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Complement activation at a particular location is determined by the balance of activating and inhibitory proteins. Factor H is a key regulator of the alternative pathway of complement, and genetic or acquired impairments in Factor H are associated with glomerular injury. The human Factor H–related proteins (FHRs) comprise a family of five proteins that are structurally related to Factor H. Variations in the genes or expression levels of the FHRs are also associated with glomerular disease, although the mechanisms of glomerular protection/injury are incompletely understood. To explore the role of the FHRs on complement regulation/dysregulation in the kidney, we expressed and purified recombinant murine FHRs (FHRs A, B, C and E). These four distinct FHRs contain binding regions with high amino acid sequence homology to binding regions within Factor H, but we observed different interactions of the FHRs with Factor H binding ligands, including heparin and C3d. There was differential binding of the FHRs to the resident kidney cell types (mesangial, glomerular endothelial, podocytes, and tubular epithelial). All four FHRs caused complement dysregulation on kidney cell surfaces *in vitro*, although the magnitude of the effect differed among the FHRs and also varied among the different kidney cells. However, only FHR E caused glomerular complement dysregulation when injected *in vivo* but did not exacerbate injury when injected into mice with ischemic acute kidney injury, an alternative pathway-mediated model. Thus, our experiments demonstrate that the FHRs have unique, and likely context-dependent, effects on the different cell types within the kidney.

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Translational Statement

Genetic variants of the factor H–related proteins (FHRs) are associated with increased risk of some kidney diseases, yet the specific functional roles of these proteins are incompletely understood. We examined the 4 murine FHRs *in vitro* and *in vivo*. Despite structural similarities among the FHRs, they exhibited distinct effects on different glomerular cell types. Future experiments can correlate the functions of the murine FHRs with those of the human FHRs. This approach should provide insight into how FHRs modulate kidney disease severity, and it may lead to new strategies for specifically blocking the molecular causes of glomerular inflammation.

The complement cascade comprises multiple different activating enzymes as well as regulatory proteins. Activation of the system at a particular anatomic site is determined by the balance of these opposing factors, and it is ordinarily a highly regulated process. Among the multiple regulatory proteins, factor H (FH) is the key inhibitor of activation through the alternative pathway and amplification loop.[1,2] FH appears to be particularly important for controlling complement activation within the glomerulus. Even though other complement inhibitory proteins are expressed on all cells throughout the body, mutations in FH are strong risk factors for complement-mediated glomerular diseases.[3] Indeed, targeted deletion of the *Cfh* gene in mice is sufficient to cause alternative pathway activation in the glomeruli, highlighting the critical role of FH at this location.[4] Furthermore, the microenvironment within the glomerulus appears to be uniquely dependent on FH, as systemic impairments in FH function often manifest with isolated injury of the kidney.[5,6]

FH is an approximately 155 kDa glycoprotein composed of 20 repeating units called short consensus repeats (SCRs;

Supplementary Figure S1A).[7] FH is primarily produced in the liver, and it is a soluble protein that circulates at high concentrations in plasma (116–562 μg/ml).[8] The complement inhibitory function of FH is contained within SCRs 1–4 at the amino terminus of the protein. The regions of the protein containing SCRs 6–8 and SCRs 19 and 20, on the other hand, enable FH to bind certain ligands, including glycosaminoglycans, sialic acid, and C3b fragments.[1] The interaction of FH with these molecules can tether it to the surface of cells and membranes, thereby providing alternative pathway regulation to those sites.[9–11] Conversely, mutations in FH that impair binding to these ligands can render particular surfaces susceptible to alternative pathway activation.[12,13] One possible explanation for the dependence of the kidney on FH is that acellular surfaces, such as the glomerular basement membrane, cannot express the other regulatory proteins (which are primarily integral membrane proteins). Consequently, these surfaces may be dependent on FH from plasma to control alternative pathway activation.

In humans, there are 5 FHRs (FHRs 1–5) whose genes are located adjacent to *CFH* on chromosome 1 (Supplementary Figure S1B). There are 4 murine paralogs, referred to as FHRs A, B, C, and E. Another possible murine FHR, FHR D, is believed to be an unprocessed pseudogene. The *CFHR* genes may have developed through partial reduplication of the *CFH* gene,[14] and they all contain regions that are structurally very similar to FH. For example, all of the human and murine FHRs contain regions with homologous sequence identity to the SCR 6–8 and SCR 19–20 binding regions of FH (Supplementary Figure S1C).[15]

Although some studies have shown complement regulatory function for the FHRs,[16–18] the proteins do not contain regions homologous to SCRs 1–4 of FH.[19] Furthermore, congenital deficiency of FHRs, especially FHR-1 and FHR-3, is relatively common in healthy individuals.[20,21] This indicates that, unlike FH, the FHRs are not essential for complement regulation. Nevertheless, genetic variants in the *CFHR* genes are associated with the same kidney diseases associated with FH defects, including C3 glomerulopathy, atypical hemolytic uremic syndrome, and IgA nephropathy.[6,22–25] Although there are structural similarities between the murine and human FHRs (Supplementary Figure S1C), the murine *FHR* genes likely developed after the separation of rodent and primate lineages.[26] The similarities and differences between the various murine and human FHRs have previously been reviewed, and it has been noted that it is difficult to determine whether mouse FHRs are direct homologues of human FHRs based on their sequence homology.[27] Thus, it may be more informative to compare the murine and human FHRs based on their function, rather than comparison of their gene loci.

Although the ability of the FHRs to modulate the alternative pathway is controversial, one possibility is that they block FH from binding some surfaces. The ability of the human FHRs to antagonize FH on surfaces has been demonstrated using several *in vitro* assays.[18,28–30] Several years ago, we also reported that murine FHRs A and B increased the lysis of sheep erythrocytes and increased complement activation on the surface of tubular epithelial cells.[31] In another study, murine FHR B was shown to bind C3b and to inhibit FH from binding this ligand.[32] Experiments using recombinant FHR B also showed that it caused complement dysregulation on several surfaces *in vitro*, including artificial cell matrix.[32] These studies suggest that some murine FHRs can antagonize the binding of FH to specific surfaces, thereby causing complement dysregulation. Consistent with this putative role of the FHRs as complement "dysregulators," deletion of FHR genes is associated with a decreased risk of some diseases, including IgA nephropathy.[33,34] Supplementary Table S1 summarizes observations regarding the functions of the human FHRs.

There are still significant gaps in our understanding of the function of the individual FHRs. First, even though the FHRs contain regions that are homologous to SCRs 6–8 and SCRs 19 and 20 of FH, the various human FHRs appear to have different disease associations. This suggests that the interactions of these proteins with different cellular and tissue surfaces are not interchangeable. Also, most of the functional examinations of the FHRs have been performed *in vitro*, and much less is known about how the proteins behave *in vivo*. To further examine the interactions between the FHRs and surfaces within the kidney, we expressed and purified recombinant forms of murine FHRs A, B, C, and E using commercially synthesized DNA constructs. We then examined side by side the interaction of these proteins with the various resident cell types of the kidney, and their ability to cause complement dysregulation on kidney surfaces *in vitro* and *in vivo*.

METHODS

Recombinant protein production

The DNA sequences for murine FH and the FHRs were codon optimized and synthesized commercially as previously described.[31] The sequences were inserted into pcDNA3.2 plasmids, along with IgG leader sequence and a histidine tag at the amino terminus. The plasmids were transiently transfected into Expi293 cells (ThermoFisher) in our laboratory. We then purified the recombinant protein by running the supernatant over a HisTrap HP column (GE Healthcare). Recombinant murine FH[35] and rH19–20[36] were produced and purified as previously described. Recombinant human and murine C3d were produced in *Escherichia coli* using the pGEX expression system (GE Healthcare) as previously described.[37] Lipopolysaccharide levels in the recombinant proteins were measured using a Pierce Chromogenic Endotoxin Quant Kit and were between 50 and 100 EU/mg of each protein. Supplementary Table S2 shows the approximate molar ratio of the FHRs added to each experiment compared with FH present in the serum, and this table also shows the estimated amount of endotoxin added.

In vitro complement activation assays

In vitro assays were performed to compare the ability of the FHRs to bind to different glomerular surfaces, and their ability to cause complement dysregulation on the target surfaces. Assays included a guinea pig erythrocyte lysis assay (GP_{Er}, a standard complement

activation assay), murine cell lines (endothelial cells, podocytes, mesangial cells, and tubular epithelial cells), and the extracellular matrix (ECM). These assays are described in detail in the Supplementary Methods.

