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ORIGINAL RESEARCH

Myocardial Cytoskeletal Adaptations in Advanced Kidney Disease

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BACKGROUND: The myocardial cytoskeleton functions as the fundamental framework critical for organelle function, bioenergetics and myocardial remodeling. To date, impairment of the myocardial cytoskeleton occurring in the failing heart in patients with advanced chronic kidney disease has been largely undescribed.

METHODS AND RESULTS: We conducted a 3-arm cross-sectional cohort study of explanted human heart tissues from patients who are dependent on hemodialysis (n=19), hypertension (n=10) with preserved renal function, and healthy controls (n=21). Left ventricular tissues were subjected to pathologic examination and next-generation RNA sequencing. Mechanistic and interference RNA studies utilizing in vitro human cardiac fibroblast models were performed. Left ventricular tissues from patients undergoing hemodialysis exhibited increased myocardial wall thickness and significantly greater fibrosis compared with hypertension patients ($P<0.05$) and control ($P<0.01$). Transcriptomic analysis revealed that the focal adhesion pathway was significantly enriched in hearts from patients undergoing hemodialysis. Hearts from patients undergoing hemodialysis exhibited dysregulated components of the focal adhesion pathway including reduced β-actin ($P<0.01$), β-tubulin ($P<0.01$), vimentin ($P<0.05$), and increased expression of vinculin ($P<0.05$) compared with controls. Cytoskeletal adaptations in hearts from the hemodialysis group were associated with impaired mitochondrial bioenergetics, including dysregulated mitochondrial dynamics and fusion, and loss of cell survival pathways. Mechanistic studies revealed that cytoskeletal changes can be driven by uremic and metabolic abnormalities of chronic kidney disease, in vitro. Furthermore, focal adhesion kinase silencing via interference RNA suppressed major cytoskeletal proteins synergistically with mineral stressors found in chronic kidney disease in vitro.

CONCLUSIONS: Myocardial failure in advanced chronic kidney disease is characterized by impairment of the cytoskeleton involving disruption of the focal adhesion pathway, mitochondrial failure, and loss of cell survival pathways.

Key Words: cardiovascular ■ chronic kidney disease ■ cytoskeleton ■ dialysis ■ end stage kidney disease ■ heart failure ■ mitochondria

The failing heart is a leading cause of mortality in patients with chronic kidney disease (CKD).[1] Adverse cardiac changes occur early in the course of CKD and include left ventricular hypertrophy (LVH), LV dilation, myocardial fibrosis, and calcification leading to a greater prevalence of LV diastolic then systolic dysfunction.[2,3] These changes have been described as CKD-associated cardiomyopathy and attributed in part to volume overload, mineral disorders, and circulating toxins that accumulate with uremia.[4–6] Additionally, chronic hemodialysis causes LV segmental myocardial stunning and hibernation, thereby, contributing to progression of myocardial adaptations.[7]

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CLINICAL PERSPECTIVE

What Is New?

- Myocardial failure in patients with advanced chronic kidney disease is characterized by impairment of the cytoskeleton and disruption of the focal adhesion pathway.
- Cytoskeletal changes can be driven by components of uremia including inflammatory and mineral stressors, and this involves the focal adhesion pathway.

What Are the Clinical Implications?

- The present study unveils novel mechanisms involved in myocardial failure in chronic kidney disease and also provides the first set of gene profile data from human hearts of hemodialysis patients, which will help inform future studies.

Nonstandard Abbreviation and Acronym

FAK	focal adhesion kinase

Molecularly, heart failure studies in the non-CKD population have shown that alterations of the cytoskeleton is a cardinal process in the pathogenesis of cardiomyopathy.[8,9] The cytoskeleton of cardiac myocytes and cardiac fibroblasts is composed of a complex network of microtubules, microfilaments, and intermediate filaments. Both cell-types contain a rich network of cytoskeletal components that interact with other groups of closely related proteins, including membrane-associated proteins, sarcomeric skeleton proteins, and proteins of the intercalated disc; together, they contribute to morphological integrity, contractility, mechanical resistance, and transmit mechanical and chemical stimuli within and between cells of the heart.[10] Cytoskeletal dysfunction is intricately involved in phenotypic transformation of cardiac fibroblasts to secretory myofibroblasts, resulting in accumulation of extracellular matrix and fibrosis of the heart.[11] Additionally, interactions between the cytoskeleton and the mitochondria are critical for normal mitochondrial bioenergetics. Given the high oxidative phosphorylation demand of cardiac cells, uncoupling the cytoskeleton and mitochondrial intracellular systems contributes to progressive myocardial failure in the non-CKD population.[12]

Whether alterations of the myocardial cytoskeleton occur in CKD is unknown. In fact, the molecular mechanisms underlying the development of the failing heart in CKD are still largely undefined. Furthermore, there is a severe and concerning paucity of human translational studies investigating these alterations utilizing human cardiac tissues in the field of nephrology. This is likely due in part to the difficulty of obtaining human heart tissues for research purposes, particularly heart tissues from patients with CKD whom also rarely undergo myocardial biopsy. To that end, we collected a rare cohort of human heart tissues from patients with advanced CKD. Herein, we postulated that impairment of the cytoskeleton is involved in the pathogenesis of the failing heart in advanced CKD. We present critical data demonstrating that hearts from advanced patients with CKD exhibit severe cytoskeletal dysregulation, dysfunction of mitochondrial bioenergetics, loss of cell survival pathways and that these changes involve the focal adhesion pathway.

METHODS

Study Design

Upon reasonable request, all supporting data and materials that are not already within the article, supplementary files, or NCBI GEO repository are available from the corresponding author. We collected a rare cohort of explanted whole human hearts as part of The CAIN (Cardiac Aging IN CKD) Cohort. Donor hearts were collected through the National Disease Research Interchange (NDRI). Healthy and diseased organs included in this study were collected from donor participants that were not transplanted and donated for research purposes; requirements for informed consent were waived. A total of 109 hearts from deceased participants were recruited to-date and screened for eligibility for this a priori 3-arm cross-sectional cohort study design (Figure 1). Fifty donors met inclusion criteria: the primary experimental group of interest were advanced CKD donors on hemodialysis (n=19). Peritoneal dialysis patients were excluded from the present study. We also included explanted heart tissues from patients who had predominantly hypertensive heart disease (n=10) as diagnosed by geometric changes on gross pathologic examination as described below. The hypertensive group was included to facilitate differentiation between the effects of hypertension, which is nearly universally prevalent in CKD, and the direct effects of reduced kidney function (uremia). Donor hearts from healthy (n=21) subjects who had no significant comorbidities, were included as our controls. All the participants that were included were aged ≥18 years. Clinical and demographic data, body mass index (BMI) and tobacco smoking status (ever or never) were recorded from data provided by the NDRI. Assessment of co-morbidities included a history of any of the following: (1) CVD (previous cardiovascular event defined as non-fatal myocardial infarction, acute coronary syndrome requiring hospitalization, percutaneous coronary intervention, or coronary artery bypass graft or stroke) and (2) diabetes. The most recent laboratory

measures including biochemistry, hematology and cardiac function measures were collected from the donor prior to organ harvesting (denoted baseline). Kidney function was assessed using the CKD-epidemiology collaboration equation for estimated glomerular filtration rate (eGFR).

Processing of Human Heart Samples

Explanted whole human hearts were transported on ice. All organs underwent gross pathologic examination, including measurements of heart weight, left atrial and LV wall thickness. LV free wall was excised for analyses that included frozen sections for histologic analysis, and RNA and protein extraction as described in detail in Data S1.

Next Generation RNA Sequencing

Total RNA was extracted from 10 to 20 mg of tissue blocks using the NucleoSpin miRNA isolation kit (Cat. No. 740971.50; Macherey-Nagel, Bethlehem, PA). cDNA libraries were synthesized and fragmented using SMART-seq2[13] and Nextera XT (Illumina, San Diego, CA), respectively. Sequencing was performed with 2×38 bp paired-end configuration on NextSeq500 at Biopolymers Facility of Harvard University. The sequenced data were mapped to the hg38genome using STAR RNA-seq aligner. Uniquely mapped sequencing reads were assigned to hg38 refGene genes using featureCounts. Samples with low total reads ($<1\times10^6$) were removed from further analyses.

