Dysregulated expression but redundant function of the long non-coding RNA HOTAIR in diabetic kidney disease

Syamantak Majumder^{1,2} · Mitchell J. Hadden¹ · Karina Thieme^{1,3} · Sri N. Batchu¹ · Divya Niveditha² · Shibashish Chowdhury² · Veera Ganesh Yerra¹ · Suzanne L. Advani¹ · Bridgit B. Bowskill¹ · Youan Liu¹ · Hana Vakili^{1,4} · Tamadher A. Alghamdi¹ · Kathryn E. White⁵ · Laurette Geldenhuys⁶ · Ferhan S. Siddiqi⁷ · Andrew Advani¹

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Abstract

Aims/hypothesis Long non-coding RNAs (lncRNAs) are garnering increasing attention for their putative roles in the pathogenesis of chronic diseases, including diabetic kidney disease (DKD). However, much about *in vivo* lncRNA functionality in the adult organism remains unclear. To better understand lncRNA regulation and function in DKD, we explored the effects of the modular scaffold lncRNA HOTAIR (HOX antisense intergenic RNA), which approximates chromatin modifying complexes to their target sites on the genome.

Methods Experiments were performed in human kidney tissue, in mice with streptozotocin-induced diabetes, the *db/db* mouse model of type 2 diabetes, podocyte-specific *Hotair* knockout mice and conditionally immortalised mouse podocytes.

Results HOTAIR was observed to be expressed by several kidney cell-types, including glomerular podocytes, in both human and mouse kidneys. However, knockout of *Hotair* from podocytes had almost no effect on kidney structure, function or ultrastructure. Glomerular HOTAIR expression was found to be increased in human DKD, in the kidneys of mice with streptozotocin-induced diabetes and in the kidneys of *db/db* mice. Likewise, exposure of cultured mouse podocytes to high glucose caused upregulation of *Hotair* expression, which occurred in a p65-dependent manner. Although HOTAIR expression was upregulated in DKD and in high glucose-exposed podocytes, its knockout did not alter the development of kidney damage in diabetic mice. Rather, in a bioinformatic analysis of human kidney tissue, *HOTAIR* expression closely paralleled the expression of its genic neighbour, *HOXC11*, which is important to developmental patterning but which has an uncertain role in the adult kidney.

Conclusions/interpretation Many lncRNAs have been found to bind to the same chromatin modifying complexes. Thus, there is likely to exist sufficient redundancy in the system that the biological effects of dysregulated lncRNAs in kidney disease may often be inconsequential. The example of the archetypal scaffold lncRNA, HOTAIR, illustrates how lncRNA dysregulation may be a bystander in DKD without necessarily contributing to the pathogenesis of the condition. In the absence of *in vivo* validation, caution should be taken before ascribing major functional roles to single lncRNAs in the pathogenesis of chronic diseases.

Keywords Diabetic kidney disease · Diabetic nephropathy · Epigenetics · Histone · HOTAIR · Kidney · lncRNA · Podocyte

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✉ Andrew Advani
advania@smh.ca

¹ Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St Michael's Hospital, 209 Victoria Street, Toronto, ON M5C 1T8, Canada

² Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Rajasthan, India

³ Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

⁴ Present address: Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁵ Electron Microscopy Research Services, Newcastle University, Newcastle upon Tyne, UK

⁶ Department of Pathology, Dalhousie University, Halifax, NS, Canada

⁷ Department of Medicine, Dalhousie University, Halifax, NS, Canada

Research in context

What is already known about this subject?

- Long non-coding RNAs (lncRNAs) are gaining increasing attention for their potential roles in the pathogenesis of diabetes and its complications, including diabetic kidney disease (DKD)
- One of the primary mechanisms of action of lncRNAs is the regulation of epigenetic processes, which themselves have also been linked to the development of diabetes complications
- The lncRNA HOTAIR regulates epigenetic processes by approximating chromatin-modifying complexes to their target sites on the genome

What is the key question?

- What is the role of the lncRNA, HOTAIR, in DKD?

What are the new findings?

- HOTAIR is present in human and mouse glomerular podocytes and is upregulated in DKD
- Deletion of *Hotair* from mouse podocytes has minimal effect on kidney function under normal conditions or in diabetes
- *HOTAIR* expression parallels that of its genic neighbour *HOXC11* in human kidney disease

How might this impact on clinical practice in the foreseeable future?

- lncRNA dysregulation may be a biomarker of diabetes complications without necessarily contributing to their pathogenesis

Abbreviations

CoREST	Corepressor for element-1-silencing transcription factor
ChIP	Chromatin immunoprecipitation
ChIRP	Chromatin immunoprecipitation by RNA purification
CKD	Chronic kidney disease
DKD	Diabetic kidney disease
EZH2	Enhancer of zeste homologue 2
GEO	Gene Expression Omnibus
HOTAIR	HOX antisense intergenic RNA
<i>Hotair</i> ^{Ctrl}	Cre positive wild-type mice
<i>Hotair</i> ^{podKO}	Podocyte-specific <i>Hotair</i> knockout mice
lncRNA	Long non-coding RNA
LSD1	Lysine-specific histone demethylase 1A
PAS	Periodic acid–Schiff
PRC2	Polycomb repressive complex 2
qRT-PCR	Quantitative reverse transcription-PCR
REST	Repressor element-1 silencing transcription factor
siRNA	Short interference RNA
α-SMA	α-Smooth muscle actin
STZ	Streptozotocin