In vivo kidney targeting experiments

To determine whether FH, FHR B, and FHR E bind to the kidney *in vivo*, we injected C57BL/6J mice (Jackson Laboratories), mice with targeted deletion of the gene for FH ($fH^{-/-}$ mice), mice doubly deficient in FH and factor B ($fH^{-/-}fB^{-/-}$ mice),[35] or mice withpartial FH deficiency ($fH^{+/-}$) with fluorescently proteins. The proteins were labeled with Alexa-fluor 647 using a SAIVI protein/antibody labeling kit (Invitrogen). Male C57BL/6J, $fH^{-/-}$, and $fH^{-/-}fB^{-/-}$ mice were injected with 450 μg of FH-Alexa 647 by tail vein injection, and wild-type mice were injected retro-orbitally with an equimolar quantity of FHR B-Alexa 647 (presumed to be monomeric). As FHR E appears to be predominantly dimeric, the injected FHR E-Alexa 647 was approximately half the molar amount compared with FH and FHR B. To examine whether the FHRs activate complement in the kidneys *in vivo*, $fH^{+/-}$ mice were injected retro-orbitally with approximately equimolar quantities of FHR A (135 μg), FHR B (100 μg), FHR C (255 μg). These FHRs were presumed to be monomeric. Another group of mice were injected with FHR E (114 μg). This quantity of FHR E is approximately half-molar compared with the other FHRs, as it appears to be predominantly dimeric (Figure 1b). All mice were killed after 24 hours. Kidneys from each animal were collected in the optimal cutting temperature medium (Sakura Finetek) and immediately frozen in liquid nitrogen. In another experiment, mice were injected before induction of kidney ischemia/reperfusion. The mice were injected with FHR E (228 μg) or an equal volume of phosphate-buffered saline. The kidney pedicles were clamped for 24 minutes and then reperfused for 24 hours as previously described.[38] Kidney function was assessed by measurement of blood urea nitrogen.

Statistical analysis

Data were analyzed and graphs were created using GraphPad Prism software (GraphPad Prism). Comparisons between the 2 groups were performed using the unpaired *t* test. Multiple group comparisons were performed using analysis of variance with *post hoc* Tukey's *t* test. Experiments with a factorial design were analyzed using analysis of variance, and interaction effects and simple effects were tested. A P value of less than 0.05 was considered statistically significant.

RESULTS

Recombinant murine FHRs A, B, C, and E

We produced recombinant forms of murine FH and FHRs A, B, C, and E. The proteins were purified, and their size and purity were confirmed by Coomassie staining (Figure 1a) and Western blot analysis (Figure 1b). We used heparin chromatography to confirm that the proteins bind to glycosaminoglycans (Figure 1c), and we also tested binding to murine and human C3d in an enzyme-linked immunosorbent assay (Figure 1d). These assays confirmed that these recombinantly expressed FHRs bind to heparin and to C3d, as expected. In spite of sequence homology among FH and all 4 FHRs, however, the proteins displayed differences in their relative binding strengths for these ligands. FHRs A and B eluted off the heparin column at much higher salt concentrations than did FH, FHR C, or FHR E. In the C3d enzyme-linked immunosorbent assays, FHR A showed the strongest binding, and FHRs C and E showed the weakest. Pairwise comparisons are shown in Supplementary Figure S2.

Complement dysregulation by the FHRs

We previously used a GP_{Er} lysis assay to study complement dysregulation by human FHR 1.[30] A salient feature of this assay is that it has sufficient range to detect complement inhibition as well as dysregulation. Using the normal mouse serum in this assay, we found that the addition of FHR B increased erythrocyte lysis in a dose-dependent fashion (Figure 2a). The addition of recombinant murine FH to the mouse serum almost completely suppressed lysis (Figure 2b), consistent with its role as an alternative pathway regulator. In contrast, the addition of an FH antagonist (rH19–20) increased lysis, presumably by blocking binding of FH in the serum to GP_{Er} via the carboxy-terminal 19–20 domains.[36] FHR A caused greater dysregulation than FHR B, whereas FHRs C and E did not have a discernable effect (Figure 2b). Interestingly, the magnitude of the effects of FHRs A and B were similar to rH19–20 at a lower molar concentration (1 μM vs. 25 μM), indicating that SCRs 6–8 contribute to binding of FH to GP_{Er}.

Complement dysregulation by the FHRs on glomerular endothelial cells

FH is important for regulating complement activation on glomerular endothelial cells (GEnCs), and dysfunction of FH is a major risk factor for atypical hemolytic uremic syndrome.[3] Similarly, several genetic variants in the *CFHR* genes are associated with atypical hemolytic uremic syndrome, suggesting that the mutant proteins facilitate complement dysregulation on GEnCs.[24,39] We tested binding of the murine FHRs to murine GEnCs grown *in vitro*, and we examined whether the proteins bind to the cells by flow cytometry. Binding of FHRs A and C to the cells was similar to FH, whereas FHRs B and E did not detectably bind (Figure 3a and b). When the GEnCs were exposed to the FHRs and then incubated with serum, exposure of the cells to FHR A, B, or C increased deposition of C3b on the cell surface (Figure 3c).

When the cells were treated with H_2O_2 to induce oxidative stress, the binding of FHR A to the cells decreased (804 vs. 524 relative fluorescent units (RFU); $P < 0.001$ by the unpaired t test, 95% confidence interval (CI) for the difference −361 to −199), whereas FHR C still bound strongly (Figure 3d and e). Treatment of the GEnCs with H_2O_2 before exposure to serum also caused greater complement activation on the GEnC surface when exposed to FHRs A and B and rH19–20, but FHRs C and E did not have a detectable effect (Figure 3f).

Of note, we did not detect binding of FHR B or E to the GEnCs. For FHR B we tested an antibody to the hexahistidine tag (6X His tag) as well as a polyclonal anti-FH antibody, both of which recognize the protein by Western blot analysis and enzyme-linked immunosorbent assay. Interestingly, even though we did not detect binding of FHR B to the GeNC