Data were analyzed for statistical significance and visualization with *R* software 3.5.1. Counts data were processed using edgeR following its default normalization method (trimmed mean of M values) and converted to count-per million counts. Lowly expressed genes were filtered using filterByExpr. Differential gene expression analysis was performed using the glmQLFTest between control and hemodialysis groups with

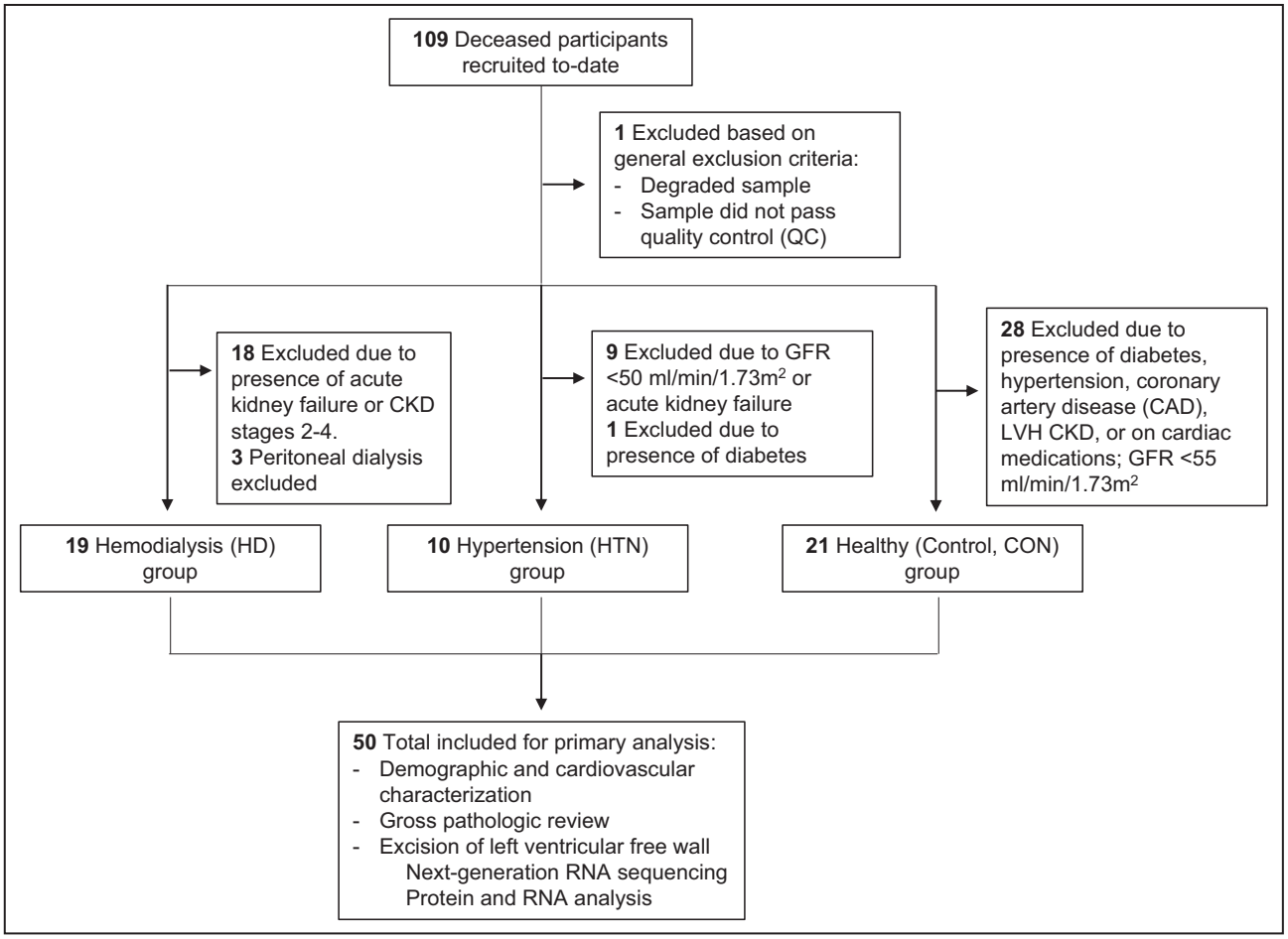


Figure 1. The Cardiac Aging in CKD (CAIN) Cohort and Study Protocol.
A total of 109 deceased donor participants have been recruited to-date in the CAIN cohort. Patients in the hypertension (HTN) group were included based on a documented history of diagnosed hypertension. All donor hearts underwent quality control (QC) screening for RNA purity. RNA quality was determined by nucleic acids absorbance ratio by spectrophotometry and a ratio ≈2.0 was considered as high purity. One patient was excluded due to poor RNA quality. Strict exclusion criteria for each group were used as illustrated. A total of 50 participants were included in the final analysis. CKD, chronic kidney disease; GFR, glomerular filtration rate; LVH, left ventricular hypertrophy.

the following formula: model.matrix(~0+gender+sequencing batch+group). *P* values were adjusted with the false discovery rate method to account for multiple comparisons. Pathway enrichment analysis was performed using limma::topGO, topKEGG. Mapping to Kyoto Encyclopedia of Genes and Genomes (KEGG) focal adhesion pathway (hsa04510) was done using pathview. RNA sequencing data were deposited in the NCBI's Gene Expression Omnibus database (GEO GSE160145). All RNA-seq data have been deposited into the NCBI Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160145).

Cell Culture

All in vitro experiments were repeated using primary human ventricular cardiac fibroblasts from 3 donors (Cat. No. 6310, 6320, 6330; ScienCell Research Laboratories, Carlsbad, CA). The n number corresponds to the number of total repeat experiments performed collectively by using all cell sources. Cardiac fibroblast cells were treated with either pooled uremic serum collected from end-stage renal disease prior to hemodialysis, TNF-α at 20 ng/mL (Cat. No. T0157; Sigma Aldrich, St Louis, MO) or mineral stress (DMEM supplemented with β-glycerophosphate [Cat. No. G9422; Sigma, St. Louis, MO] and/or calcium chloride [Cat. No. 223506; Sigma Aldrich, St Louis, MO]) in time-course experiments (0–48 hours), in vitro.

siRNA Transfection

Human ventricular cardiac fibroblasts were serum starved for 24 hours prior to transfection with either focal adhesion kinase (FAK) siRNA (Cat. No. sc-29310; Santa Cruz Biotechnology, Dallas, TX) or siRNA-B (Cat. No. sc-44230; Santa Cruz Biotechnology, Dallas, TX). Cells were treated daily with or without mineral stressors for 5 days. Cells were then harvested and processed for immunoblot analyses. Further details are provided in Data S1.

Antibodies

Focal Adhesion Protein Antibody Kit (Cat. No. 13430; Cell Signaling Technology, Danvers, MA) includes: FAK, paxillin, talin-1, tensin 2, vinculin; Cytoskeletal Marker Antibody Kit (Cat. No. 8614; Cell Signaling Technology, Danvers, MA) includes: keratin 17, pan-keratin, β-tubulin, vimentin, β-Actin; and GAPDH (Cat. No. 8884; Cell Signaling Technology, Danvers, MA).

Statistical Analysis

Baseline characteristics were summarized using means with SD, median with interquartile range or frequencies (percentage). Two group comparisons were performed using independent-samples *t* test and three group comparisons were performed using ANOVA or χ^2 tests as appropriate. Multiple group comparisons underwent post hoc Tukey-Kramer HSD. Statistical analysis was conducted using STATA (version 14) and JMP Pro (version 16.0.0). An alpha of <0.05 was regarded as statistically significant. Further details on statistical analysis for RNA-seq data is provided in Data S1.

Study Approval

The study was approved by the Partners Institutional Review Board (IRB) (protocol # 2017P001244/PHS) and adhered to the Declaration of Helsinki.

Detailed descriptions for cell culture, immunohistochemistry, SDS-PAGE, actin polymerization assays, immunoblotting, and real-time polymerase chain reaction (RT-PCR) protocols and data analyses are provided in Data S1.

RESULTS

Study Participants

Donor hearts from 19 patients undergoing hemodialysis, 10 patients with hypertension and 21 healthy controls were included in this study (Table 1). Demographics were well-matched: 11 (57.9%) of patients undergoing hemodialysis, 6 (60%) hypertension and 12 (57.1%) healthy control subjects were male (*P*=0.8). There was no statistical difference in age between the groups (mean age: 46.9±11.2 hemodialysis, 56.1±5.2 hypertension, 49.7±15.1 years control) (*P*=0.16). Patients with hypertension had a higher BMI (32.3±6.4 kg/m^2) compared with patients undergoing hemodialysis (27.1±4.6 kg/m^2) and healthy controls (26.5±5.7 kg/m^2) (*P*<0.05). 14 (73.7%) of the patients undergoing hemodialysis were diabetic which was expected given the high prevalence of diabetes-related kidney disease. No participants in the hypertension or control groups had diabetes.

Hemodialysis and hypertension groups had similar use of cardiac medications, whereas healthy controls were not on any cardiac medications. Patients with hypertension had preserved GFR (75.9[53.4, 97.1] mL/min per 1.73 m^2) compared with control group (91.9[76.6136.0] mL/min per 1.73 m^2) (*P*<0.0001) (Table 2). CKD group had markedly elevated phosphate levels (6.1[5.4,7.2] mmol/L) compared with the hypertension (3.0[2.5,3.6] mmol/L) and healthy control (2.6[2.1,3.3] mmol/L) groups (*P*<0.0001). Hemodialysis group had lower hemoglobin levels (11.1[9.7,12.2] g/dL=L) compared with the hypertension (13.7[12.2,15.7] g/dL) and control groups (12.2[11.1,13.8] g/dL) (*P*<0.05).

Cardiac Characterization

Cardiac biomarkers were similar between hemodialysis and hypertension groups (Table 2). Pathologic gross specimen examination of explanted hearts revealed that heart weight (normalized to body surface area [BSA], $P<0.0001$) and LV wall thickness (normalized to BSA, $P<0.001$) was higher in hemodialysis and patients with hypertension compared with healthy controls (Figure 2A). There was no statistical difference in left atrial size between the groups.

Histologic examination for tissue architecture of LV sections demonstrated disorganized muscle fibers and expanded extracellular matrix on hematoxylin and eosin (H&E) staining (Figure S1). Masson's trichome staining demonstrated severe and diffuse fibrosis (blue staining) in hearts from the hemodialysis group (Figure 2B). Hearts from the hemodialysis group had significantly greater myocardial fibrosis on quantification compared with hypertension ($P<0.05$) and control ($P<0.01$) hearts (Figure 2C).