Introduction

The overwhelming majority of gene transcription events do not result in protein translation. Whereas some non-protein-coding transcripts represent bona fide ‘transcriptional noise’ [1], others are functional non-coding RNAs, such as transfer RNAs (tRNAs), ribosomal RNAs, small RNAs (including micro-RNAs) and long non-coding RNAs (lncRNAs). Arbitrarily greater than 200 nucleotides in length, lncRNAs, like their protein-coding counterparts, are commonly transcribed by RNA polymerase II, spliced, 5′ capped and polyadenylated [2]. Recent years have witnessed a tremendous upsurge in interest in lncRNAs amongst all disciplines ranging from fundamental biology to human disease, including diabetes and its complications. lncRNAs may contribute to islet cell dysfunction [3] and they appear to play important roles in the regulation of metabolism [4]. However, being in its relative infancy, the lncRNA field has also experienced its share of controversy and contradiction [5, 6]. A case in point is the archetypal *trans*-acting modular scaffold lncRNA, HOX antisense intergenic RNA (HOTAIR) [7].

HOTAIR is a 2,148 nucleotide lncRNA that is transcribed in an antisense direction from an intergenic region between

HOXC11 and *HOXC12* in the *HOXC* cluster on chromosome 12 in humans [7]. *HOXC* is one of four gene clusters (*HOXA-D*) that encode transcription factors necessary for the determination of positional identity along body axes during development [7, 8]. HOTAIR is the first lncRNA reported to function in *trans*, regulating the expression of genes encoded from the *HOXD* locus located on chromosome 2 [7]. It is considered to influence gene transcription by acting as a modular scaffold, approximating chromatin modifying complexes to their target sites on the genome. The 5' domain of HOTAIR binds polycomb repressive complex 2 (PRC2), which includes the histone methyltransferase, enhancer of zeste homologue 2 (EZH2), that catalyses the trimethylation of histone H3 on lysine residue 27 (H3K27me3), a marker of gene repression [7]. Concurrently, a 3' domain of HOTAIR has been reported to bind the LSD1/CoREST/REST complex, which contains lysine-specific histone demethylase 1A (LSD1) (together with corepressor for element-1-silencing transcription factor [CoREST] and repressor element-1 silencing transcription factor [REST]), an enzyme that demethylates H3K4me2, which is typically found at sites of active gene transcription [9].

Unlike some other lncRNAs [10], orthologues of HOTAIR are present in other mammals, offering an opportunity to gauge the biological importance of a single lncRNA at the level of the whole organism. *Hotair* has 90% nucleotide conservation in mice [7], it is transcribed from the micro-syntenic location corresponding to its cognate human counterpart [11], and it similarly binds both EZH2 and LSD1 [12]. Targeted deletion of *Hotair* has been reported to cause homeotopic transformation of the spine and malformations of the wrist bone [12]. However, the variable phenotypes reported have led some researchers to call into question the importance of the *in vivo* actions of *Hotair*, at least during embryological development [6, 8, 11–14].

As the explication of lncRNA biology matures through the study of model transcripts such as HOTAIR, lncRNA dysregulation has been increasingly linked to the pathogenesis of complex chronic diseases, amongst them diabetic kidney disease (DKD) [15–22]. Given the trajectory of discovery, it seems highly likely that new candidates and new mechanisms will be unearthed and proposed in the future. Our earlier work has served to define a pivotal role for the EZH2-regulated histone mark, H3K27me3 in preventing diabetes-induced damage to glomerular podocytes [23, 24]. In light of the reported biological importance of HOTAIR–EZH2 interaction and of the importance of EZH2 to the maintenance of podocyte health, we set out to examine the actions of HOTAIR in podocytes both in the normal state and when challenged with diabetes.

Methods

Human studies Human kidney tissue was obtained at the time of nephrectomy for conventional renal carcinoma and the

clinical characteristics of the patients have been described before [24]. The study was approved by the Nova Scotia Health Authority Research Ethics Board (Halifax, NS, Canada) and the Research Ethics Board of St Michael's Hospital. A waiver of consent based on impracticability criteria was provided by the Nova Scotia Health Authority Research Ethics Board.

Mouse studies Podocin-*cre*⁺ (B6.Cg-Tg(*NPHS2-cre*)295Lbh/J) mice [25] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were bred with *Hotair*^{LoxP/+} mice [12] obtained from H. Chang (Stanford University School of Medicine, Stanford, CA, USA) to generate Podocin-*cre*⁺*Hotair*^{+/+} (*Hotair*^{Ctrl}) and Podocin-*cre*⁺*Hotair*^{fl/fl} (*Hotair*^{podKO}) mice. These mice were studied at 10–14 weeks of age under non-diabetic conditions. To measure glomerular *Hotair* expression in experimental DKD, C57BL/6 mice (The Jackson Laboratory) were rendered diabetic with i.p. injections of streptozotocin (STZ; 55 mg/kg in 0.1 mol/l citrate buffer, pH 4.5) after a 4 h fast for 5 consecutive days. Non-diabetic control mice received an equal volume of citrate buffer. Blood glucose was measured 12 days after the first i.p. injection and mice were followed for a further 12 weeks. Additionally, male *db/m* and *db/db* mice (The Jackson Laboratory) were studied at 20 weeks of age. To examine the effects of podocyte-specific *Hotair* knockout under diabetic conditions, male *Hotair*^{Ctrl} and *Hotair*^{podKO} mice were rendered diabetic with STZ as described above and were studied 12 weeks after the first i.p. injection of STZ (i.e. 10 weeks after confirmation of diabetes). Mouse phenotyping methods are described in electronic supplementary material (ESM) Methods. All experimental procedures adhered to the guidelines of the Canadian Council on Animal Care and were approved by St Michael's Hospital Animal Care Committee.