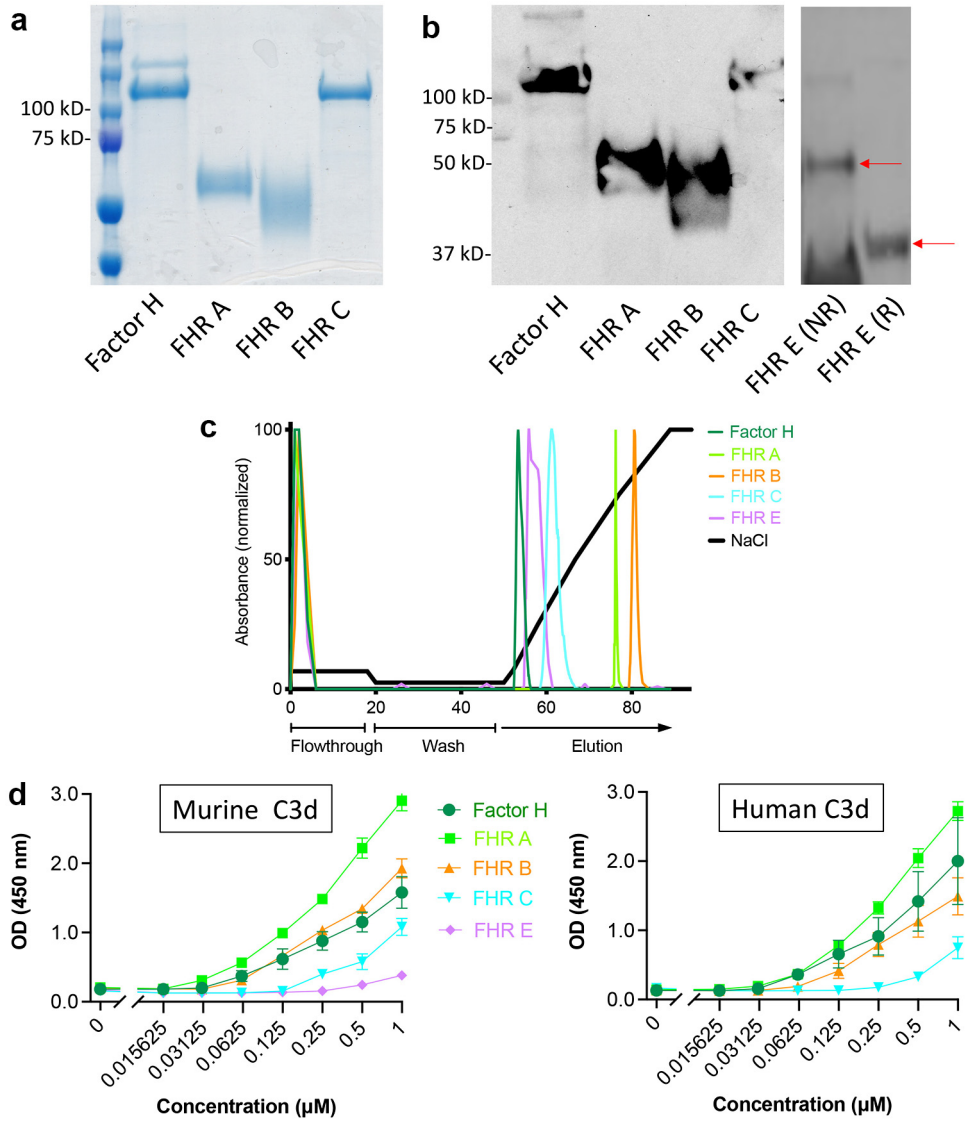

Figure 1 | Characterization of recombinant murine factor H (FH) and factor H–related proteins (FHRs). We produced recombinant murine FH (approximately 155 kDa), FHR A (approximately 50 kDa), FHR B (approximately 35 kDa), FHR C (approximately 100 kDa), and FHR E (approximately 40 kDa). (**a**) The size and purity of each protein was evaluated by Coomassie staining. (**b**) FHRs A, B, and C were also detected by Western blot analysis under reducing conditions using an antibody against the HIS tag. FHR E, which did not have a HIS tag, was detected with a polyconal antibody to FH. In nonreducing conditions (NR), FHR E had a higher apparent molecular weight, suggesting that it was dimerized. (**c**) Purified proteins were run over a heparin column and then eluted using a NaCl gradient. The proteins eluted off the column at different salt concentrations. The black line indicates the salt concentration (0–2 M, pH 7.4), and the colored lines indicate the absorbance (280 nm) of the proteins as they were eluted off the column. (**d**) Binding of FH and the FHRs to murine and human C3d was examined by an enzyme-linked immunosorbent assay. The relative binding was FHR A > FHR B > FHR C > FHR E. The graph shows the mean of 3 replicates, and error bars indicate SD. The results were analyzed using analysis of variance, and pairwise comparisons with the Tukey method for multiple comparisons are shown in Supplementary Figure S2. OD, optical density.

surface, it did cause functional complement dysregulation on the cells in the presence of serum. This discrepancy suggests that FHR B may not need to bind to the cell in order to influence complement activity on the cell surface.

The FHRs also cause complement dysregulation on other kidney cell types

Increased plasma levels of the FHRs are associated with faster disease progression in IgA nephropathy,[40,41] suggesting that they might also contribute to complement dysregulation on mesangial cells and other glomerular cell types. To test this, we grew immortalized murine mesangial cells, podocytes, and tubular epithelial cells *in vitro* and examined whether the FHRs bound to these cells. FHRs A and C bound to all 3 cell types, although the relative binding varied (Figure 4a–c). FHR B bound to epithelial cells at a low level (197.8 ± 32.5 RFU; 95% CI: 117.1–278.5), but it did not bind to the other types. FHR E only bound to podocytes (13,087 ± 10,745 RFU; 95% CI: 4828–21,347). FHR A caused complement dysregulation on all 3 cell types, FHR B affected mesangial and tubular cells,

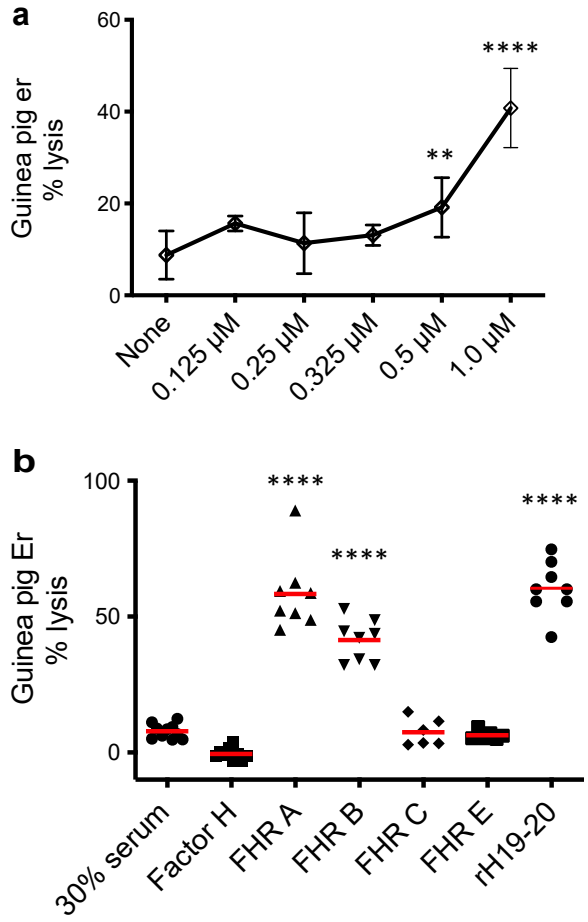

Figure 2 | Factor H–related proteins (FHRs) increase the lysis of guinea pig erythrocytes (Er) by mouse serum. Guinea pig Er were incubated with 30% normal mouse serum, and the percent lysis was calculated. (**a**) The addition of FHR B to the reaction increased the degree of lysis in a concentration-dependent fashion. The graph shows the mean ± SD for each concentration. (**b**) The assay was repeated with the addition of 1.0 μM of murine factor H or FHR A, B, or C. In addition, 25 μM of recombinant murine factor H short consensus repeats 19–20 (rH19–20) was used as a positive control. The percent lysis for each condition was compared with serum alone using analysis of variance ($P < 0.0001$). $^{**}P = 0.0077$, $^{****}P < 0.0001$ versus serum alone. The mean of 6–9 replicates for each protein is indicated with a red line.

and FHR E only caused dysregulation on podocytes. FHR C did not increase complement activation on any of the cell types.

The FHRs cause complement dysregulation on extracellular matrix

Complement is activated on the glomerular basement membrane in the setting of FH deficiency, indicating that this surface requires FH to control alternative pathway activation.[4] FH and FHR A bound directly to the ECM generated by GEnCs grown *in vitro*, as well as to Matrigel (Figure 5a). Binding of FHRs A, B, and C to Matrigel was significantly greater when added in the presence of serum. FHRs A, B, and C increased deposition of C3 onto the ECM when added to serum, but FHR E did not affect complement activation on this surface (Figure 5b). Although FHRs are believed to competitively inhibiting binding of FH to some surfaces, the addition of the FHRs did not significantly reduce binding of FH to the ECM (Figure 5c). Furthermore, although serum increases binding of FHR A to the ECM, the addition of serum (either C3-sufficient or C3-deficient) did not increase the ability of FHR A to inhibit FH binding to the ECM (Figure 5d).

FHR B binds to glomerular and tubular structures *in vivo*

To test whether FH binds to kidney surfaces *in vivo*, we fluorescently tagged recombinant FH and injected wild-type, FH-deficient mice (to minimize competition with endogenous protein) and mice doubly deficient in FH and factor B (to prevent alternative pathway activation). We found that FH bound prominently in the glomerular capillary loops of all 3 strains of mice (Figure 6a–d). Faint staining on the tubules was also seen in both FH-deficient strains (Figure 6c and d).

To examine binding of FHRs B and E to kidney surfaces *in vivo*, we fluorescently tagged the proteins and injected them into wild-type mice. We were able to detect FHR B within glomeruli, but fluorescence was not as prominent in the glomerular capillaries as it was for FH (Figure 6e and f). FHR B also bound to the basolateral surface of some tubules. Labeled FHR E could not be detected in the kidneys of injected mice (not shown). Staining of a phosphate-buffered saline injected mouse was used as a negative control (Supplementary Figure S3). We also examined the spleens of mice injected with FHR B, demonstrating that the protein also binds in tissues outside of the kidney (Supplementary Figure S4).