Transcriptomic Profiling of Human Hearts

We took an a priori hypothesis driven methodologic approach with RNA sequencing (RNA-seq) to examine whether cytoskeletal associated genes are dysregulated in hearts from the hemodialysis group. Among the top 1000 differentially expressed genes between hemodialysis and healthy control hearts, 5 were significantly upregulated and 21 were significantly downregulated in hearts from the hemodialysis group compared with control ($P<0.05$) (Figure 3A). KEGG pathway enrichment analysis showed among the significant differentially expressed genes were genes involved in the cytoskeleton and associated components such as focal adhesion, adherens junctions, and tight junctions (Figure 3B). The focal adhesion pathway was one of the most significantly perturbed pathways in hearts from the hemodialysis group versus control (Figure 3C).

To further validate our findings from bulk RNA-seq data of human hearts, we performed RNA-seq on cardiac fibroblasts treated with and without mineral stressors, in vitro. Within the most significant 106 downregulated genes involved were the focal adhesion and actin cytoskeleton regulation pathways, consistent with our findings in human heart tissues described above (Figure S2).

Maladaptations of Cytoskeletal Components in the Focal Adhesion Pathway Is Involved In Myocardial Failure in CKD, Ex Vivo

Because the cytoskeletal network and the focal adhesion pathway involves numerous genes and

Table 1. Baseline Characteristics and Demographics of Study Population

Variables	Hemodialysis	Hypertension	Control	*P* value
Subjects, n	19	10	21	…
Male, n (%)	11 (57.9)	6 (60)	12 (57.1)	0.8
Ethnicity, n (%)				
White	12 (63.2)	7 (70)	16 (76.2)	…
Black	7 (36.8)	2 (20)	4 (19.1)	…
Asian	0 (0)	1 (10)	1 (4.8)	…
Age, years	46.9±11.2	56.1±5.2	49.7±15.1	0.16
BMI, kg/m^2	27.1±4.6	32.3±6.4	26.5±5.7	0.02*
Smoking, n (%)	7 (36.8)	3 (30)	8 (42.1)	0.9
Comorbidities				
Hypertension, n (%)	18 (94.7)	10 (100)	0 (0)	0.6†
Diabetes, n (%)	14 (73.7)	0 (0)	0 (0)	<0.0001*,†
CAD, n (%)	7 (36.8)	1 (10)	0 (0)	0.13†
Prior CVD, n (%)	9 (56.3)	2 (20)	0 (0)	0.11†
Medications, n (%)				
ACEi or ARB	1 (5.3)	1 (10)	0 (0)	0.09†
Statin	1 (5.3)	1 (10)	0 (0)	0.09†
Beta blocker	1 (5.3)	0 (0)	0 (0)	<0.01*,†
Aspirin	1 (5.3)	1 (10)	0 (0)	0.09†
Blood thinner	0 (0)	0 (0)	0 (0)	…

Data are presented as mean with 95% CI, median with interquartile range or frequencies (%). *P* value was obtained by ANOVA for 3-group comparison and $\chi 2$ (categorical variables). ACEi indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; and CVD, cardiovascular disease.

*P-value reaches statistical significance threshold of $P<0.05$.

†P-value by independent t-test for 2-group comparisons.

Table 2. Baseline Laboratory Values of Study Population

Variables	Hemodialysis	Hypertension	Control	*P* value
Biochemistry				
Sodium, mmol/L	139±7.5	141.6±4.0	139.5±5.0	0.6
Potassium, mmol/L	5.0±1.0	3.6±3.4	3.4±0.8	<0.0001*
Bicarbonate, mmol/L	23.7±6.9	23.7±6.9	21.2±4.7	0.3
Blood urea nitrogen, mmol/L	57.5±32.8	17.5±6.5	13.3±5.9	<0.0001*
Creatinine, mg/dL	…	0.95 [0.75, 1.45]	0.8 [0.7, 1.0]	<0.0001*
GFR, mL/min per 1.73 m^2	…	75.9 [53.4, 97.1]	91.9 [76.6, 136.0]	<0.0001*
Corrected calcium, mg/dL	8.9±1.3	8.8±1.2	8.6±0.9	0.64
Phosphate, mg/dL	6.1 [5.4, 7.2]	3.0 [2.5, 3.6]	2.6 [2.1, 3.3]	<0.0001*
Magnesium, mmol/L	2.2 [2.1, 2.6]	2.0 [1.8, 2.1]	1.9 [1.6, 2.0]	<0.01*
LDH, U/L	239 [153, 428]	254 [223, 339]	284 [192, 783]	0.5
Lipase, U/L	89.3±126.7	117.9±134.5	142.5±352.1	0.9
Liver function tests				
Albumin, g/L	3.3 [2.8, 4.2]	4.1 [3.3, 4.5]	3.6 [3.2, 4.1]	0.3
AST, U/L	39.6±39.9	69.4±75.0	52.4±43.9	0.4
ALT, U/L	35±35.9	30.6±16.9	38.2±25.3	0.8
ALP, U/L	113.5±43.3	90.8±41.2	78.6±27.2	<0.05*
Hematology				
Hemoglobin, g/dL	11.1 [9.7, 12.2]	13.7 [12.2, 15.7]	12.2 [11.1, 13.8]	<0.05*
Hematocrit, %	32.4 [30.6, 36.6]	40.1 [36.8, 45.0]	37.1 [34.1, 39.8]	<0.05*
Platelets, 10^5/L	217.4±100.7	202.8±108.5	203.4±84.5	0.9
Cardiac Biomarkers				
Troponin T, ng/L	0.1±0.15	0.01±0.0	…	0.7
Troponin I, ng/L	0.7±1.0	0.05±0.06	…	0.3
Creatine phosphokinase (CPK), U/L	145.3±63.5	167.2±89.0	…	0.3
CK-MB, IU/L	5.5±4.9	9.0±11.3	…	0.3

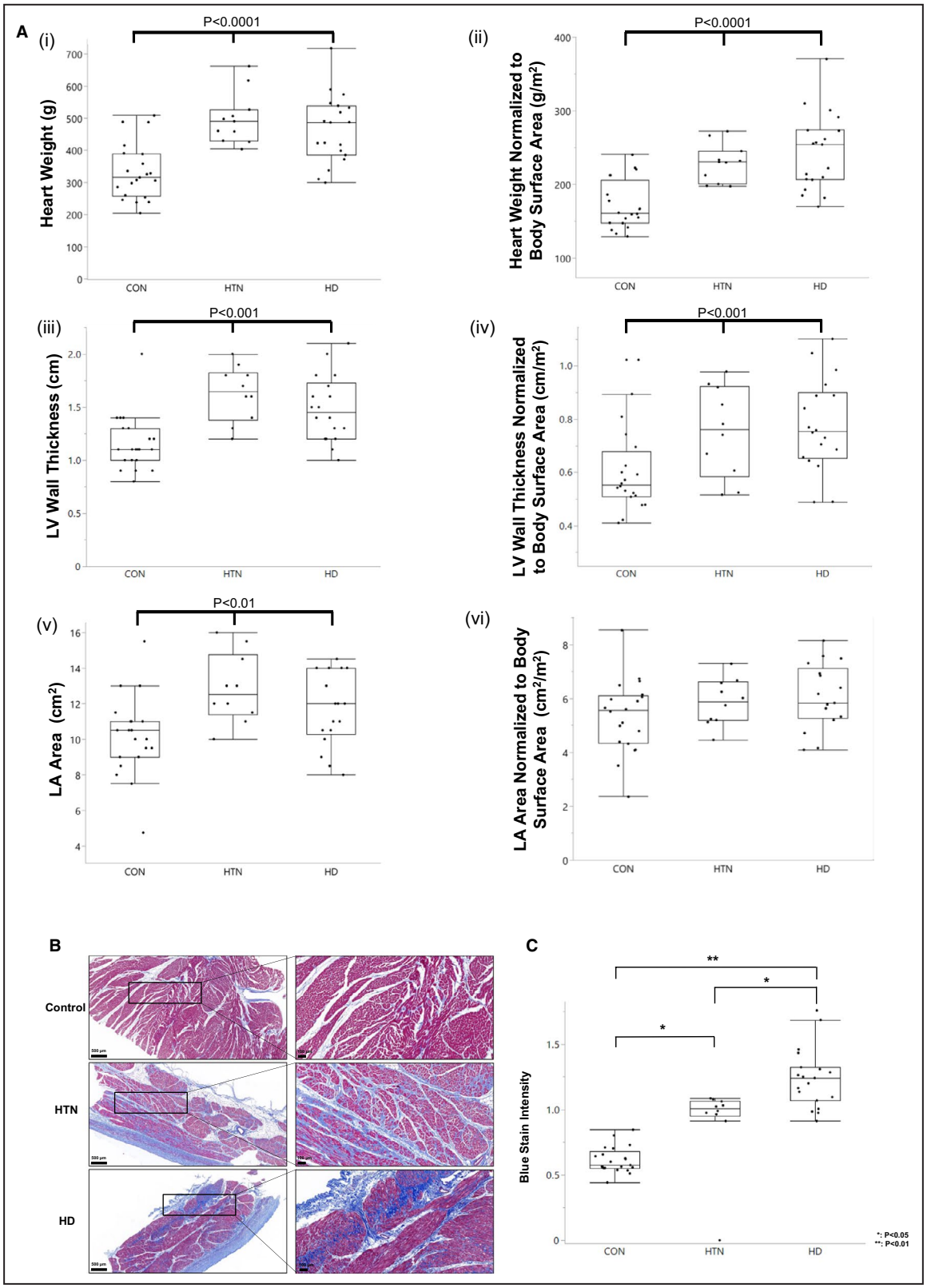
Data are presented as mean with 95% CI, median with interquartile range or frequencies (%). *P* value was obtained by ANOVA for 3-group comparison and $\chi 2$ (categorical variables). ACEi indicates angiotensin-converting enzyme inhibitor; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK-MB, creatine kinase MB; GFR, glomerular filtration rate; and LDH, lactate dehydrogenase.