Digoxigenin-labelled in situ hybridisation and RNAscope in situ hybridisation Digoxigenin-labelled in situ hybridisation was performed on formalin-fixed, paraffin-embedded human or mouse kidney tissues as described in ESM Methods. RNAscope in situ hybridisation (Advanced Cell Diagnostics, Hayward, CA, USA) was performed according to the manufacturer's instructions and using custom software as previously reported [26] and as described in ESM Methods.

Isolation of podocyte-enriched mouse glomeruli Podocyte-enriched glomerular cell extracts were isolated from mouse kidneys using the CELlection Biotin Binder Kit (Thermo Fisher Scientific, Rockford, IL, USA) as described in ESM Methods.

Immunofluorescence microscopy For immunofluorescence microscopy, mouse slides were stained with antibodies in the following concentration: mouse nephrin 1:100 (AF3159, R & D Systems, Minneapolis, MN, USA), secondary antibody

Alexa Fluor 488 donkey anti-goat 1:100 (Thermo Fisher Scientific). Co-staining was with the following antibodies: EZH2 1:100 (#5246, Cell Signaling Technology, Danvers, MA, USA), LSD1 1:100 (#2139, Cell Signaling Technology), H3K27me3 1:100 (#9733, Cell Signaling Technology), H3K4me1 (ab8895, Abcam, Cambridge, MA, USA), H3K4me2 1:100 (ab7766, Abcam), H3K4me3 1:100 (ab213224, Abcam), secondary antibody Alexa Fluor 647 donkey anti-rabbit 1:100 (Thermo Fisher Scientific). Slides were viewed using a Zeiss LSM 700 confocal microscope and analysis was conducted on approximately 30 podocytes from at least six glomeruli per slide using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA).

Transmission electron microscopy Images of the entire glomerular profile were taken through three randomly selected glomeruli from 5–8 mice per group (Philips CM100, Electron Microscopy Research Services; Newcastle University, Newcastle, UK). Podocyte density was estimated by the method of Weibel and Gomez [27]. Podocyte density was multiplied by mean glomerular volume (determined in periodic acid–Schiff [PAS] stained kidney sections [28]) to give an estimate of podocyte number per glomerulus. Glomerular basement membrane thickness and podocyte foot process width were measured in a masked manner in approximately ten micrographs (magnification $\times 5800$) from each glomerular profile using ImageJ (National Institutes of Health).

Quantitative reverse transcription-PCR Primers were obtained from Integrated DNA Technologies and had the following sequences: *Hotair* forward AGGGTCCCCAACATCGGTAGA, reverse TGCGGTGGAGATAGATGTGC or forward AGCTGAGAAGGCCTGAATGA, reverse AAGGGGTGAACAGTGATCTG; *Rplp0* forward GCGTCCTGGCATTGTCTGT, reverse GAAGGCCTTGACCTTTTCAGTAAG; *Rpl13a* forward GCTCTCAAGGTTGTTTCGGCTGA, reverse AGATCTGCTTCTTCTCCGATA. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed on a Viia 7 real-time PCR system (Thermo Fisher Scientific) using SYBR Green and data analysis was performed using the comparative C_t method.

Conditionally immortalised mouse podocytes Differentiated, conditionally immortalised mouse podocytes were cultured as previously described [29]. Cells were serum starved for 4 h and incubated in normal media (5.6 mmol/l glucose) or in 25 mmol/l glucose (high glucose) for 48 h. Mannitol (19.4 mmol/l) added to normal media served as the osmotic control. For p65 knockdown, cells were transfected with 50 nmol/l mouse sequence-specific short interference RNA (siRNA) for p65 (sc-29411, Santa Cruz Biotechnology, Dallas, TX, USA) or scrambled siRNA (AM4611, Thermo Fisher Scientific) for 4 h prior to exposure to high glucose

for 48 h. Chromatin immunoprecipitation by RNA purification (ChIRP) was performed using an EZ-Magna ChIRP Interactome Kit (EMD Millipore, Etobicoke, ON, Canada). Biotinylated *Hotair* probes were designed and synthesised by Biosearch Technologies (Petaluma, CA, USA) to cover the entire length of *Hotair* (ESM Table 1). The LacZ probeset was from Millipore. Primer sequences for *Hotair* targets (*Hoxd1*, *Hoxd3*, *Hoxd11*, *Dlk1*, *Plagl1*, *Dcn* and *H19*) and control genes (*Vamp5*, *Emp2*) were as reported in [12] and were from Integrated DNA Technologies (Coralville, IA, USA). Values were normalised relative to LacZ binding compared with input DNA [30]. Chromatin immunoprecipitation (ChIP) was performed using a Magna ChIP kit (EMD Millipore) with an antibody against p65 (1:100 dilution; #8242, Cell Signaling Technology) or an equal concentration of normal rabbit IgG control (sc-2027, Santa Cruz Biotechnology). qRT-PCR was performed using primers specific for the mouse *Hotair* promoter (forward CCCAGCCA GGTAGGTAGAGT, reverse GAAGGGGCTGATGG ATGCTT).