Injection of mouse FHR E causes glomerular complement dysregulation

We next injected separate cohorts of mice with partial deficiency of FH ($fH^{+/-}$ mice) with FHR A (135 μg), FHR B (100 μg), FHR C (255 μg), and FHR E (228 μg) to test whether the proteins affected complement activation/regulation in the kidney. We have previously found that homozygous deficiency of FH is associated with greater glomerular C3 deposition than heterozygous deficiency,[42] but that exogenous proteins can antagonize FH in partially deficient mice and increase complement deposition.[12] The mice were killed after 24 hours, and we immunostained the kidneys for C3b, iC3b/C3d, and C9. C3b and iC3b/C3d were deposited within the glomeruli and along the tubules of all mice (Figure 7a). Granular C9, as a marker of C5b-9, was also identified in these locations, although to a lesser degree (Figure 7b). When we quantified complement fragment deposition, injection of the mice with FHR E increased glomerular C3b and C3d deposition (Figure 8). We did not observe a significant effect of FHR A, B, or C on glomerular complement deposition, although FHR A was associated with a reduction in tubulointerstitial C3d. To test whether the reduction of

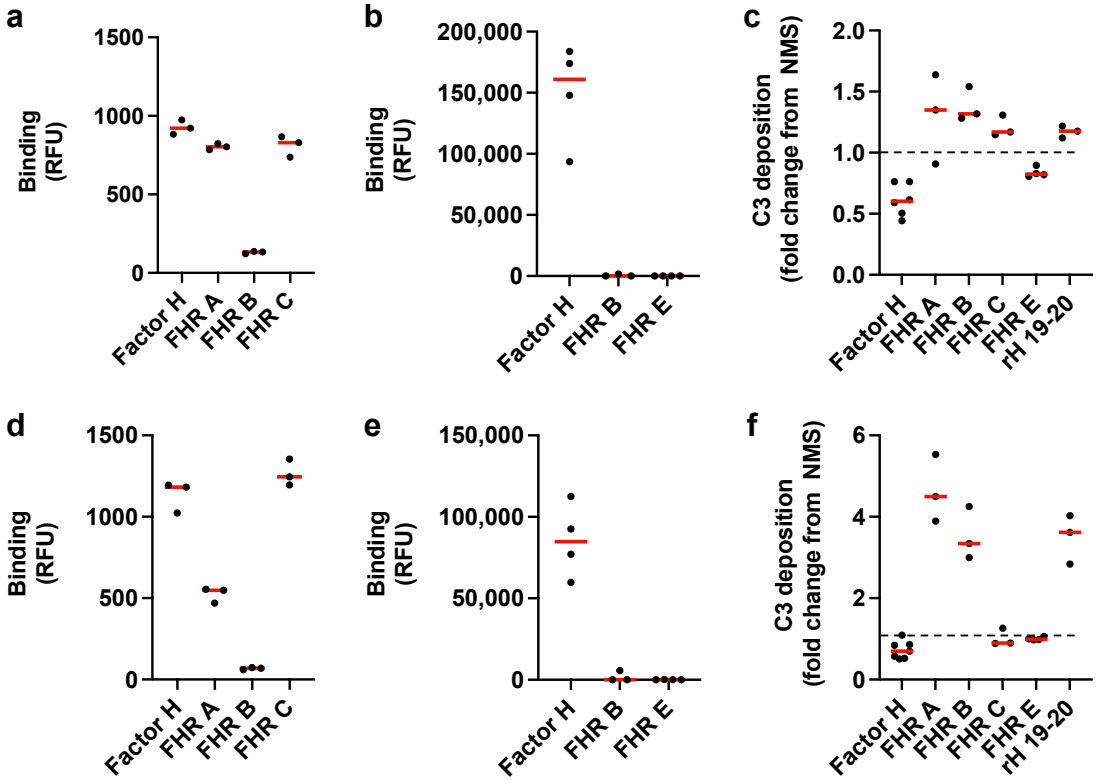

Figure 3 | A subset of factor H–related proteins (FHRs) increase complement activation on murine glomerular endothelial cells (GEnCs). Murine GEnCs were incubated with 1.0 μM of factor H (FH) or the FHRs. (**a**) When stained with an anti-HIS antibody, FH, FHR A, and FHR C were detected on the cell surface. (**b**) A polyclonal antibody was used to detect FHR E as this protein does not have a HIS tag. FHRs B and E were not detected on the cell surface using this antibody. (**c**) GEnCs were incubated with the FHRs before exposure to normal mouse serum (NMS), and C3 deposition on the cells was measured by flow cytometry. The results are compared with those obtained with serum alone (normalized to 1 and indicated with a dashed line) and are reported as fold change. C3 deposition increased with FHR B (95% confidence interval [CI]: 1.032–1.729), FHR C (95% CI: 0.99–1.422), and rH19–20 (95% CI: 1.049–1.293). C3 deposition decreased when cells were treated with FH (95% CI: 0.386–0.751) and FHR E (95% CI: 0.774–0.902). In a separate set of experiments, the cells were exposed to H_2O_2 before incubating them with the proteins. (**d**) When stained with an anti-HIS antibody, FH, FHR A, and FHR C were detected on the cell surface of H_2O_2-treated cells. (**e**) A polyclonal antibody was used to detect FH, FHR B, and FHR E in this reaction. FH was detected bound to the surface of H_2O_2-treated cells, but FHRs B and E were not detected using this antibody. (**f**) H_2O_2-treated GEnCs were incubated with the FHRs before exposure to NMS, and C3 deposition on the cells was measured by flow cytometry. The results are compared with those obtained with serum alone and are reported as fold change. C3 deposition increased with FHR A (95% CI: 2.586–6.696), FHR C (95% CI: 1.918–5.146), and rH19–20 (95% CI: 1.998–4.992). C3 deposition decreased when cells were treated with FH (95% CI: 0.528–0.929). The mean of 3–6 replicates for each protein is indicated with a red line. RFU, relative fluorescent units.

tubulointerstitial C3 was caused by consumption of C3 in plasma, we performed a C3a enzyme-linked immunosorbent assay. C3a levels were undetectable in the FHR A injected mice (data not shown), arguing against extensive activation of plasma C3.

Injection of mice with FHR E does not exacerbate ischemic acute kidney injury

Ischemic acute kidney injury is associated with alternative pathway activation in the kidney tubulointerstitium,[43] and mice with reduced levels of FH or those treated with FH antagonists develop worse injury.[38,44] Furthermore, a recent study showed that mice deficient in FHR E develop worse acute kidney injury after injection with lipopolysaccharide.[45] To test whether exogenous FHR E would affect complement activation and injury in acute kidney injury, we injected mice i.v. with 288 μg of FHR E or an equal volume of phosphate-buffered saline and subjected the mice to 24 minutes of ischemia. At 24 hours after ischemia, blood urea nitrogen levels in the phosphate-buffered saline and FHR E groups were 113.9 ± 15.3 mg/dl and 116.5 ± 4.7 mg/dl, respectively, indicating that injection of the mice with FHR E did not exacerbate kidney injury. C3b was deposited on the tubules of the outer medulla in both groups of mice (Figure 9a and b). When we quantified the deposits in the glomerular and tubulointerstitial compartments, however, there was no significant difference between the 2 groups of mice.