* *P*-value reaches statistical significance threshold of $P<0.05$.

proteins, we narrowed our approach by using the RNA-seq results as a guide to screen for dysregulation of major and centric components of the cytoskeleton. This led to observations that in addition to dysregulation of cytoskeletal components, the components of focal adhesion and cell survival pathways were also involved in cytoskeletal impairment.

Figure 2. Cardiac characterization.

A, Gross pathologic characterization. All heart samples underwent gross pathologic examination. Hemodialysis (HD) heart weight in grams (i) and normalized to body surface area (BSA) (ii) was significantly greater in HD and hearts from the hypertension group (HTN). HD and HTN hearts had increased left ventricular wall thickness (iii) expressed in centimeters (cm) and (iv) normalized to BSA. While HD and HTN hearts appeared to have increased left atrial area (v) expressed in cm^2, this was not statistically significant following normalization to BSA (vi). Box-and-whisker plot denote extremes, interquartile range, and median. Displayed data points include outliers. *P* value was obtained by ANOVA for 3-group comparison. Post hoc analysis by Tukey-Kramer Honestly Significant Differences (HSD) test. **B**, Masson's trichrome stain of fibrotic changes in HD and HTN heart tissues: Left ventricular tissue sections were stained with Masson's trichome stain. Left panels: 500 µm scale; Right panels: 100 µm scale of enlarged areas shown in the yellow boxes in the top panel. Left ventricular tissues from HD participants had severe and widespread fibrosis compared with patients with hypertension, as illustrated by the alinine blue color which stains for collagen. Little to no significant fibrosis were found in left ventricular tissue from control patients. **C**, Quantification of Masson's trichrome stain of fibrosis in Control, HTN, and HD hearts: Quantification of fibrosis, as indicated by intensity of alinine blue staining, showed that left ventricular tissues from patients undergoing hemodialysis had significantly greater fibrosis compared with HTN and control groups. Box-and-whisker plot denote extremes, interquartile range, and median. Displayed data points include outliers. *P* value was obtained by ANOVA for 3-group comparison. Post hoc analysis by Tukey-Kramer HSD. Con indicates control; LA, left atrial; and LV, left ventricular.



We first validated findings from RNA-seq by screening protein expression of major cytoskeletal components involved in the focal adhesion pathway in LV tissues using immunoblotting (Table S1). Significantly altered major cytoskeletal genes found on RNA-seq showed detectable expression of β-tubulin, vimentin, vinculin, β-actin, and FAK. Other major cytoskeletal components, such as paxillin, talin-1, talin-2, tensin-2,

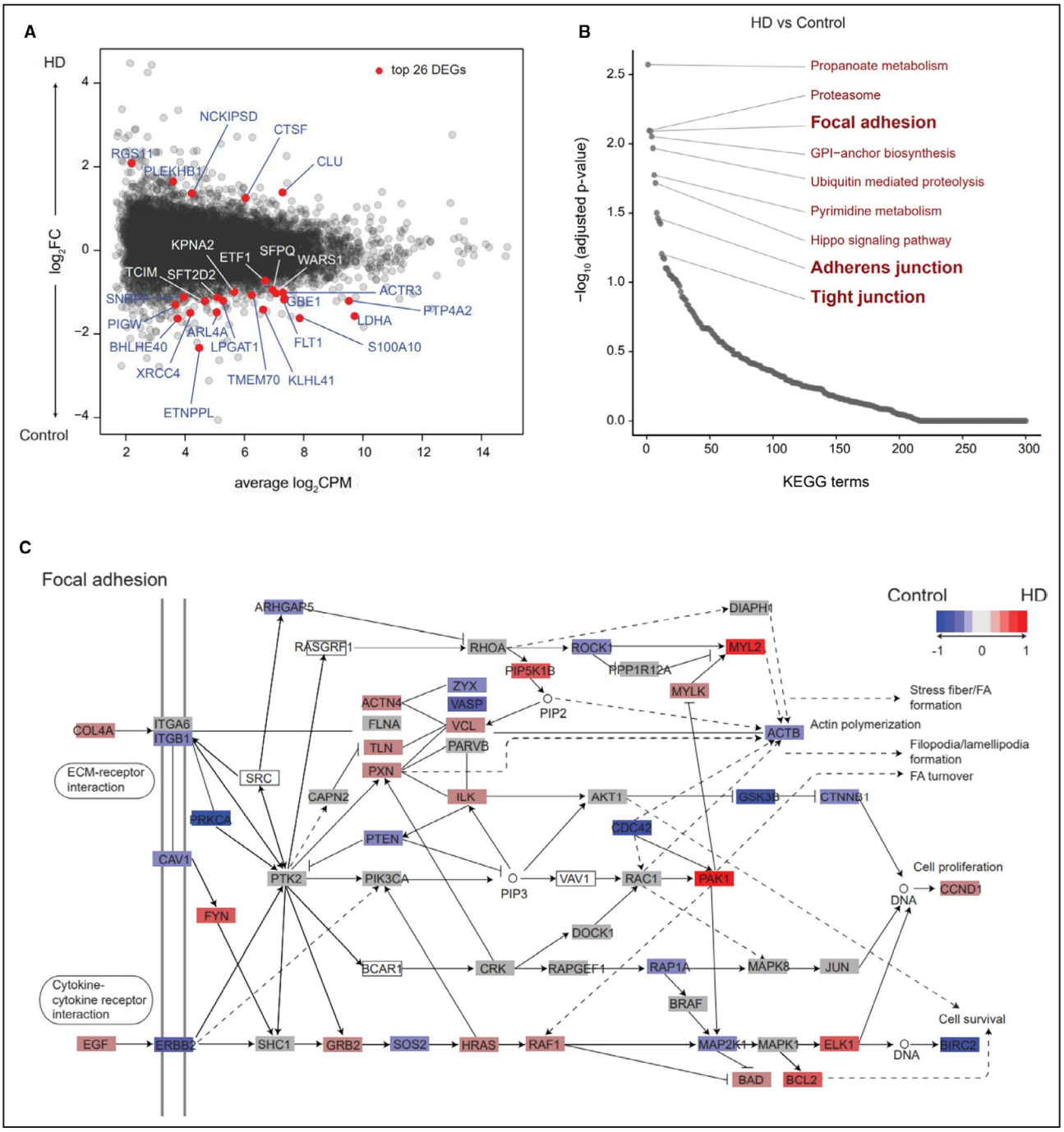


Figure 3. Transcriptomic profiles of heart tissues, ex vivo.
A, RNA-seq analysis of heart tissues (left ventricular [LV] free wall) comparing hemodialysis (HD) and control groups. Top 26 significantly differentially expressed genes are highlighted in red (false discovery rate [FDR] adjusted *P* values <0.05). The x-axis denotes mean log counts per million (log_2CPM) and the y-axis denotes log fold change (log_2FC). **B**, Transcriptomics pathway enrichment analysis. Approximately 300 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are aligned in the order of statistical significance. **C**, Gene expression ratios mapped to the focal adhesion pathway and pseudo-colored according to the indicated scale. Genes with blank rectangles had insufficient counts. Dotted lines denote indirect effect. CPM indicates counts per million; and FC, fold-change.

keratin 17, and pan-keratin, were not identified within the top 1000 differentially expressed genes and had undetectable protein expression.

Cross-sectional analysis of detectable cytoskeletal proteins was then performed using RT-PCR (Figure 4A) and immunoblotting (Figure 4B). β-actin, the major microfilament, exhibited reduced mRNA expression in LV tissues from patients with hypertension (P<0.05), but was more severely reduced in hearts from the hemodialysis group (P<0.01). However, β-actin protein

expression was significantly suppressed in hearts from the hemodialysis group ($P<0.01$) but not in hearts from the hypertension group. β-tubulin mRNA and protein expression were also reduced in hearts from the hypertension group ($P<0.05$, $P<0.05$), and further suppressed in the hemodialysis group ($P<0.05$, $P<0.01$).

Vinculin is a membrane-associated cytoskeletal anchor protein involved in focal adhesion that links the cell membrane and actin filaments. There were no significant vinculin mRNA alterations between the three groups (Figure 4A). However, vinculin protein expression was uniquely increased in hearts from the hemodialysis group ($P<0.05$) (Figure 4B). There was no statistical difference in vinculin protein expression between hypertensive and control hearts.

The Cytoskeletal Environment of Hearts From the Hemodialysis Group Is Not Optimal for Mitochondrial Function, Ex Vivo

Normal cytoskeletal integrity is required for efficient mitochondrial bioenergetics, in part due to its critical role in regulating mitochondrial dynamics.[12] Therefore, we examined mitochondrial function reflected by fusion and fission activity, regulated by mitofusins-1 (MFN1) and dynamin-like GTPase, optic atrophy 1 (OPA1). RNA-seq identified significant alterations in OPA1 and MFN1 expression (Table S2). Using RT-PCR, we found that both OPA1 and MFN1 were significantly suppressed in hemodialysis and hearts from the hypertension group ($P<0.01$) (Figure 5A).