Immunoblotting was performed with antibodies in the following concentrations: nephrin 1:1000 (AF3159, R & D Systems), p65 1:100 (#8242, Cell Signaling Technology), β -actin 1:10,000 (#A1978, Sigma-Aldrich, Oakville, ON, Canada). Densitometry was performed using ImageJ.

Mesangial matrix index Mesangial matrix accumulation was calculated in approximately 30 glomeruli from PAS-stained kidney sections by investigators masked to the study groups, as previously described [31].

Immunohistochemistry Immunostaining of mouse kidney tissue was performed as previously described [31] with the following antibodies: collagen IV 1:500 (AB756P, EMD Millipore), α -smooth muscle actin (α -SMA) 1:400 (ab5694, Abcam), nephrin 1:200 (AF3159, R&D Systems). The proportion of glomerular area positively immunostaining was determined in 30 random glomerular profiles in each kidney section using ImageScope for collagen IV and α -SMA and HALO version 2.3.2089.23 (Indica Labs, Corrales, NM, USA) for nephrin immunostaining.

Bioinformatics Transcriptomic data were extracted from the NCBI Gene Expression Omnibus (GEO) database, accessible under the series number GSE66494. The details of the dataset are described in [32]. In brief, we studied the ‘discovery cohort’ consisting of transcriptomic data derived using the Agilent Whole Human Genome Microarray 4x44K from biopsy tissue from 48 patients with chronic kidney disease (CKD) compared with tissue from five control participants. Differential expression of *HOTAIR*, *HOXC11*, *TGF- β 1* (also known as *TGF β 1*), *CTGF* (also known as *CCN2*) and *CCL2*

was assessed using limma, an R/Bioconductor software package. The false discovery rate was controlled using the Benjamini–Hochberg method. A p value of ≤ 0.05 was used to identify differentially expressed genes. Pearson correlation coefficient was calculated using Microsoft Excel. Boxplots and heatmaps were generated using limma.

Statistics Data are expressed as means $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Fisher least significant difference post hoc test for multiple groups comparison or by two-tailed Student's t test for comparison between two groups, unless otherwise stated. All statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA, USA) unless otherwise stated.

Results

HOTAIR is expressed by human glomerular podocytes in vivo

To determine whether HOTAIR is expressed in adult human kidneys, we first performed digoxigenin-labelled in situ hybridisation on normal human kidney tissue. HOTAIR

riboprobe binding was observed in scattered glomerular cells, parietal epithelial cells of Bowman's capsule and tubule epithelial cells, especially tubule epithelial cells of distal convoluted tubules and cortical collecting ducts (Fig. 1a, b). Next, by combining RNAscope in situ hybridisation for HOTAIR and immunohistochemistry for the slit pore protein, nephrin, we confirmed HOTAIR expression by adult human glomerular podocytes (Fig. 1c). Finally, diminishing the likelihood of non-specific probe binding, we observed a significantly larger number of RNAscope puncta using the HOTAIR probeset compared with a probeset directed against the bacterial gene, *dapB* (negative control) where glomerular puncta were almost completely unapparent (Fig. 1d).

Generation of podocyte-specific *Hotair* knockout mice As we had observed in adult human kidneys, when labelling mouse kidneys with an RNAscope probeset, we observed *Hotair* expression in adult mouse glomerular podocytes (Fig. 2a, b). In both human (Fig. 1) and mouse (Fig. 2) kidneys, *Hotair* was observed to be primarily nuclear in its localisation. To determine the function of *Hotair* in glomerular podocytes, we generated podocyte-specific *Hotair* knockout mice. We studied

Fig. 1 HOTAIR is expressed by kidney cells in normal adult human kidney tissue, including glomerular podocytes. (a, b) Representative digoxigenin-labelled in situ hybridisation for HOTAIR (purple) in normal human kidney tissue (a, scale bar, 100 µm; b, scale bar, 50 µm; $n = 12$). HOTAIR is present in tubule epithelial cells (especially distal tubules and cortical collecting ducts), parietal epithelial cells and glomerular cells. (c) Representative RNAscope in situ hybridisation for HOTAIR (red puncta) and immunohistochemistry for nephrin (brown) in a human glomerulus (scale bar, 50 µm). The black arrow labels RNAscope HOTAIR probeset

binding in the nucleus of a peripherally arranged nephrin-positive cell (podocyte) in the zoomed-in image ($n = 6$). (d) Representative RNAscope in situ hybridisation (red puncta) in human glomeruli for HOTAIR, the bacterial transcript *dapB* (negative control) or the housekeeper *PPIB* (positive control) and quantification of RNAscope puncta ($n = 6$ /probeset). Original images taken with a $\times 63$ objective (scale bars, 20 µm); blue is DAPI. The white arrows point to HOTAIR RNAscope puncta. Values are mean $\pm$ SD. * $p < 0.05$ by two-tailed Student's t test

two groups of mice, Podocin-cre⁺*Hotair*^{fl/fl} mice and Podocin-cre⁺*Hotair*^{+/+} mice, henceforward referred to as *Hotair*^{podKO} and *Hotair*^{Ctrl}, respectively. Comparison of *Hotair* expression levels in podocyte-enriched glomerular cell fractions with *Hotair* expression in whole kidneys revealed an approximate 70% reduction in glomerular cell *Hotair* in

Hotair^{podKO} mice (Fig. 2c–e). Further confirming efficient excision of *Hotair* from podocytes, by dual immunofluorescence we observed absence of *Hotair* probeset binding in nephrin-positive glomerular cells in the kidneys of *Hotair*^{podKO} mice, accompanied by a reduction in glomerular *Hotair* levels (Fig. 2f).