DISCUSSION

Although the physiologic roles of the FHRs are incompletely understood, genetic variants of these proteins and increased expression levels of wild-type protein are associated with glomerular disease. In the current study, we made recombinant forms of the 4 murine FHRs and directly compared their

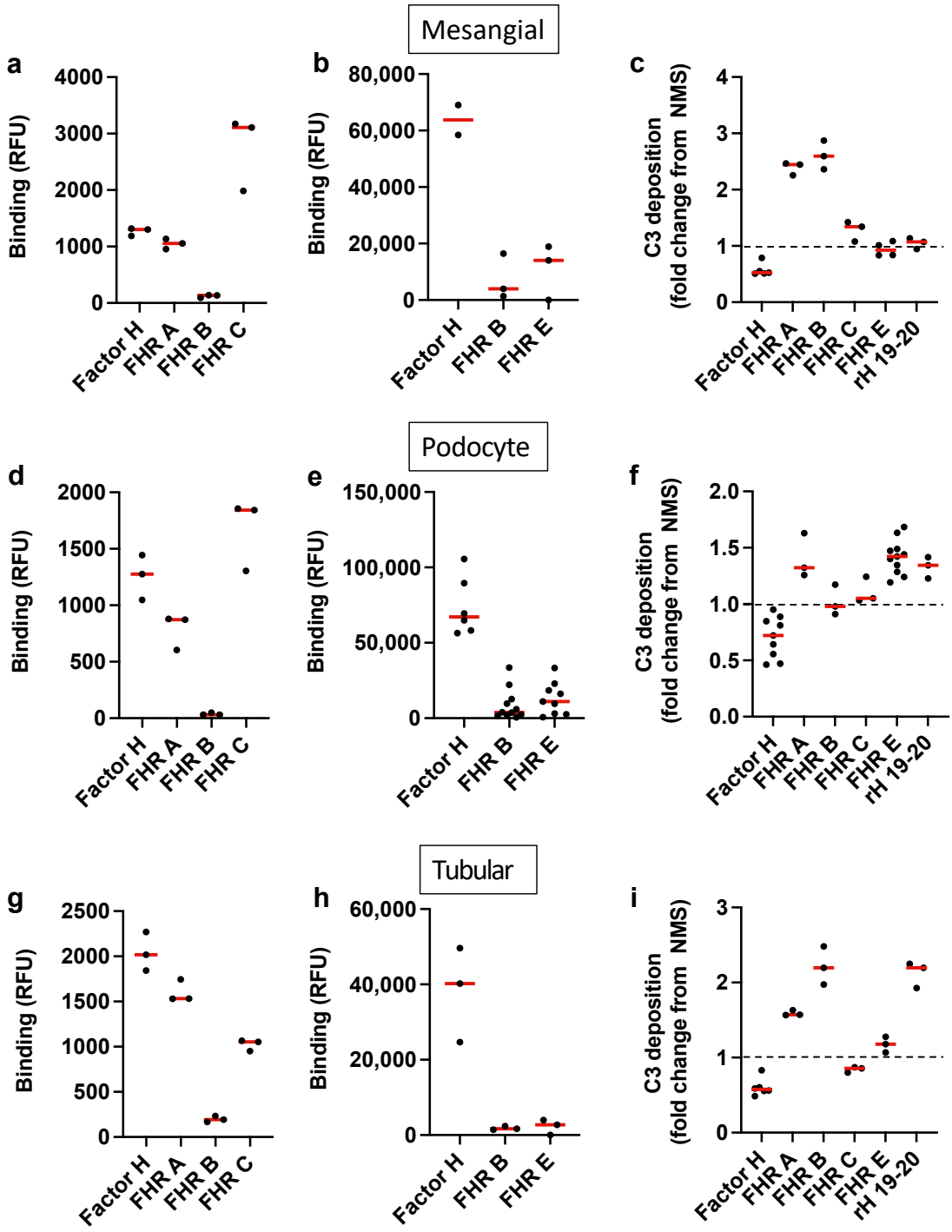

Figure 4 | Factor H–related proteins (FHRs) increase complement activation on murine mesangial cells, podocytes, and tubular epithelial cells. Murine mesangial cells were incubated with 1.0 μM of factor H (FH) or the FHRs, and binding of the proteins was evaluated by flow cytometry. (**a**) FH, FHR A, and FHR C bound to mesangial cells. (**b**) A polyclonal antibody was also used to detect FH, FHR B, and FHR E. Only FH was detected on the cells. (**c**) Mesangial cells were incubated with the FHRs before exposure to normal mouse serum (NMS), and C3 deposition on the cells was measured by flow cytometry. The results are compared with those obtained with serum alone (normalized to 1 and indicated with a dashed line) and are reported as fold change. C3 deposition decreased with FH (95% confidence interval [CI]: 0.429–0.725), and it increased with FHR A (95% CI: 2.098–2.680) and FHR B (95% CI: 1.975–3.245). (**d**) The FHRs were also incubated with murine podocytes, and FHR A and C bound to the cells. (**e**) FHR E showed a low level of binding to the podocytes (95% CI: 4828–21,347 random fluorescence units). (**f**) C3 deposition on podocytes decreased with FH (95% CI: 0.565–0.846) and increased with FHR E (95% CI: 1.317–1.521). (**g**) Binding experiments were performed using tubular epithelial cells. FH, FHR A, and FHR C bound to the cells. (**h**) FHR B and FHR E were not detected on the cell surface by a polyclonal antibody. (**i**) C3 deposition on tubular epithelial cells decreased when incubated with FH (95% CI: 0.481–0.729) and FHR C (95% CI: 0.754–0.931). C3 deposition increased with FHR A (95% CI: 1.499–1.679) and FHR B (95% CI: 1.586–2.484). For each experiment, the mean for 3–11 replicates of each condition is shown in red. RFU, relative fluorescent units.

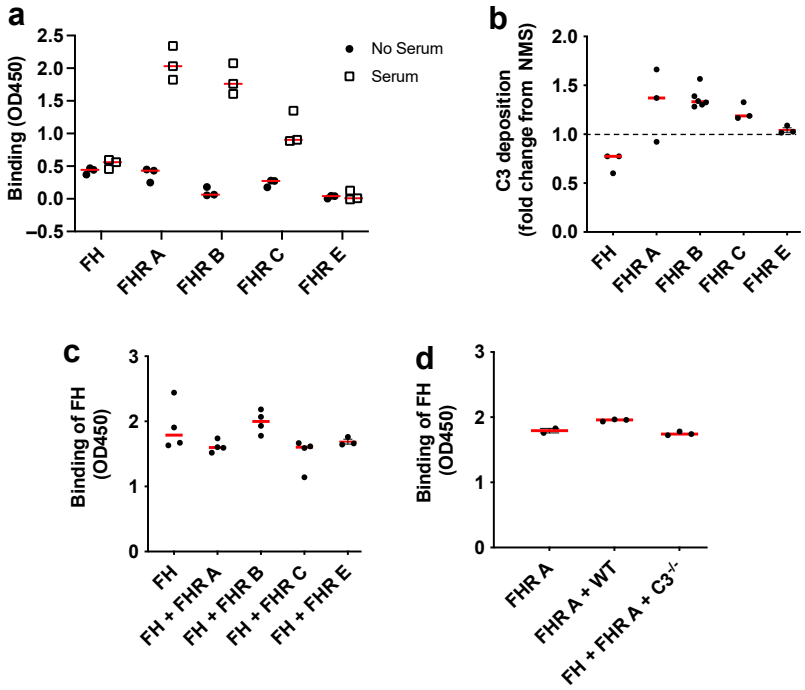

Figure 5 | Factor H–related proteins (FHRs) increase complement activation on the extracellular matrix (ECM). (**a**) Recombinant factor H (FH) and the FHRs were incubated with the ECM (Matrigel), and the bound protein was detected using an enzyme-linked immunosorbent assay. There was positive binding of FH to the ECM (95% confidence interval [CI]: 0.302–0.554 OD_{450}), FHR A (95% CI: 0.101–0.651 OD_{450}), and FHR C (95% CI: 0.099–0.386 OD_{450}). When the FHRs were added to the ECM in the presence of serum (shown in boxes), there was a significant increase in binding of FHR A ($P < 0.001$, 95% CI for difference 1.237–2.142 OD_{450}), FHR B ($P < 0.001$, 95% CI for difference 1.315–2.116 OD_{450}), and FHR C ($P < 0.01$, 95% CI for difference 0.373–1.23 OD_{450}). (**b**) We next added serum to the ECM and incubated it at 37°C for 30 minutes, and then measured the amount of C3 deposited on the ECM surface. The dashed line indicates C3 deposition when serum was used (set at 1.0), and the data are shown as the fold change relative to serum. When recombinant FH was added to the reaction, it decreased the amount of deposited C3 (95% CI: 0.468–0.966). FHR B increased it (95% CI: 1.259–1.476), FHR C increased it (95% CI: 1.009–1.445). (**c**) We tested whether the FHRs reduced binding of FH to the ECM when added in a 2-fold excess. The addition of the FHRs did not significantly alter binding of FH to the ECM. (**d**) We examined whether a 2-fold excess of FHR A affected binding of FH to the ECM in the presence of wild-type or C3-deficient serum ($C3^{-/-}$). The presence or absence of C3 in the serum did not affect the ability of FHR A to competitively inhibit binding of FH. The mark represents the mean of 3–6 replicates. NMS, normal mouse serum; OD, optical density.

binding and function on several cellular and noncellular surfaces. Similar to FH, all of the FHRs bound to heparin (a glycosaminoglycan surrogate) and FHRs A, B, and C also bound to immobilized C3d, although they exhibited different relative binding strengths for these ligands. We also found that FH suppressed complement-mediated hemolysis of GP_{Er}, whereas FHRs A and B caused complement dysregulation in this assay. This presumably occurred because the FHRs competitively inhibited FH present in the normal mouse serum from controlling alternative pathway activation on the surface of the GP_{Er}. A recombinant construct encompassing SCRs 19–20 of FH also increased hemolysis.