Vimentin is a type III intermediate filament protein that can regulate focal adhesions[14] and mitochondrial membrane potential to promote oxidative phosphorylation,[15] and is an established marker of myocardial fibroblast cellularity.[16] Vimentin mRNA expression was significantly suppressed in hemodialysis ($P<0.05$) but not in hearts from the hypertension group (Figure 4A). Vimentin protein expression was severely suppressed in hearts from the hemodialysis group compared with hypertensive and control hearts ($P<0.001$) (Figure 4B). This suggests dysregulated mitochondrial membrane potential and reduced myocardial fibroblast cellularity despite widespread fibrosis (Figure 2B).

Hearts From the Hemodialysis Group Exhibit Loss of Cell Survival Pathways

RNA-seq identified dysregulated expression profiles of pro-apoptotic genes (caspase 3; CYCS, cytochrome c; BAX; and P53) and anti-apoptotic genes (BCL2 and BAG1) in LV tissues of hemodialysis and hearts from the hypertension group (Table S3). RT-PCR showed significant upregulation of caspase 3 ($P<0.05$), P53 ($P<0.05$), CYCS ($P<0.05$), and BAX ($P<0.05$) in hearts from the hemodialysis group (Figure 5B). Interestingly, hearts from the hypertension group had downregulation of caspase 3 ($P<0.05$), upregulation of P53 ($P<0.05$) and CYCS ($P<0.01$), but no significant changes in BAX. Both groups exhibited downregulation of BCL2 ($P<0.01$) and BAG1 ($P<0.05$) compared with healthy controls (Figure 5C).

Cytoskeletal Maladaptations Are Driven by Uremic Stress In Cardiac Fibroblasts, In Vitro

Cardiac fibroblasts contain well-developed contractile apparatus and play a critical role in extracellular matrix remodeling such as aberrant fibrosing responses to disease.[17] Previous studies have shown that uremic factors such as mineral stressors and pro-inflammatory cytokines are significant drivers of cardiovascular disease and are highly elevated in CKD and hemodialysis patients.[18–21] Therefore, we sought to investigate if these uremic factors can promote similar cytoskeletal adaptions seen in hearts from the hemodialysis group within cardiac fibroblasts.[4,22] We validated that uremic factors such as mineral stressors and proinflammatory cytokines can exhibit a fibrotic phenotype in cardiac fibroblasts that was similarly reported in previous studies (Figure S3).[23–27] We treated human cardiac fibroblasts with 10% pooled human uremic serum, in vitro. β-actin ($P<0.05$), β-tubulin ($P<0.05$), and vimentin ($P<0.05$) were significantly suppressed, but there was no significant difference in vinculin relative to controls (Figure 6A). Pro-inflammatory cytokine TNF-α significantly suppressed β-actin ($P<0.05$), β-tubulin ($P<0.05$), vimentin ($P<0.05$), and vinculin ($P<0.05$) in ventricular fibroblasts (Figure 6B).

Dysregulated Mineral Stressors Can Induce Cytoskeletal Dysfunction in Cardiac Fibroblasts, In Vitro

We conducted further mechanistic studies to assess whether abnormalities in mineral metabolism found in uremic environments can directly regulate cytoskeletal changes in cardiac fibroblasts, in vitro. We treated ventricular cardiac fibroblasts with high calcium, high phosphate, or both high phosphate and high calcium at concentrations known to stimulate calcification as demonstrated in prior studies (Figure 6C).[27] High phosphate suppressed β-actin ($P<0.05$) and β-tubulin ($P<0.01$), but not vimentin or vinculin. High calcium reduced β-actin ($P<0.05$) but not β-tubulin, vimentin, or vinculin expression. Combined high calcium and high phosphate stress downregulated vimentin ($P<0.01$), upregulated vinculin ($P<0.01$), but did not significantly modulate β-actin or β-tubulin.

Secretory myofibroblasts are characterized to have a strong contractile phenotype, which is useful for closure in wound healing but can also contribute to

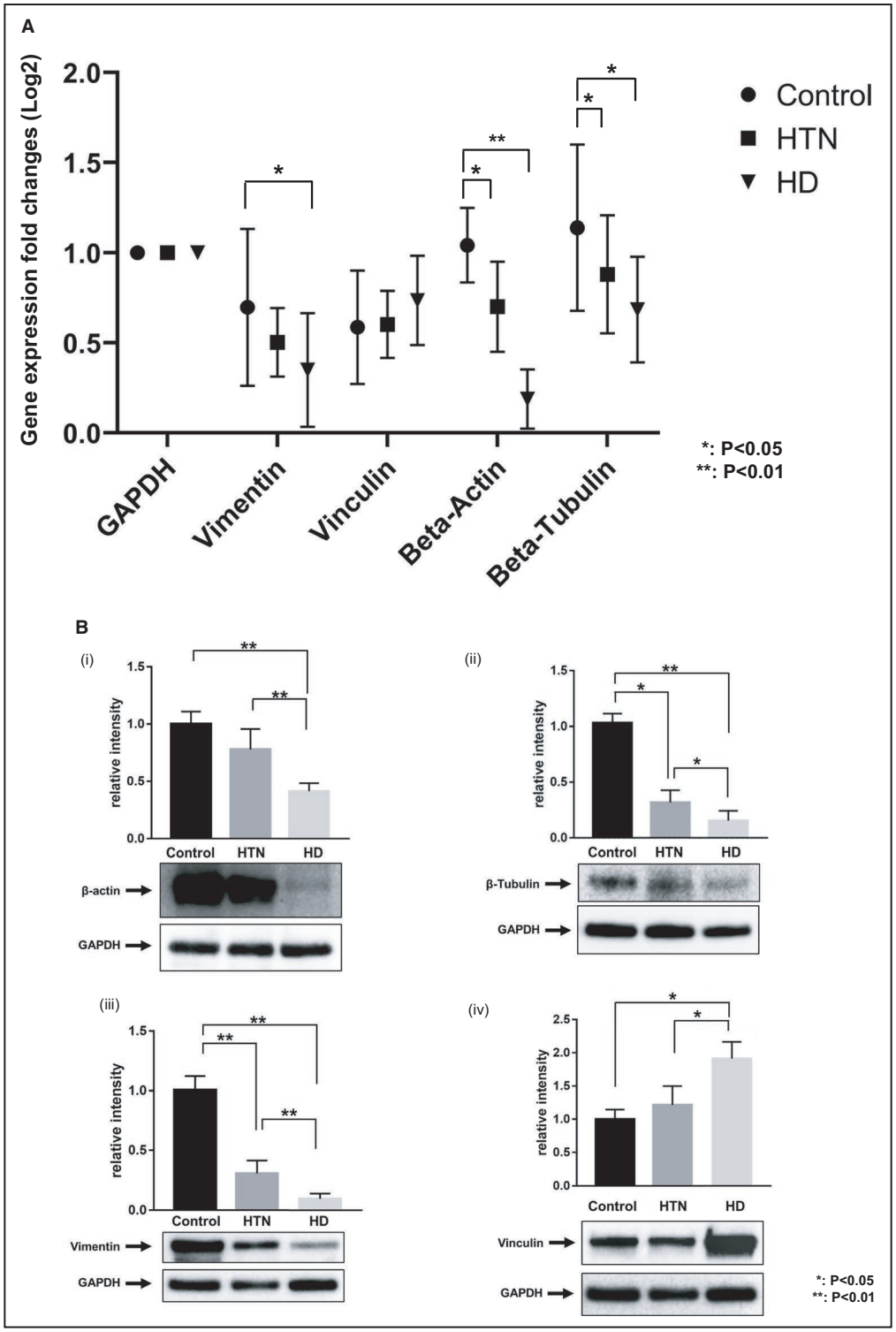


Figure 4. Dysregulation of the cytoskeleton is a cardinal feature of myocardial failure in patients undergoing hemodialysis, ex vivo.

A, Cytoskeletal genes are significantly altered in hemodialysis (HD) hearts. mRNA expression of major cytoskeletal proteins β-actin, β-tubulin, vimentin and vinculin as assessed by quantitative polymerase chain reaction (PCR). β-actin, β-tubulin, and vimentin was significantly dysregulated in hearts from patients undergoing hemodialysis compared with control. Hypertensive (HTN) hearts had dysregulation of β-actin and β-tubulin, but not vimentin compared with control. GAPDH was used as a standard. **B**, Cytoskeletal protein expressions in left ventricular heart tissues. (i) β-actin, (ii) β-tubulin, and (iii) vimentin expression was significantly suppresed in HD hearts. However, only β-tubulin and vimentin expression was reduced in HTN hearts; (iv) vinculin expression was significantly upregulated in HD hearts but not in HTN hearts. (*P<0.05; **P<0.01).