Knockout of *Hotair* from podocytes has no effect on albuminuria, glomerular structure, podocyte number or podocyte ultrastructure Next, we examined the renal phenotype of otherwise normal adult *Hotair*^{podKO} and *Hotair*^{Ctrl} mice (aged approximately 10–14 weeks). There was no difference in body weight (Fig. 3a), systolic BP (Fig. 3b) or kidney weight (Fig. 3c) between *Hotair*^{Ctrl} and *Hotair*^{podKO} mice, although kidney weight:body weight ratio was slightly reduced in *Hotair*^{podKO} mice (Fig. 3d). Albuminuria (Fig. 3e, f), plasma creatinine (Fig. 3g) and blood urea nitrogen (Fig. 3h) did not differ between *Hotair*^{Ctrl} and *Hotair*^{podKO} mice. Light microscopic appearance of the kidneys of *Hotair*^{podKO} mice was unremarkable (Fig. 3i),

as was glomerular volume (Fig. 3j). By transmission electron microscopy, we saw no overall change in podocyte density (Fig. 3k), podocyte number (Fig. 3l) or podocyte foot process width (Fig. 3m). Likewise, there was no difference between *Hotair*^{Ctrl} and *Hotair*^{podKO} mice in podocyte levels of the *Hotair* binding partners EZH2 and LSD1 or the histone marks that they regulate (H3K27me3 and H3K4me1-3) (ESM Fig. 1).

HOTAIR expression is increased in the kidneys of humans and mice with diabetes Having detected that *Hotair* is present in (but dispensable to) normal mouse podocytes, we next queried whether the expression pattern of HOTAIR is altered in DKD.

Fig. 3 Knockout of *Hotair* from podocytes has minimal renal phenotypic effects. (a) Body weight, (b) systolic BP, (c) mean kidney weight, (d) kidney weight:body weight ratio, (e) 24 h urine AER and (f) urine albumin:creatinine ratio in *Hotair*^{Ctrl} (n = 8) and *Hotair*^{podKO} (n = 8) mice aged 10–14 weeks. (g) Plasma creatinine and (h) blood urea nitrogen (BUN) in *Hotair*^{Ctrl} (n = 7) and *Hotair*^{podKO} (n = 8) mice. There was insufficient sample for measurement of plasma variables in one *Hotair*^{Ctrl} mouse. (i) Representative H&E (scale bars, 100 μm; *Hotair*^{Ctrl} [n = 8], *Hotair*^{podKO} [n = 8]) and PAS (scale bars, 25 μm;

Hotair^{Ctrl} [n = 7], *Hotair*^{podKO} [n = 7]) stained kidney sections from *Hotair*^{Ctrl} and *Hotair*^{podKO} mice showing unremarkable kidney architecture in *Hotair*^{podKO} mice. (j) Glomerular volume, (k) podocyte density, (l) podocyte number and (m) podocyte foot process width in *Hotair*^{Ctrl} (n = 7) and *Hotair*^{podKO} (n = 5) mice. The representative transmission electron micrographs in (m) illustrate normal foot process architecture in the glomerulus from a *Hotair*^{podKO} mouse (scale bars, 2 μm). Values are mean ± SD. **p* < 0.05 by two-tailed Student's *t* test

We determined the magnitude of HOTAIR transcript levels in glomerular cells after digoxigenin-labelled in situ hybridisation of kidney tissue from individuals without diabetes and from individuals with pathologically confirmed diabetic glomerulosclerosis, observing an approximate 80% increase in glomerular HOTAIR in the setting of human diabetic glomerulosclerosis (Fig. 4a). Next, we examined Hotair levels in experimental diabetes in mice. By qRT-PCR, we observed an increase in Hotair expression in the kidneys of mice with STZ-induced diabetes (ESM Table 2) or *db/db* mice (ESM Table 3) (Fig. 4b). Likewise, quantification of glomerular Hotair labelling by in situ hybridisation also revealed an increase in Hotair abundance in the setting of STZ-induced diabetes (Fig. 4c) or in *db/db* mice (Fig. 4d).

Exposure to high glucose increases Hotair expression by mouse podocytes in a p65-dependent manner To explore the possible mechanisms by which Hotair expression is upregulated by podocytes in the diabetic setting, we studied immortalised cultured mouse podocytes [29]. ChIRP demonstrated Hotair enrichment at known genomic sites [12] (Fig. 5a), whereas when podocytes were exposed to high glucose we observed a greater than doubling in Hotair levels (Fig. 5b). In considering how high glucose may upregulate HOTAIR, we were cognisant of work reporting a role for the p65 subunit of NF- κ B in regulating the expression of human HOTAIR

[33, 34]. We performed in silico analysis of the mouse *Hotair* promoter and identified three potential binding sites for NF- κ B (ESM Fig. 2). By ChIP, we found p65 enrichment at the *Hotair* promoter in mouse podocytes (Fig. 5c). Supporting a role for p65 in mediating Hotair upregulation, siRNA directed against p65 negated the upregulation of Hotair induced by high glucose (Fig. 5d, e).