When we specifically tested the effects of the FHRs on glomerular surfaces *in vitro*, we found that the FHRs bound to the various cell lines tested and caused complement dysregulation on the cell surfaces. This was true for FHRs C and E, which had no discernable effect on GP_{Er} lysis, whereas FHR B increased C3 deposition on some surfaces to which it did not directly bind. Nevertheless, even with the high sequence homology among the 3 FHRs, they behaved distinctly in these assays. Furthermore, the effects of each individual FHR varied among the different cell surfaces. FHRs A, B, and C also increased C3 deposition on the ECM, although they did not competitively inhibit FH from binding. This suggests that complement dysregulation by these proteins may not be as simple as competitive inhibition of binding by FH to a surface. Interestingly, binding of FHRs A, B, and C to the ECM was significantly greater in the presence of serum, possibly due to deposition of C3 fragments on the target surface. These *in vitro* results are summarized in Supplementary Table S3.

When we injected mice with fluorescently labeled FH and FHR B to test binding to glomerular surfaces *in vivo*, we observed higher levels of signal for FH than for FHR B within the glomerular capillary loops. This may reflect either greater binding of FH to the glomerular basement membrane, or it is possible that FHR B binds elsewhere in the body, limiting the amount of protein available for binding in the kidney. Interestingly, although FH binds to C3 fragments, we saw similar glomerular binding of FH in mice that had abundant glomerular C3 deposits ($fH^{-/-}$ mice) and in mice with scant glomerular C3 (wild-type and $fH^{-/-}fB^{-/-}$ mice). FH is not

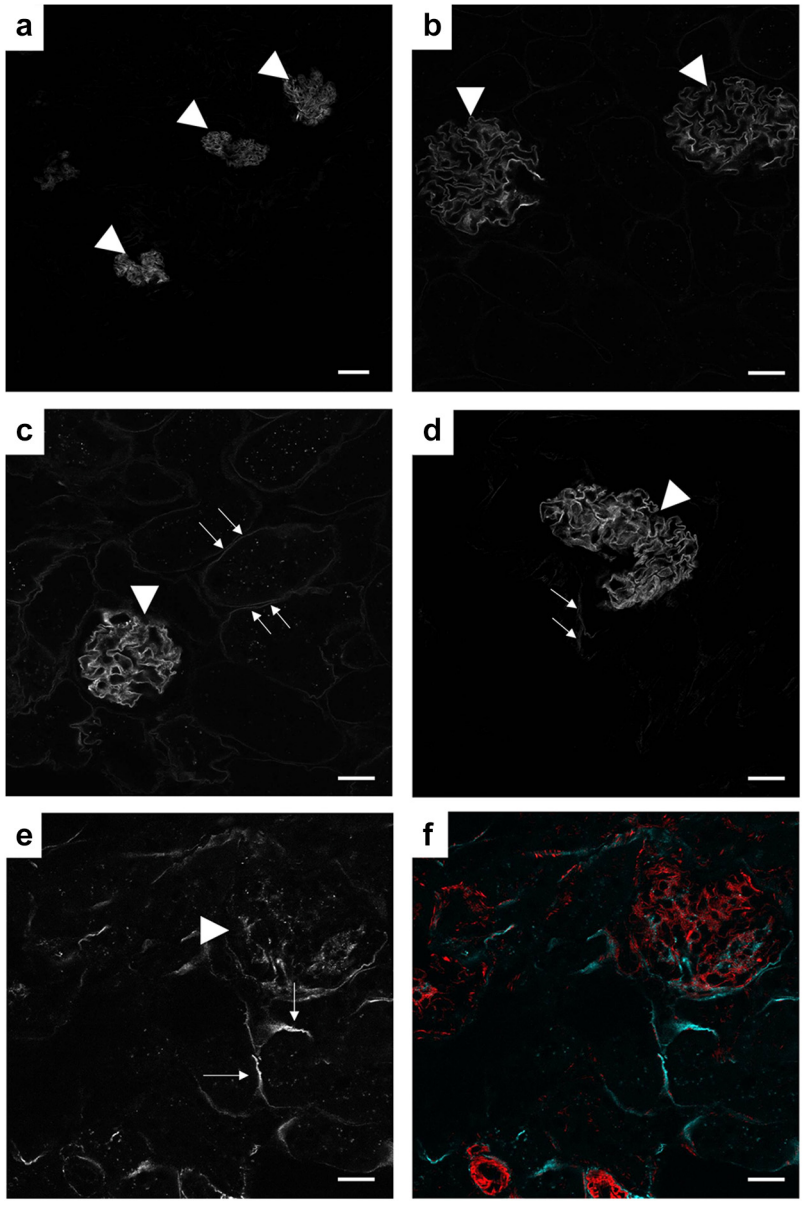

Figure 6 | Factor H (FH) and factor H–related protein B (FHR B) bind to discrete locations within the kidney. (a,b) Wild-type mice (n = 3) were injected with fluorescently labeled FH, and the mice were killed after 24 hours. The kidneys were examined using confocal microscopy, and representative images are shown. (**a**) A ×20 view shows that FH presents in 3 glomeruli (arrowheads). (**b**) A ×60 view shows that FH localizes within the glomerular capillaries. Injection of labeled FH into (**c**) FH-deficient ($fH^{-/-}$ mice; n = 3) and (**d**) mice doubly deficient in FH and factor B ($fH^{-/-}fB^{-/-}$ mice; n = 3) displayed similar patterns of glomerular FH deposition. (**e,f**) Wild-type mice were injected with fluorescently labeled FHR B, and the mice were killed after 24 hours (n = 3). The kidneys were examined using confocal microscopy. (**e**) A ×60 view shows that FHR B is present in the glomerulus (arrowhead), but fluorescence in the capillaries is not as intense as for FH. FHR B is also seen on Bowman's capsule, and along the basolateral aspect of the tubules (arrows). (**f**) Dual imaging of the kidneys for FHR B (aqua) and F-actin (red). Bar = 50 μm for panel (**a**) and 20 μm for all other panels. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

always detected in biopsy tissue from patients with glomerulonephritis.[46,47] The variance between our results and those studies could be due to differences between mouse and human FH, but the differences could also be due to differences in tissue fixation or staining protocols.

Although the FHRs caused complement dysregulation on all glomerular cell types *in vitro*, only FHR E was associated with a dysregulatory effect *in vivo*. It is possible that the FHRs cause complement dysregulation outside of the kidney, reducing the availability of complement proteins in the kidney. We did not see increased levels of C3a in the plasma of injected mice, however, arguing against this possibility. It is also possible that the kidney cell phenotypes are altered *in vitro*. For example, in the *in vitro* experiments, the cells were enzymatically detached from the tissue culture flasks using a reagent that contains proteases. This may have

