney.

To interrogate renin functionality in our adult mutants, our collaborators (Attoquant Diagnostic GmbH, Vienna, Austria) developed an indirect renin assay and demonstrated a complete absence of Ang I and Ang II in the mesonephric kidneys of adult *ren*$^{-/-}$ zebrafish. Normal levels of the metabolites were found in mesonephric kidneys from *ren*$^{\Delta 9/\Delta 9}$ and *ren*$^{KI/KI}$ confirming that neither removal nor alteration of this glycosylation site dramatically affects protein folding or activity of renin. The glycosylation sites are located towards the surface of the renin protein, which may explain the apparent flexibility in protein conformation.

Loss of a functional *ren* gene resulted in alterations to mesonephric kidney morphology—specifically cytoplasmic vacuolation of proximal tubular epithelium, but not renal degeneration.[44] Vacuolation of proximal tubule cells often indicates an osmotic imbalance due to increased solute transport across the cells.[45] However, an almost complete absence of detectable Ang II may have adverse effects on the proximal tubule where it normally stimulates sodium and water reabsorption.[46] Since vacuolation of proximal cells was also observed in the *Ren1*$^{c-/-}$ mouse, this suggests a conserved response to the lack of renin, reflecting a common action of Ang II on proximal tubule ion/water transport across species and deserving further investigation. This contrasts with the absence of such a mechanism in the aglomerular teleost.[47]

Table 3. Gene Ontology Analyses Giving Fold Enrichment of Differentially Expressed Gene Transcripts Between Wild-Type or Renin Knockout Mouse and Zebrafish Libraries

GO term	Mus *Ren1*$^{+/+}$	ZF *ren*$^{+/+}$	Mus *Ren1*$^{-/-}$	ZF *ren* $^{-/-}$	Associated genes
Thyroid receptor binding	6.38	…	5.07	…	*Trip12, Arid5a,* Thrap3, Tacc1,* Brd8*
Integrin binding	3.78	…	4.29	…	*Utrn,* Cd81, Cd9,* Emp2, Dmd, Lamb2,* Mfge8,* Nisch,* Gsk3b, S1pr3**
Integrin complex	3.74	…	4.6	…	*Itgb1,* Itgb5,* Itgav,* 1tga7**
Regulation of focal adhesion assembly	5.59	…	5.8	…	*Rac1, Nrp1,* Vegfa, Iqgap1, Limch1,* Clasp2, Pten, Lrp1,* Actg1,* Ptk2,* Dusp3,* Macf1,* S100a10, Map4k4**
Positive regulation of blood pressure	4.3	…	4.33	…	*Glp1r,* Wnk1,* Agtr1a, id2, Nr2f2,* Atp5j,* Rarres2**
Actin cap	22.3	…	15.23	…	*Cald1,* Gsn, Actr2**
Actin filament depolymerization	9.93	…	8.45	…	*Wdr1,* Gsn, Mical3,* Dstn**
Actin filament polymerization	…	5.5	…	5.8	*capza1b, pfn2, pfn1, pfn2l,* tmsb4x,* lmod1b*
relaxation of smooth muscle	16.55	…	9.02	…	*Rgs2, Prkg1,* Slc8a1,* Gucy1a1, Mrvi1*
Sprouting angiogenesis	…	4.16	…	4.82	*pgfb, pkma, cxcl12b, vegfaa, rtn4a, atf4b, crema,b, lgals2a,* twist1a**

GO indicates gene ontology; and ZF, zebrahfish.
*Only transcribed in mouse or zebrafish, respectively.

During early mammalian kidney development, renin cells are expressed throughout the renal vasculature—only after birth are renin-expressing cells spatially restricted to the juxtaglomerular apparatus.[48] However, RLCs retain the ability to switch to an endocrine renin phenotype in response to physiological challenge.[18,33,49,50] We questioned whether the lack of functional renin might simulate such a challenge and crossed the double-transgenic reporter line *Tg(ren:LifeAct-RFP, acta2:EGFP)* with *ren*$^{+/+}$ and *ren*$^{-/-}$ fish to assess the extent of RLCs in the mesonephric kidney. There was a dramatic recruitment of RLCs along the renal vasculature with concomitant decrease of EGFP expression in the mesonephric kidney of the *ren*$^{-/-}$ zebrafish, as seen in the mouse.[48] RLCs may be recruited along the arterioles in an attempt to control ion concentration and/or blood volume. Obligate heterozygous fish carrying deletion or substitution of the exon 2 glycosylation site, in combination with the knockout allele complemented for loss of renin, as shown by the restoration of the banding pattern of afferent arterioles. This, together with the biochemical data, confirms that renin glycosylation in exon 2, though highly conserved, is not essential for renin activity.