Hotair knockout from podocytes does not affect the development of glomerular injury in diabetic mice Because we had found HOTAIR to be upregulated in DKD, we set out to determine whether it plays a role in diabetes-associated glomerular injury. We rendered *Hotair*^{Ctrl} and *Hotair*^{podKO} mice diabetic with STZ. As expected, glomerular Hotair levels were increased in STZ-diabetic *Hotair*^{Ctrl} mice in comparison with their non-diabetic counterparts, whereas glomerular Hotair expression was lower in STZ-diabetic *Hotair*^{podKO} mice in comparison with STZ-diabetic *Hotair*^{Ctrl} mice (ESM Fig. 3). Body weight was decreased (Fig. 6a) and HbA_{1c} was increased (Fig. 6b) equivalently in STZ-diabetic *Hotair*^{Ctrl} and STZ-diabetic *Hotair*^{podKO} mice. Kidney weight was unchanged across experimental groups (Fig. 6c), whereas kidney weight:body weight ratio was reduced in non-diabetic *Hotair*^{podKO} mice in comparison with STZ-diabetic *Hotair*^{Ctrl} or STZ-diabetic *Hotair*^{podKO} mice (Fig. 6d). Urine albumin excretion (Fig. 6e), glomerular basement membrane

Fig. 4 HOTAIR expression is increased in human and experimental DKD. **(a)** Digoxigenin-labelled in situ hybridisation for HOTAIR and quantification of glomerular HOTAIR in kidney tissue from individuals without diabetes (h_Control, $n = 12$) and individuals with DKD (h_DKD, $n = 11$) (scale bars, 50 μ m). **(b)** qRT-PCR in STZ-diabetic C57BL/6 mice (STZ, $n = 6$) in comparison with age-matched controls ($n = 6$) or in *db/db* mice ($n = 8$) in comparison with non-diabetic *db/m* mice ($n = 8$). **(c)**

Digoxigenin-labelled in situ hybridisation for Hotair and quantification of glomerular Hotair in kidney tissue from control ($n = 6$) and STZ-diabetic ($n = 6$) mice (scale bars, 25 μ m). **(d)** Digoxigenin-labelled in situ hybridisation for Hotair and quantification of glomerular Hotair in kidney tissue from non-diabetic *db/m* ($n = 6$) and diabetic *db/db* ($n = 6$) mice (scale bars, 25 μ m). Values are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ by two-tailed Student's t test

Fig. 5 *Hotair* expression is increased in a p65-dependent manner in cultured mouse podocytes exposed to high glucose. **(a)** ChIP for *Hotair* in cultured mouse podocytes. In comparison with LacZ binding (control), *Hotair* is enriched at genomic sites previously identified as being affected by *Hotair* binding (*Hoxd1*, *Hoxd3*, *Hoxd11*, *Dlk1*, *Plag1*, *Dcn* and *H19*), whereas there is no enrichment at unaffected sites (*Vamp5*, *Emp2*). **(b)** qRT-PCR for *Hotair* in mouse podocytes under control conditions (5.6 mmol/l glucose) or after exposure to high glucose (HG, 25 mmol/l) for 48 h or mannitol (osmotic control) ($n = 3$ /condition). **(c)** ChIP for

p65 at the *Hotair* promoter (IgG, $n = 2$; p65, $n = 6$). **(d)** Immunoblotting for p65 after transfection of mouse podocytes with p65 siRNA or scramble control for 48 h (scramble, $n = 3$; p65 siRNA, $n = 3$). **(e)** qRT-PCR for *Hotair* in mouse podocytes transfected with p65 siRNA for 4 h before exposure to HG for 48 h (control, $n = 7$; p65 siRNA, $n = 8$; HG, $n = 8$; p65 siRNA + HG, $n = 8$; mannitol, $n = 7$). Values are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA followed by Fisher's least significant difference post hoc test (**b**, **e**), two-tailed one-sample t test (**c**) or two-tailed Student's t test (**d**)

thickness (Fig. 6f) and mesangial matrix accumulation (Fig. 6g) were each equivalently increased in STZ-diabetic *Hotair*^{Ctrl} and STZ-diabetic *Hotair*^{podKO} mice. Likewise, glomerular collagen IV expression (ESM Fig. 4a) and α -SMA levels (ESM Fig. 4b) were increased to the same magnitude in STZ-diabetic *Hotair*^{Ctrl} and STZ-diabetic *Hotair*^{podKO} mice, whereas glomerular nephrin levels did not differ between the four experimental groups (ESM Fig. 4c).

HOTAIR expression parallels the expression of the adjacent HOXC11 gene in human CKD Finally, to further understand the role (if any) that HOTAIR may play in human CKD, we performed a bioinformatic analysis of transcriptomic data from a publicly available dataset of 48 biopsy samples obtained from individuals with CKD (including but not limited to DKD) compared with five control kidney RNA samples (GSE66494) [32]. We examined the expression patterns of HOTAIR and the adjacent *HOXC11* gene, together with three other genes important in the pathogenesis of CKD: *TGF- β 1*, *CTGF* and *CCL2* [35–37]. Differential gene expression analysis also showed upregulation of HOTAIR expression in these CKD samples (Fig. 7a). Furthermore, heatmapping revealed

that HOTAIR expression patterns closely parallel those of the *HOXC11* gene in human CKD, whereas other genes implicated in CKD pathogenesis (e.g. *TGF- β 1*, *CTGF* and *CCL2*) did not exhibit such a coordinated pattern of expression (Fig. 7b). Indeed, by Pearson correlation analysis we observed an extremely high correlation between the expression levels of HOTAIR and *HOXC11* in human CKD ($r = 0.87$, $p \leq 0.001$; Fig. 7c).