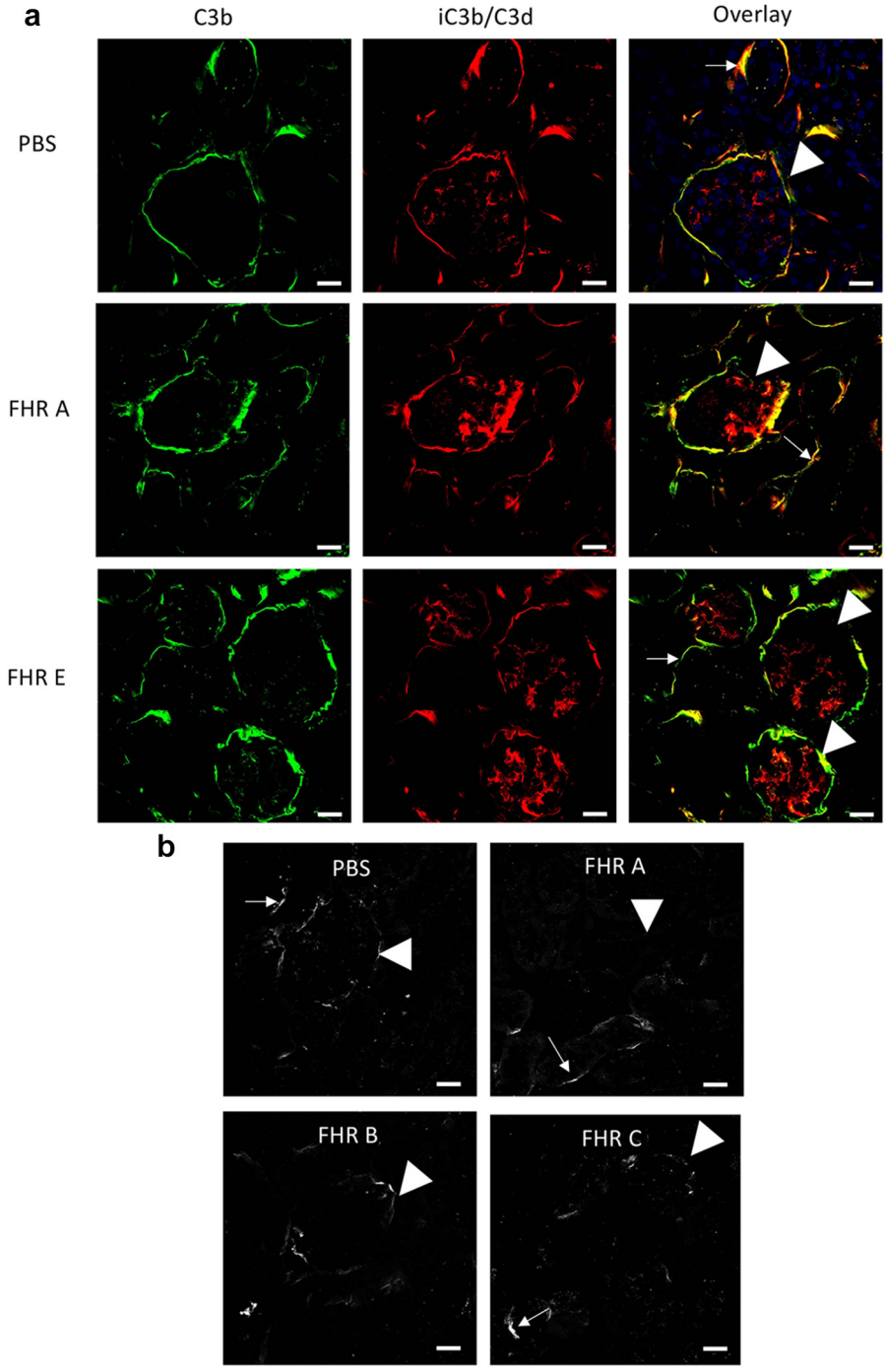

Figure 7 | Complement activation in kidneys of mice injected with the factor H–related proteins (FHRs). Mice with partial deficiency of factor H ($fH^{+/-}$ mice) were injected with approximately equimolar concentrations of FHR A, B, C, or E, and the mice were killed after 24 hours (n = 4–8 for each protein). Complement deposits in the kidneys were examined using confocal microscopy. (**a**) Kidneys were stained for C3b (green) and iC3b/C3d (red). Representative images are shown for mice injected with phosphate-buffered saline (PBS), FHR A, and FHR E. (**b**) Kidneys were also stained for C9, and representative images are shown for mice injected with PBS, FHR A, FHR B, and FHR C. Glomeruli are indicted with arrowheads, and tubules are indicated with arrows. Original magnification ×60 for all images. Bar = 20 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

affected surface proteoglycans, altering the interactions of FH and/or the FHRs with the cell surface. Finally, it is possible that binding of FHRs at sites of complement activation blocked our ability to detect tissue C3 by steric hindrance, although one of the antibodies used to detect C3 fragments is a polyclonal serum.

Although injection of FHR E was associated with increased glomerular complement activation in $fH^{+/-}$ mice, it did not

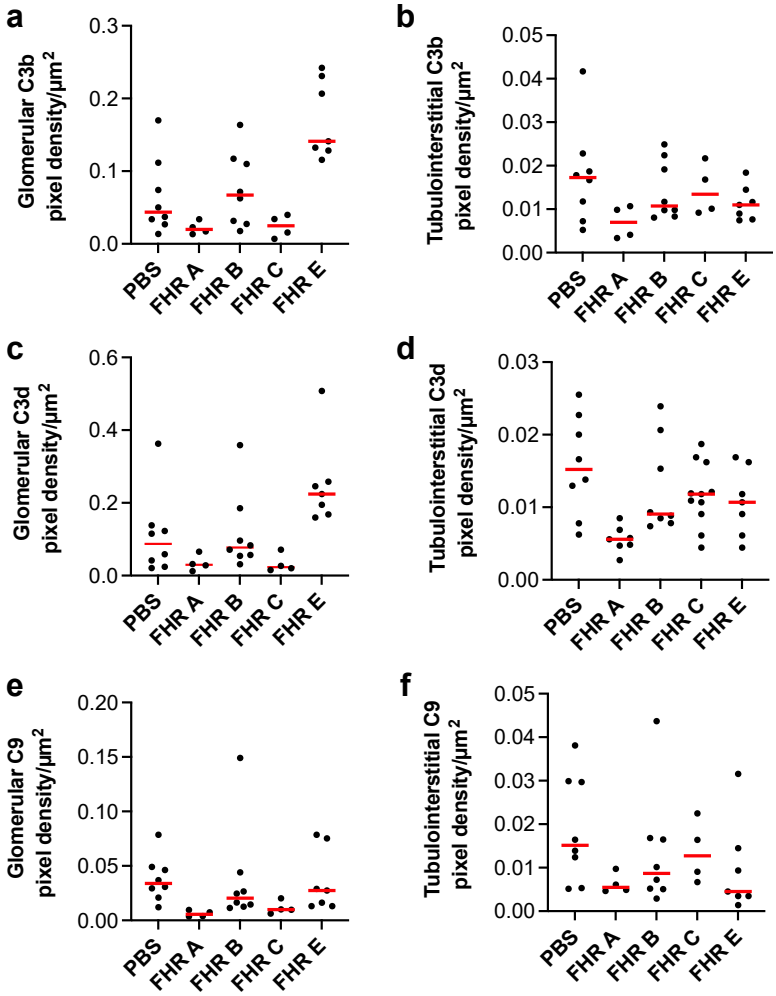

Figure 8 | Quantification of complement deposits in kidneys of mice injected with the factor H–related proteins (FHRs). Mice with partial deficiency of factor H ($fH^{+/-}$ mice) were injected with approximately equimolar concentrations of FHR A, B, C, or E, and the mice were killed after 24 hours (n = 4–8 for each protein). The abundance of C3b, C3d, and C9 were evaluated based on fluorescent signal intensity in the glomeruli or tubulointerstitium. (**a**) C3b levels in the glomeruli were compared using analysis of variance (ANOVA) ($P < 0.0001$), and levels increased in mice injected with FHR E versus phosphate-buffered saline (PBS) ($P < 0.001$). (**b**) Injection with the FHRs did not significantly affect C3b in the tubulointerstitium. (**c**) C3d levels in the glomeruli were compared using ANOVA ($P < 0.01$), and levels increased in mice injected with FHR E versus PBS ($P < 0.05$). (**d**) Tubulointerstitial C3d was decreased in mice injected with FHR A ($P < 0.01$ for ANOVA, $P < 0.01$ FHR A vs. PBS). Injection of the mice with FHRs did not significantly affect C9 deposition in the glomeruli (**e**) or the tubulointerstitium (**f**). For each experiment, mean for each condition is shown in red.

significantly increase tubulointerstitial complement activation or worsen injury in a model of ischemic acute kidney injury. We previously found that injection of mice with rH19–20 exacerbates injury in this model,[44] indicating that antagonism of binding of the SCR 19–20 region of FH on ischemic tubular epithelial cells is deleterious. C3 deposition in the tubulointerstitium appeared worse to a blinded observer, and it is possible that patchy nature of complement activation in the kidney after ischemia reduced the sensitivity of our analysis to quantitatively detect increased tubulointerstitial activation. In spite of the sequence homology between SCRs 3–5 of FHR E and SCRs 19–20 of FH, however, FHR E did not appear to function as an FH antagonist. Our *in vitro* studies demonstrated stronger dysregulation of FHR E on podocytes than on tubular epithelial cells, whereas rH19–20 caused dysregulation on both cell types. It is therefore possible that the dysregulatory effects of FHR E are context-specific, and that this protein will have a stronger effect in diseases in which the alternative pathway is activated on podocytes compared with diseases where it is activated on tubular cells.