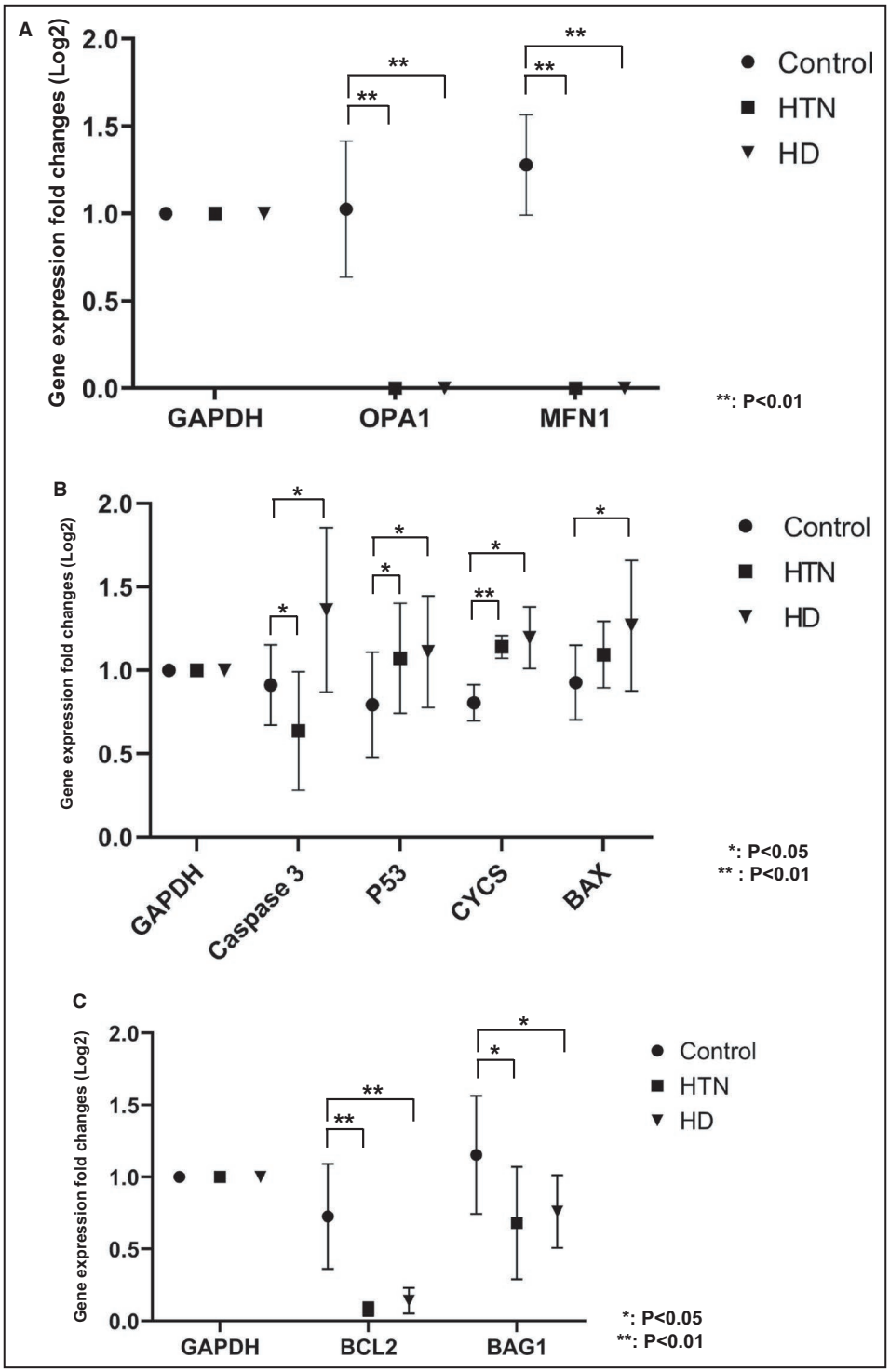


Figure 5. Disruption of mitochondrial bioenergetics and cell survival pathways occurs in the failing myocardium in patients undergoing hemodialysis, ex vivo.
quantitative polymerase chain reaction was used to assess mRNA levels of genes identified on RNA sequencing related to mitochondrial function, cell survival and apoptosis. **A**, Mitochondrial function is significantly altered in hearts from HD and patients with HTN: Mitochondrial function, reflected by expression of OPA1 and MFN1, critical genes that regulate mitochondrial division and fusion activities was severely suppressed in both hearts from HD and patients with HTN. **B**, Pro-apoptotic pathways are activated in hearts from HD and patients with HTN. In HD hearts, caspase 3, as well as P53, cytochrome C (CYCS) and BAX was upregulated. In HTN hearts, caspase 3 was downregulated but expression of P53, CYCS and BAX was increased. **C**, Anti-apoptotic genes are suppressed in HD and HTN hearts. Anti-apoptotic genes, BCL2 and BAG1 were down-regulated in both HD and HTN hearts. (*:*P*<0.05; **:*P*<0.01).

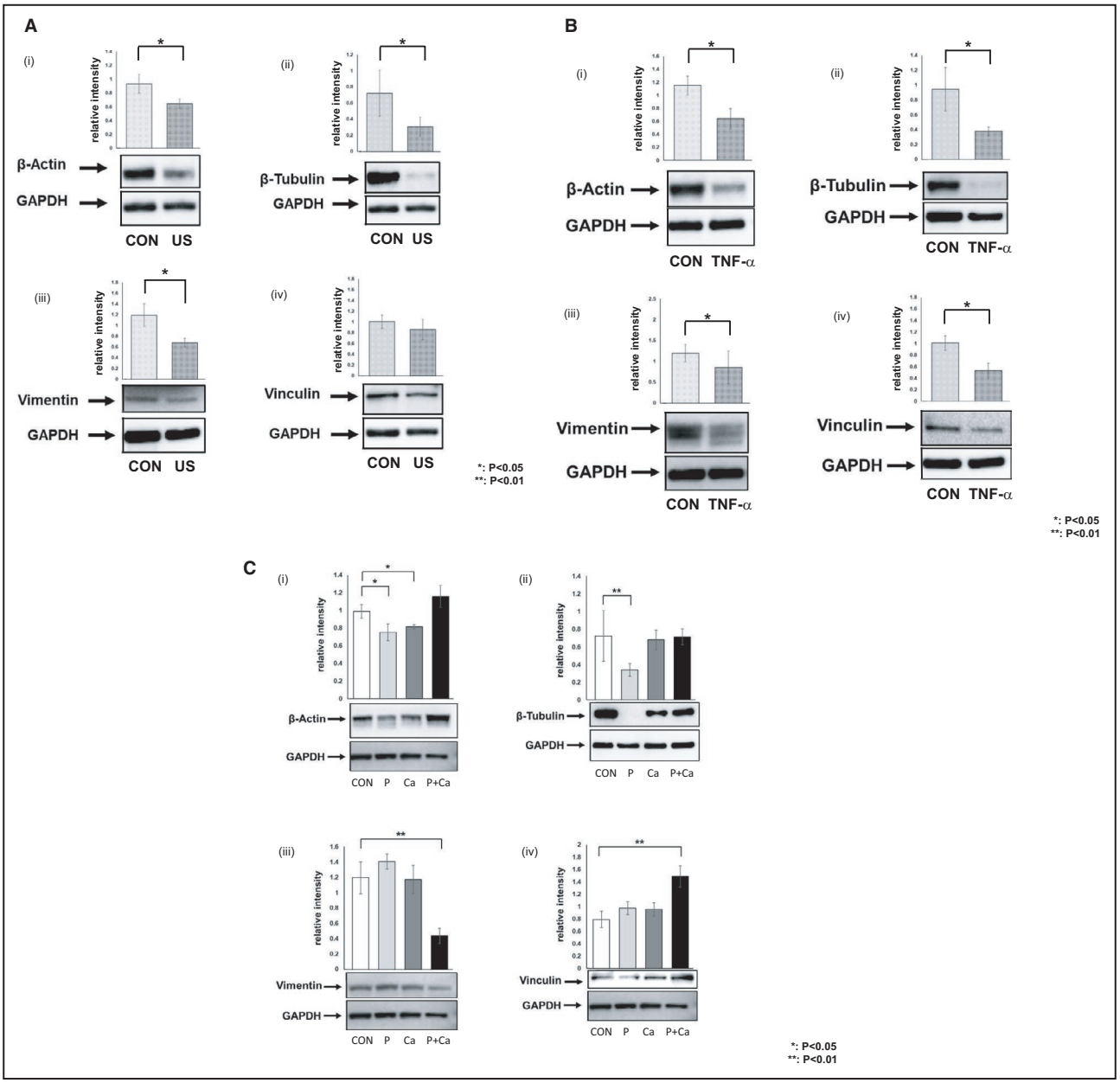


Figure 6. Cytoskeletal dysregulation can be driven by uremic stress, in vitro.
Primary human cardiac fibroblasts were cultured from the ventricle. Expression of target proteins was assesed by immunoblotting. **A**, Dysregulation of all major cytoskeletal compartments following treatment with pooled uremic serum (US). Cardiac fibroblasts were treated with 10% pooled human uremic serum or 10% human serum from healthy donors for 48 hours. (i) β-actin, (ii) β-tubulin, and (iii) vimentin was significantly suppresed in cardiac fibroblasts from the ventricle following uremic treatment. Vinculin (iv) expression did not significantly change with uremic treatment. **B**, Dysregulation of all major cytoskeletal compartments following treatment with TNF-α. Cardiac fibroblasts were treated with TNF-α at 20 ng/mL for 48 hours. (i) β-actin, (ii) β-tubulin, (iii) vimentin, and (iv) vinculin were all suppressed in cardiac fibroblasts from the ventricle following treatment. **C**, Cytoskeletal dysregulation can be driven by uremic mineral stress. β-actin (i) was significantly suppressed with either phosphate or calcium treatments, but not with combined phosphate and calcium treatment. β-tubulin (ii) was significantly suppressed only with phosphate treatment. Combined phosphate and calcium treatment significantly suppressed vimentin (iii) but upregulated vinculin (iv). Primary human cardiac fibroblasts treated for 48 hours with either high phosphate (P: 5 mmol/L β-glycerolphosphate), high calcium (Ca: 5 mmol/L calcium chloride), or both high calcium and high phosphate (P+Ca, 5 mmol/L+5 mmol/L). Cytoskeletal components were differentially regulated by high calcium and high phosphate. GAPDH was used as a standard. N=6 for all treatments. (*P<0.05, **P<0.01). Con indicates control.

progressive fibrosis.[28–30] We assessed the impact of mineral stressors and proinflammatory cytokines on polymerization of actin, which is essential to forming actin filaments for the contractile activity of myofibroblasts. TNF-α significantly promoted actin polymerization (P<0.05), but there was no significant difference in

actin polymerization with mineral stressors relative to control (Figure S4).