Discussion

In the search for new treatment modalities for DKD, recent interest has turned to the potential impact of therapeutically targeting non-coding RNAs, including lncRNAs [38]. Here, we report that the lncRNA HOTAIR is expressed by glomerular podocytes and upregulated in DKD. However, genetic knockout of *Hotair* has little effect on podocyte ultrastructure, glomerular health or renal physiology, either under normal conditions or when mice are challenged with experimental diabetes. Rather HOTAIR expression parallels that of its genic neighbour, *HOXC11*. At a time when interest in lncRNA

Fig. 6 Knockout of *Hotair* from podocytes has minimal effect on the renal phenotype of STZ-diabetic mice. Male *Hotair*^{Ctrl} and *Hotair*^{podKO} mice were rendered diabetic with STZ (or injected with citrate buffer, control) and were followed for 10 weeks. **(a)** Body weight, **(b)** HbA_{1c}, **(c)** mean kidney weight, **(d)** kidney weight:body weight ratio and **(e)** 24 h urine AER. **(f)** Representative transmission electron micrographs with measurement of glomerular basement membrane thickness below (scale bars, 2 μm). **(g)** Representative PAS-stained kidney sections with semi-

quantitative mesangial matrix index below (scale bars, 25 μm). In **(a–e, g)**, non-diabetic *Hotair*^{Ctrl}, *n* = 7; STZ-diabetic *Hotair*^{Ctrl}, *n* = 7; non-diabetic *Hotair*^{podKO}, *n* = 5; STZ-diabetic *Hotair*^{podKO}, *n* = 9. In **(f)**, *Hotair*^{Ctrl}, *n* = 7; STZ-diabetic *Hotair*^{Ctrl}, *n* = 5; non-diabetic *Hotair*^{podKO}, *n* = 5; STZ-diabetic *Hotair*^{podKO}, *n* = 8. AU, arbitrary units. Values are mean ± SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by one-way ANOVA followed by Fisher's least significant difference post hoc test

biology is escalating rapidly, the results serve as a counterweight to emphasise that, in complex chronic diseases like DKD, lncRNA dysregulation may be a bystander without necessarily contributing to disease pathogenesis.

HOTAIR was originally described as a *trans*-acting lncRNA that is required for PRC2 occupancy at the *HOXD* locus [7], its subsequent interaction with the H3K4me2-demethylating LSD1/CoREST/REST complex further arguing in favour of a gene regulatory function [9]. However, although histone patterning at the *HOX* loci was originally studied as a means of discerning how lncRNAs may affect gene transcription, it has become apparent that the *HOXD* locus is not the only site of HOTAIR binding on the genome. For instance, over 800 genome-wide HOTAIR occupancy sites have been identified in human breast cancer cells and

hundreds of genes are induced or repressed with HOTAIR overexpression in cancer cells [39, 40]. We observed HOTAIR expression in normal adult mouse and human kidney tissue and in mouse podocytes grown in culture, and we observed that HOTAIR expression is consistently increased in human and mouse DKD. This prompted us to explore the regulatory control of HOTAIR in podocytes and to inquire as to whether HOTAIR upregulation may be causatively implicated in the pathogenesis of DKD. We found that podocyte HOTAIR levels are increased by high glucose and that this upregulation is dependent on the p65 subunit of the proinflammatory transcription factor, NF-κB. However, *Hotair* knockout from podocytes is largely inconsequential either under normal conditions or in diabetes, with the exception of a small but statistically significant diminution in kidney weight:body

Fig. 7 *HOTAIR* expression closely mimics the expression pattern of the adjacent *HOXC11* gene in human CKD. Transcriptomic data were analysed from dataset GSE66494 (discovery cohort) consisting of RNA extracted from kidney biopsy tissue from 48 individuals with CKD and five control participants [32]. **(a)** Relative gene expression in CKD tissue (in comparison with control) was examined for *HOTAIR*, the adjacent *HOXC11* gene and three genes implicated in the development of CKD (*TGF-β1*, *CTGF* and *CCL2*). The boxplot displays the median values (bold lines) of each gene with interquartile ranges (upper and lower limit of the boxes). The whiskers show the maximum and minimum, excluding

outlying values. CKD vs control: *HOTAIR* $p = 3.61 \times 10^{-5}$, *HOXC11* $p = 1.98 \times 10^{-7}$, *TGF-β1* $p = 0.352$, *CTGF* $p = 0.17 \times 10^{-5}$, *CCL2* $p = 7.11 \times 10^{-8}$ by two-tailed Student's *t* test. **(b)** Heatmap of expression of the five genes relative to control showing a similar pattern of expression of *HOTAIR* and *HOXC11* across the 48 CKD samples, that is not shared amongst the three comparator genes (*TGF-β1*, *CTGF* and *CCL2*). Colour key shows Log₂ fold change. **(c)** Correlation between expression levels of *HOTAIR* and *HOXC11*, $r = 0.87$, $p \leq 0.001$ (Pearson correlation coefficient)

weight ratio in *Hotair*^{podKO} mice. This subtle change could be a consequence of podocyte *Hotair* deletion or it could be a consequence of local *cis* effects at the *HoxC* locus. Either way, although numerically significant, its biological importance is uncertain in light of the absence of change in other parameters of renal, glomerular or podocyte structure or function.