Fluorescence-activated cell sorting of vascular and renin fluorescent reporter-expressing cells on *ren*$^{+/+}$ or *ren*$^{-/-}$ backgrounds proved to be a very effective method for isolating JG cells from the former and RLCs from the latter, where over 3-fold more cells expressed the RFP reporter. Cluster analysis of the merged zebrafish libraries clearly distinguished RLCs from smooth muscle cells, identifying numerous upregulated renin cell-specific transcription factors, including *cnot4b*, *twist1a*, *nr2f5*, *nkx3.2*, and *sox6*, or downregulated transcription factors (*cited4a*), regulators of signaling pathways including *angptl3* and *rgs5b*, and also *hmox1a*, *tnmd*, and *tfpia*. Several of these have been recognized previously as RLC-specific in the mouse.[18]

Sub-cluster analysis allowed us to identify differentially expressed genes distinguishing JG cells from RLCs. Increased expression of *cited4a, id1, dio1, nrarpa nkx3.2*, and *tnfaip8l3* was seen in JG cells relative to RLCs, while RLCs exhibited increased *twist1a, cnot4b, cremb* and *fosl12* transcription-related factors and a large group of signaling factors including *igfbp5b, cnpy1, alkal1, rgl1, angptl3, gpr137ba, vegfaa, pgfb,* and *gng2.* Pseudotime mapping indicated that cluster 0 may represent a transition stage between JG cells and RLCs. This suggests a possible de-differentiation and re-differentiation of RLC-derived smooth muscle cells as they become recruited. The overall response to renin knockout was confirmed by canonical correlation analysis of the merged libraries.

Comparison with mouse expression matrices allowed us to look for conserved and divergent expression profiles from respective *Ren*$^{+/+}$ and *Ren*$^{-/-}$ cells of the zebrafish and mouse. Irrespective of genotype, JG cells and RLCs from the zebrafish mesonephric kidney libraries expressed *snai1a*, *snai2*, *twist1a,* and *sox6*, all of which enable DNA-binding of transcription factors, but none of these were transcribed to a significant extent in the mouse libraries. Equally irrespective of genotype, JG cells and RLCs from the mouse metanephric kidney libraries expressed transcripts associated with integrins and regulation of focal adhesion. This is highly suggestive that such functions have evolved in RLCs of higher mammals, along with their exposure to increasing blood pressure.[51] Transcripts from the recently identified nuclear mechano-transducer, lamin A (*Lmna*), associated with the mouse JG cell baroreceptor,[52] were also noticeably absent from zebrafish-derived RLCs, suggesting an additional control in land-based mammals.

In conclusion, despite the control of ion balance afforded to the zebrafish through ionocytes of the gills and skin, these studies reveal conservation of functions such as RLC recruitment and RAS involvement in proximal tubule function between the zebrafish and the mouse, while other functions, related to increased blood pressure, have evolved. The zebrafish is proving a valuable and tractable, vertebrate model for exploring mechanisms in the RAS and renin cell biology.

PERSPECTIVE

Ablation of renin lineage cells in the AMA of zebrafish larvae reveals dual functionality of the cells—both renin expression and contractility. This should be explored in the mesonephric and metanephric kidney. Targeted knockout of renin function is not lethal in zebrafish larvae, because sodium homeostasis is achieved by ionocytes in the gills. Following renin knockout, adult zebrafish show vacuolation of the proximal tubule and renin lineage cell recruitment in the mesonephric vasculature, both of which are seen in the metanephric kidney of the mouse, suggesting an ongoing requirement for renin in both species. Transcriptome analysis of renin lineage cells from zebrafish and mice reveal significant differences however—with mouse RLCs expressing transcripts associated with integrins and focal adhesion regulation. This suggests that such functions have evolved with transition to land, which created additional homeostatic challenges including increased blood pressure.

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Affiliations

Centre for Cardiovascular Science (S.H., L.M., S.R., C.B., C.B.B., A.A., Z.L., J.M.) and Centre for Inflammation Research (M.S.B., N.H.), The Queen's Medical Research Institute, The University of Edinburgh, United Kingdom. Now with DSM Nutritional Products Ltd, Switzerland (S.R.). Now with Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom (C.B.B.). MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, United Kingdom (N.H.). Veterinary Pathology, Royal (Dick)School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Easter Bush Campus, United Kingdom (J.d.P.). Department of Pediatrics, School of Medicine, University of Virginia, Charlottesville (A.D.G.M., M.L.S.S.-L., R.A.G.).

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Disclosures

None.

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