Myocardial Cytoskeletal Dysregulation Involves the Focal Adhesion Pathway in Patients Undergoing Hemodialysis

We next analyzed the expression of FAK, an upstream cytosolic tyrosine kinase that is a central regulator of cytoskeletal dynamics and stabilization. RT-PCR showed significant upregulation of FAK in hearts from the hemodialysis group ($P<0.05$) and significant downregulation in hypertensive ($P<0.05$) hearts relative to control (Figure 7A). FAK protein expression was also significantly upregulated in LV tissues from patients undergoing hemodialysis ($P<0.05$) but downregulated patients with hypertension ($P<0.05$) relative to control (Figure 7B). Mechanistic studies showed that suppression of FAK could occur in ventricular cardiac fibroblasts following treatment with pooled uremic serum or TNF-α ($P<0.05$) (Figure S5). Furthermore, FAK expression was significantly reduced by high phosphate or combined high phosphate and high calcium ($P<0.05$), but not high calcium alone, in ventricular fibroblasts (Figure S6).

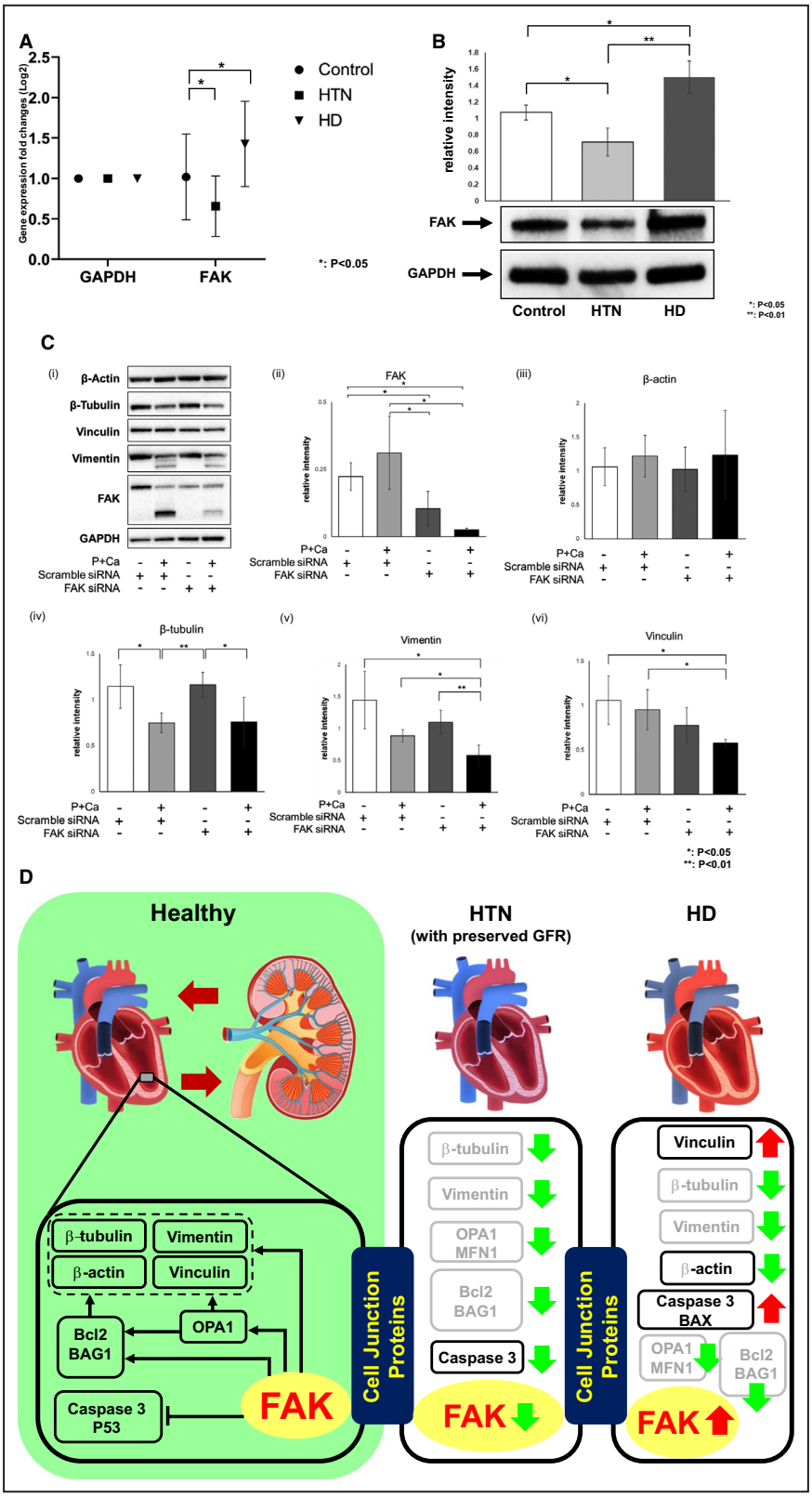
To determine if FAK is centric to cytoskeletal impairment in CKD, we performed FAK knockdown using interference RNA (siRNA). Optimization studies demonstrated that FAK silencing was the most effective at 5 days post-transfection (Figure S7). We then transfected ventricular fibroblasts with either FAK siRNA or scrambled siRNA. Cells were also concomitantly treated with mineral stressors (high phosphate and high calcium). Interestingly, mineral stressors promoted FAK cleavage with detection of 92-94 kDA N-terminal fragment (Figure 7C). Vimentin was reduced with mineral stressor treatment, and was further suppressed in the combined FAK siRNA and mineral stress group ($P<0.05$). Vinculin expression was significantly suppressed in the combined FAK siRNA and mineral stress treatment group ($P<0.05$), but unchanged in other groups. β-actin expression did not significantly change across the groups. β-tubulin expression was downregulated with mineral stressors ($P<0.05$), but unchanged with FAK siRNA transfection.

DISCUSSION

To the best of our knowledge, the present study is the first cross-sectional controlled study to investigate myocardial adaptations in human hearts from donors with advanced CKD. The multi-arm cross-sectional approach allows us to compare phenotypic and molecular changes of the myocardium in dialysis-dependent patients, compared with patients with hypertensive heart disease, and a healthy donor group. We present evidence for severe dysregulation of all major components of the cytoskeleton, including microtubular, microfilament and intermediate filament systems in the failing heart of advanced patients with CKD on hemodialysis. Furthermore, using well-validated models, we have demonstrated similar molecular changes in cardiac fibroblasts treated with uremic serum, and found uremic factors such as mineral stressors and proinflammatory cytokines that can drive these changes.

The pattern of cytoskeletal adaptations in hearts from the hemodialysis group differed strikingly from hearts from the hypertension group, exhibiting generally more severe cytoskeletal adaptations. This reflects the involvement of different pathogenic mechanisms (Figure 7D). The cytoskeleton plays a central pleiotropic role in regulating the intrinsic function of both cardiac myocytes and fibroblasts, and is a primary site of cellular damage in heart failure within the general population.[31,32] Cytoskeletal dysregulation has been implicated in the development of cardiac myofibroblast differentiation and the fibrotic response,[11] as well as in cardiac hypertrophy, myocardial decompensation, and contractile dysfunction in heart failure.[33] Cytoskeletal adaptations are highly specific to the type of pathologic stress, species, and the specific chamber of the heart affected. These findings highlight the caution needed when extrapolating cardiomyopathy findings from experimental models and human studies involving the general population and patients with CKD.[34]

The absence of β-actin is incompatible with life and results in cytoskeletal collapse and apoptotic cell death.[35] β-actin dysfunction is involved in the pathogenesis of dilated cardiomyopathy,[36] but its involvement in other types of cardiomyopathy is currently unknown. We found severe suppression of β-actin in hearts from the hemodialysis group, suggestive of significant loss of cytoskeletal integrity and maladaptation of the myocardium in patients undergoing hemodialysis. We postulate that hemodynamic alterations resulting from extracorporeal hemodialysis therapy may exert significant myocardial stress beyond uremic stress alone. Indeed, chronic dialysis can cause LV segmental myocardial stunning and hibernation, thereby, contributing to the process of chronic heart failure.[7] β-actin is also an important component of Z-discs and costameres of cardiac myocytes, and governs tangential forces and cytoskeletal recruitment of signaling elements for hypertrophic cellular response.[37] We show that uremic factors such as proinflammatory cytokines and mineral stressors differentially affect actin polymerization, suggesting individual uremic factors act upon different pathways that contribute to cardiac remodeling. Our results also show that β-actin cannot be used as a loading control in studies examining myocardial tissues



in CKD due to its altered expression (Figure S8). We suggest GAPDH could be used as a loading control, based on its consistency in our results, for studies examining uremic cardiomyopathy using human tissues.