Regarded together, these experiments demonstrate that the murine FHRs have functional effects on complement activation and regulation within the kidney. However, the side-by-side comparison of the FHRs revealed that, in spite of homology between the binding regions of all 3 FHRs, there are marked differences in how they interact with a given cell type or surface. Furthermore, the individual FHRs behave differently on each cell type, and their ability to dysregulate complement on GEnCs increases after the cells are injured.

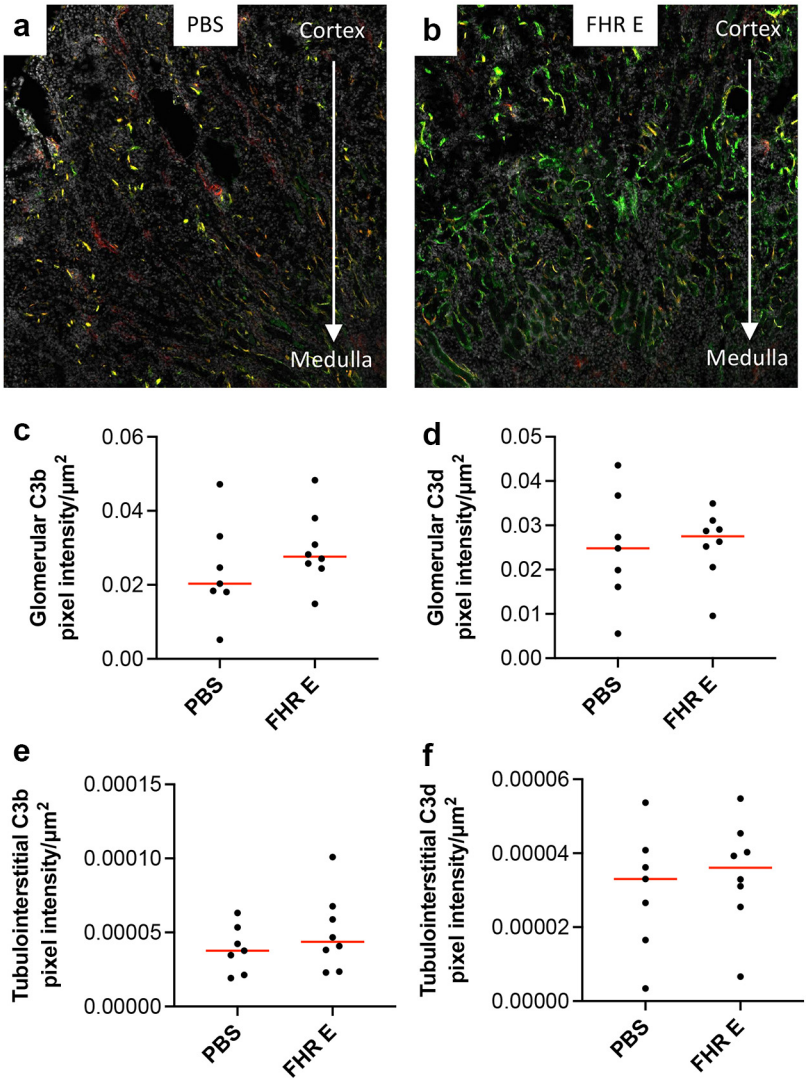

Figure 9 | Injection of mice with factor H–related protein E (FHR E) does not exacerbate ischemic acute kidney injury. Mice were injected i.v. with FHR E or with phosphate-buffered saline (PBS) as a vehicle control. They were then subjected to 24 minutes of bilateral ischemia followed by 24 hours of reperfusion. Kidneys were immunostained for C3b (green) and C3d (red) and examined using confocal microscopy (**a**,**b**). The intensities of glomerular C3b (**c**), glomerular C3d (**d**), tubulointerstitial C3b (**e**), and tubulointerstitial C3d (**f**) were quantified in a blinded fashion. Treatment with FHR E was not associated with increased deposition of these fragments. The mean for each group is shown in red. The kidney cortex and medulla in the displayed fields are indicated. Original magnification ×100. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

These findings indicate that complement regulation/dysregulation by FH and the FHRs is dynamic throughout the body, and it can change depending on the health of a tissue. The functional differences among the murine FHRs also make it challenging to draw conclusions about human disease from these studies. The human FHRs are structurally similar to the mouse FHRs, although it is difficult to determine whether the murine FHRs are homologues of the human proteins based on their structure (Supplementary Figure S1).[27] Nevertheless, given the heterogeneity of the function of different FHRs, it is important to determine which human FHRs are functionally similar to each of the murine proteins. Identifying such functional similarities would help clarify what the results in mice mean regarding human physiology.

Unexpectedly, there is a discordance in the binding of some FHRs to surfaces and dysregulation by the FHRs on those surfaces. FHR B, for example, increased C3 deposition on GEnCs, mesangial, and tubular epithelial cells, even though it did not bind to those cells. *In vivo*, on the other hand, FHR B bound within the kidney but did not increase complement activation. It is possible that FHR B affects complement activation through a fluid phase effect. Other proteins, for example, have been shown to bind FH in solution, reducing its ability to regulate complement on cell surfaces.[12]

There are several limitations to our study. The addition of the recombinant FHRs to the endogenous proteins present in serum likely creates supraphysiologic concentrations. It is also unclear whether the effects of the FHRs in

the kidney are attenuated by binding of the proteins elsewhere in the body. Another limitation of our experiments is that our results indicate that there are context-dependent effects on the function of the FHRs. Thus, our results demonstrate that the FHRs modulate complement activation on different kidney surfaces, but these experiments do not indicate whether these effects are biologically important in specific disease settings. Many of the genetic disease–associated mutations in the FHRs lead to duplications in the binding regions of these proteins. These changes may lead to differences in affinity of the mutant proteins for target surfaces.[48] In addition, we examined the effects of multiple different proteins on a variety of different surfaces, and it is possible that the number of replicates in individual experiments was too low to detect the effects of the protein. Finally, it should be noted that the recombinant proteins used in these studies contained endotoxin. There are interactions between the complement system and toll-like receptors.[49] Complement proteins are also acute phase reactants, so endotoxin may increase production of complement proteins. Endotoxin was present in all of the proteins, however, so it is unlikely to explain different results obtained with the various FHRs.

In sum, our results demonstrate that the FHRs can alter the balance of activation/regulation on different resident cell types within the kidney. Although the effects of the FHRs can be modest in the context of other complement regulatory proteins, the net effect over time may be important for chronic diseases.[40,41,50] The alternative pathway is activated in multiple different glomerular and tubulointerstitial diseases.[43,51–54] Even if the FHRs are not the primary drivers of complement activation, these proteins may modulate complement-mediated injury in these diseases. Furthermore, the effect of the FHRs on complement regulation/dysregulation may be altered in damaged tissues, increasing their functional effects. The understanding of FHR roles will be greatly increased once there are *in vivo* models of the FHR absence, dysfunction, and tissue-specific expression/modulation, as well as an improved understanding of which murine FHRs serve orthologous functions to specific human FHRs.

DISCLOSURE

JMT and VMH receive royalties from Alexion Pharmaceuticals, Inc. and are consultants for Q32 Bio, Inc., a company developing complement inhibitors. Both also hold stock and will receive royalty income from Q32 Bio, Inc. VPF serves as a consultant for, and receives grant funding from, Apellis Pharmaceuticals. MCP reports consulting honoraria with Alexion, Apellis, Achillion, and Gyroscope. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Methods.

Supplementary File (PDF)

Figure S1. Comparison of the murine and human factor H–related proteins (FHRs).

Figure S2. Pairwise comparison of binding of the factor H–related proteins (FHRs) to murine and human C3d.

Figure S3. Control staining in vehicle–treated mice.

Figure S4. Factor H–related protein (FHR) B binds within the spleen.

Table S1. Summary of findings relevant to human factor H–related protein (FHR) activities or expression (with references).

Table S2. Estimated molar ratio of supplemented recombinant murine factor H–related protein (rFHR) to murine factor H (FH) present in serum of the experiments.

Table S3. Summary of *in vitro* results using murine factor H–related proteins (FHRs).

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