Residing in the intergenic region between *Hoxc11* and *Hoxc12*, in mice *Hotair* has a tissue distribution that resembles the distribution of transcripts derived from its genic neighbours

[8]. During development, *HOXC11* is found in the metanephric mesenchyme [41], the embryonic region from which podocytes are derived [42]. In an entirely separate, publicly available, human CKD dataset [32] we observed that whole kidney *HOTAIR* levels are increased and that the expression of *HOTAIR* closely parallels that of the neighbouring *HOXC11* gene. Only two of the 48 samples in this dataset were derived from patients whose primary diagnosis was DKD [32]. Thus, although we observed the p65-dependent upregulation of

Hotair by high glucose in cultured mouse podocytes, diabetes is not the sole cause of HOTAIR dysregulation in CKD and podocytes are unlikely to be the sole cell-type in which HOTAIR is dysregulated. Rather, aberrant HOTAIR expression in human CKD is more probably indicative of broader genic dysregulation, including that of the *HOX* cluster genes, the consequences of which are uncertain in the fully developed organism.

The results herein presented are generally aligned with the studies of skeletal malformations in *Hotair* knockout mice, which overall suggest that *Hotair* is not a major determinant of developmental identity [6, 8, 11, 12]. They are also aligned with recent reports that many lncRNAs are largely dispensable in either mice or zebrafish [5, 30]. However, they are generally misaligned with other contemporary studies that describe a pathogenetic role for other lncRNAs in DKD [15–22]. There are several potential reasons that may underlie the largely benign consequences of the knockout of *Hotair* from podocytes. For instance, the significance and specificity of PRC2 or LSD1/CoREST/REST binding of HOTAIR is uncertain, especially in vivo [6]. PRC2, for example, is recruited to chromatin by thousands of different RNA transcripts [43–45]. In this context, the deletion of just one interacting lncRNA partner from one cell-type may be quite insufficient to induce a discernible effect on renal physiology, and the deficiency may be compensated for by any number of other non-coding transcripts present in the target nucleus. The expression of Cre recombinase in Podocin-*cre*⁺ mice begins to occur during the late capillary loop stage of embryonic development [25] and, in some other settings, a renal phenotype resulting from the experimental disturbance of histone modifying processes in podocytes has only become apparent during the process of ageing [46]. Accordingly, the results of the present study do not preclude an important role for HOTAIR at either extreme of the life course.

Several limitations of the currently presented experiments warrant emphasis. First, our survey of the consequences of *Hotair* deletion on histone marks was limited to assessment of global levels of H3K27me3 and H3K4me1-3 in podocytes. Previous analyses have indicated that HOTAIR inactivation causes H3K4me3 gain and, to a lesser extent, H3K27me3 loss at target genes [12], and we cannot determine the extent to which the podocyte regional histone landscape was altered in *Hotair*^{podKO} mice. Second, whereas we approached the function of HOTAIR through the lens of its best-characterised mode of action, that of a modular scaffold [7, 9], HOTAIR has more recently been reported to also exert chromatin-independent effects. For instance, HOTAIR interacts with E3 ubiquitin ligases [47] and it can also function as a competing endogenous RNA (ceRNA) or micro-RNA sponge [48]. Third, our study of HOTAIR function in vivo focused on the consequences of deletion of the lncRNA from podocytes, and the importance of HOTAIR to other kidney cell-types remains

to be determined. Likewise, it is possible that inducible deletion of *Hotair* from podocytes may have yielded a different phenotype to the constitutive deletion. Finally, given that we observed upregulation of HOTAIR in humans and mice with DKD, it remains to be determined whether overexpression of *Hotair* is either sufficient to promote kidney disease development under normal conditions or to accelerate kidney disease progression in the setting of diabetes. These limitations notwithstanding, we can be confident, however, that in normal mice or mice with experimental diabetes, inactivation of the lncRNA *Hotair* does not influence the renal phenotype in a biologically meaningful manner.

In summary, despite evolutionary differences in its encoding genes [49], the lncRNA HOTAIR is expressed by glomerular podocytes in both humans and mice and its expression is upregulated in experimental and human DKD. However, in this glomerular cell-type and in this disease setting, HOTAIR appears to play a largely redundant role. LncRNAs are vast in number, low in abundance and overlapping in function [50]. The present study highlights that lncRNA dysregulation can occur in complex chronic diseases like DKD without necessarily contributing to disease pathogenesis.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request. The transcriptomic dataset was extracted from the publicly available GEO repository (GSE66494).

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Author contributions SM conceived the idea, designed and performed the experiments, analysed the data and revised and edited the manuscript. MJH performed experiments and analysed data. KT, SNB, VGY, HV and TAA designed and performed the experiments and analysed the data. DN and SC performed the bioinformatic analyses. SLA performed the histological studies. BBB and YL performed the in vivo studies. KEW performed the electron microscopy. LG and FSS contributed to the design of the human studies, sourced archived human tissue and provided the clinical data. AA conceived the idea, designed the experiments, analysed the data and wrote the manuscript. All authors reviewed, edited and approved the final version of the manuscript. AA is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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