Normal contractile properties of cardiac muscle involves constantly cyclic polymerization and depolymerization of tubulin.[38,39] Tubulin can co-localize with mitochondria to facilitate efficient intracellular energy

Figure 7. Regulation of the cardiac cytoskeleton by focal adhesion kinase (FAK).
A, Expression of FAK mRNA is significantly upregulated in left ventricular tissues from patients undergoing hemodialysis, ex vivo. Analysis of FAK mRNA expression by quantitative polymerase chain reaction revealed that FAK was significantly upregulated in hearts from patients undergoing hemodialysis. However, FAK mRNA expression was suppressed in hearts from patients with HTN. **B**, Expression of FAK protein is upregulated in left ventricular tissues from patients undergoing hemodialysis, ex vivo. Protein expression of FAK was significantly upregulated in hearts from patients undergoing hemodialysis as assessed by immunoblotting. FAK protein expression was significantly suppressed in HTN hearts. **C**, FAK silencing mediates cytoskeletal dysfunction in the presence of mineral stressors. (i) Primary human ventricular cardiac fibroblasts were transfected once with either scrambled or FAK siRNA. Cells were then treated daily with or without mineral stressors (ie P+Ca: 2 mmol/L β-glycerophosphate with 1 unit/mL alkaline phosphatase, and 2.7 mmol/L calcium chloride) for 5 days. After treatment, target cytoskeletal proteins were assessed by immunoblotting. (ii) Treatment with FAK siRNA significantly reduced full-length FAK expression at 5 days, and exposure to high calcium and high phosphate further reduced FAK expression. High calcium and high phosphate treatment resulted in detection of 80 kDa N-terminal FAK fragment, which was decreased in combined FAK siRNA and high mineral stressors treatment. There was no statistical difference in FAK expression between the control and scrambled siRNA groups. (iii) There was no significant difference in β-actin expression across the groups. (iv) β-Tubulin was significantly decreased in cardiac fibroblasts exposed to mineral stressors compared with controls, but was not significantly different in the FAK silencing groups. (v) Vimentin was decreased significantly when cardiac fibroblasts were exposed to high calcium and high phosphate, and further decreased in the FAK silenced group exposed to high calcium and high phosphate. (vi) Expression of vinculin followed a similar pattern to vimentin. **D**, Schematic diagram of ultrastructural adaptations of the failing myocardium in CKD. In the healthy individuals, interactions between the heart and kidney is critical for normal physiological function. Patients with advanced CKD on hemodialysis (HD) exhibited significant cytoskeletal maladaptations that were generally more severe than hypertensive control hearts. HD hearts exhibited a unique pattern of severely reduced β-actin, β-tubulin and upregulation of vinculin expression. Cytoskeletal dysregulation in HD and HTN hearts is associated with mitochondrial dysfunction (reduced OPA1 and MFN1 genes responsible for mitochondrial fusion and division) and loss of cell survival pathways. FAK is a cytosolic tyrosine kinase that plays a central role in regulating cytoskeletal integrity. FAK expression is upregulated in HD hearts. This may account for increased expression of anchor protein vinculin observed as a protective response. GFR indicates glomerular filtration rate.

transfer via the phosphocreatine pathway.[12] Our results showed β-tubulin was significantly reduced in hearts from the hemodialysis group, suggesting that β-tubulin deficiency may play a role in reduced contractile function in uremic states, and may impair efficient intracellular energy transfer.

Vinculin is a membrane anchor protein that also helps regulate contractile function and facilitates the connection between the actin cytoskeleton and the sarcolemma.[40] We found significantly increased vinculin expression in hearts from the hemodialysis group. Overexpression of cardiac vinculin results in reinforced cortical cytoskeleton and enhanced myofilament organization, leading to improved contractility and hemodynamic stress tolerance in healthy and myosin-deficient fly hearts.[40] It is possible that upregulation of vinculin in hearts from the hemodialysis group may be the myocardium's physiological stress response to hemodynamic changes related to hemodialysis.

Vimentin is a type III intermediate filament protein that is expressed in cardiac fibroblasts and adult cardiac myocytes in response to growth stimuli.[41] It is integral to organelle positioning,[42] epithelial-mesenchymal transition, cell motility,[43] mitochondrial physiology,[15] and an indicator of fibroblast cellularity of the interstitium.[44] Fibroblast hyperactivity results in excess collagen deposition and the development of cardiac fibrosis, a clinical feature observed in the myocardium of patients with CKD.[45] Interestingly, we found that hearts from the hemodialysis group had severely reduced vimentin, which may suggest a fibrosing response or hyperfibrotic activity in the absence of increased interstitial cellularity, and an environment conducive to dysfunction of mitochondrial membrane potential and localization.

High mitochondrial content and cytoskeletal regulation of cardiac bioenergetics are essential to fulfill the energy demands of the heart.[46] We postulated that cytoskeletal malfunction may be associated with mitochondrial dysfunction in the failing myocardium in CKD. We found suppression of MFN1 and OPA1 genes in hearts from the hemodialysis group. In the general population, mitochondrial impairment is key in the progression of heart failure.[47] Our data suggest for the first time that cytoskeletal-mitochondrial uncoupling may occur in CKD-associated cardiomyopathy and may be a critical event in its progression.

We also found differential activation of pro-apoptotic pathways in hemodialysis and hearts from the hypertension group. Increased pro-apoptotic gene expression in hearts from the hemodialysis group suggest a loss of cardiomyocyte and cardiac fibroblast cell density, as well as transformation of fibroblasts into a secretory phenotype, and enhanced myofibroblast hypertrophic and inflammatory signaling.[48,49] Additionally, prior studies have suggested that increased apoptotic gene expression is linked with impaired mitochondrial function and dynamics.[47] Our data suggest that the imbalance in expression favoring increased pro-apoptotic genes could be another compounding factor along with poor mitochondrial dynamics that would contribute to CKD-associated cardiomyopathy.

FAK is a non-receptor tyrosine kinase and a central part of focal adhesion protein complexes, which are mediators between the actin cytoskeleton and integrins, and is involved in multiple processes including adhesion, migration, survival, and myofibroblast differentiation.[50,51] RNA-seq showed significant downregulation

of focal adhesion-related genes in hearts from the hemodialysis group. FAK protein was upregulated in hearts from the hemodialysis group but downregulated in hearts from the hypertension group relative to control. This is in contrast to findings of increased FAK in chronically volume overloaded human hearts, and in pressure-overloaded and hypertensive rat hearts.[52,53] This is not surprising given that our study demonstrates that major components of the cardiac fibroblast cytoskeleton are differentially regulated by uremic, pro-inflammatory, and mineral stressors uniquely found in CKD. These data highlight the complexity of myocardial remodeling that occurs under uremic stress.

The data presented should be interpreted against the limitations of our study: (1) While our study has utilized a rare collection of human hearts in one of the largest translational studies of its kind to-date, the power of the study is limited by the small sample size; (2) due to the inherent limitations of the single-cell in vitro models utilized in this study, further studies are needed to validate our findings utilizing CKD animal models; (3) we acknowledge the limitations of our approach to RNA-seq analysis with the use of cultured human cardiac cells to help with validation of ex vivo RNA-seq data. In this study, we provide the first set of gene profile data of hearts from the hemodialysis group, and we believe that this discovery work will help inform future studies. The strength of our study includes a multi-arm-controlled approach allowing us to identify cardiac changes unique to advanced CKD versus hypertension. As hypertension is almost universally present in patients with CKD, the study design allows us to differentiate changes resulting from hypertension from those attributable to uremia. This study's findings will be critical to help advance our understanding of cardiac disease in CKD where no direct therapies with proven mortality benefits are currently available.

In conclusion, the present study provides critical evidence that: (1) myocardial failure in advanced patients with CKD on hemodialysis is a state of severe dysregulation of all major components of the cytoskeleton and dysregulation of the focal adhesion pathway; (2) LV tissues from patients undergoing hemodialysis exhibit a specific and unique pattern of severely reduced β-actin, β-tubulin, vimentin, and upregulation of vinculin. Additionally, β-actin is not an appropriate loading control for studies examining LV tissues from uremic patients; (3) cytoskeletal failure in hemodialysis is associated with dysfunction of mitochondrial bioenergetics and cytoskeletal-mitochondrial uncoupling, as well as loss of cell survival pathways; and lastly, (4) cytoskeletal changes can be driven by uremic, pro-inflammatory and mineral stressors, and occur through the focal adhesion pathway in patients undergoing hemodialysis. The present study presents evidence of complex molecular changes underlying myocardial failure in patients undergoing hemodialysis and reveals potential new therapeutic targets warranting further studies.

ARTICLE INFORMATION

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Author contributions: K.L., T.L., and R.T. designed the study and oversaw all aspects. T.L. and L.H. conducted RNA-seq and computational analysis. A.H., G.N., T.L., L.H., and F.G. conducted experiments. K.L., L.H., T.F.H., and T.L. performed data analysis. All other authors provided intellectual guidance, reviewed data analysis, and helped with the construction of the manuscript.

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Supplemental Material

Data S1
Tables S1–S3
Figures S1–S8

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