Amniotic fluid stem cell-derived vesicles protect from VEGF-induced endothelial damage

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Injection of amniotic fluid stem cells (AFSC) delays the course of progression of renal fibrosis in animals with Alport Syndrome, enhancing kidney function and improving survival. The mechanisms responsible for these protective outcomes are still largely unknown. Here, we showed that vascular endothelial growth factor (VEGF) signaling within the glomeruli of Alport mice is strongly elevated early on in the disease, causing glomerular endothelial cell damage. Intraventricular injected AFSC that homed within the glomeruli showed strong modulation of the VEGF activity, particularly in glomerular endothelial cells. To investigate this phenomenon we hypothesized that extracellular vesicles (EVs) produced by the AFSC could be responsible for the observed renoprotection. AFSC derived EVs presented exosomal and stem cell markers on their surface membrane, including VEGFR1 and VEGFR2. EVs were able to modulate VEGF in glomerular endothelial cells by effectively trapping the excess VEGF through VEGFR1-binding preventing cellular damage. In contrast, VEGFR1/sVEGFR1 knockout EVs failed to show similar protection, thus indicating that VEGF trapping is a potentially viable mechanism for AFSC-EV mediated renoprotection. Taken together, our findings establish that EVs secreted by AFSC could target a specific signaling pathway within the glomerulus, thus representing a new potential glomerulus-specific targeted intervention.

The complex local autocrine/paracrine signaling between podocytes and glomerular endothelial cells (GEC) is of critical importance for the homeostatic balance of the filtration barrier1. In particular, podocytes secrete various factors that act directly on the glomerular endothelium2-4. In recent years multiple studies have demonstrated that VEGF signaling plays a key role in the development and maintenance of glomerular capillary network and endothelial permeability5-7. An angiogenic imbalance between VEGF (specifically VEGF-A), VEGF receptor 2 (VEGFR2) and the soluble vascular endothelial growth factor receptor 1 (sVEGFR1, a truncated variant of the VEGF receptor 1, VEGFR1) has been reported in many diseases, including kidney disease where modulation of VEGF signaling correlates with impaired endothelial fenestrations, endothelial dysfunction and increased proteinuria8-9. Although the therapeutic use of compounds with anti-VEGF activity may prevent proteinuria in endothelial murine models of diabetic nephropathy10,11, the significance of VEGF/VEGFRs/sVEGFR1 modulation within the glomerular milieu, its contribution to GEC damage and progression of chronic kidney disease (CKD) is still not clearly understood.

We previously demonstrated that stem cells derived from amniotic fluid (AFSC) are renoprotective and significantly delayed disease progression in a mouse model of Alport Syndrome (AS, where a mutation in any of the collIVα3,α4,α5 genes results in the disruption of the glomerular basement membrane (GBM), podocyte effacement and renal failure) via preservation of podocyte number and maintenance of glomerular function12. The renoprotection by AFSC could possibly be ascribed to their ability to secrete various trophic mediators able to stimulate endogenous glomerular repair mechanisms. In this context, stem cell-derived extracellular vesicles (EVs), which are important cell-to-cell communication vehicles13-14, are suggested to be involved in tissue protective mechanisms15-17. At present, the mechanism(s) responsible for the therapeutic effect of AFSC on GEC damage and in particular their possible modulation of the VEGF pathway within the glomerulus has not yet been investigated.

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In the present study, we found changes in VEGF signaling activity within the Alport glomeruli, particularly during the initiation phase of the disease. Injected AFSC that lodged within glomerular capillaries modulated VEGF/sVEGFR1 levels, thus preventing further endothelial damage, possibly by activating endogenous repair mechanisms. Specifically, we confirmed that AFSC release EVs that express various surface markers, including VEGFR1 and VEGFR2, and can modulate VEGF/VEGFRs signaling in damaged GEC by decreasing the bio-availability of excess VEGF. In conclusion, our data confirm the ability of AFSC to ameliorate renal damage and establish that their secreted EVs could target a specific signaling pathway re-establishing GEC function, thus representing a potentially new glomerulus-specific targeted intervention.

**Results**

**VEGF/VEGFRs/sVEGFR1 signaling characterization within Alport glomerulus.** To investigate the role of VEGF in AS progression, we determined if VEGF signaling is altered within the glomeruli of Alport mice. The specific VEGF isoform we studied is the VEGF-A.

As shown in Fig. 1A–C, VEGF expression, mainly produced by podocytes [Suppl. Figure 1A–H], was markedly altered early on in disease and peaked at 3 months but returned to baseline level thereafter. VEGF over-activation was shown by the increased pVEGFR2/VEGFR2 ratio in Alport glomeruli [Fig. 1D]. At 3 months of age VEGF1 expression [Fig. 1E], and the VEGFR1/VEGFR2 ratio were significantly decreased in AS [Fig. 1F]. In addition, sVEGFR1 was significantly decreased in later stages (5–6 months of age) [Fig. 1G]. Of note, at 6 months of age, no major shifts in the VEGFR1 and VEGFR2 expression were detected between WT and AS glomeruli [Suppl. Figure II–K] likely indicating that glomerular cells might be counter-reacting to the VEGF signaling alteration at this advanced stage of AS by turning down the sensitivity to the signal.

**GEC characterization in Alport mice.** We next examined the impact of VEGF modulation on GEC. TEM scans showed characteristic splitting of the GBM consistent with AS pathology, whereas WT mice had normal
basement membrane thickness [Fig. 2A,C, arrows]. In addition, GEC presented an altered morphology characterized by disruption of fenestrations when compared to WT [Fig. 2A–D, arrowheads] at 3 months of age. Fenestration size was significantly increased in Alport mice further confirming changes in GEC morphology [Fig. 2B,D,E] during VEGF peak expression. In addition, AS glomeruli showed de novo expression of plasma-lemma vesicle associated protein-1 (PV1, which plays a key role in diaphragm formation in fenestrae and maintenance of endothelial integrity16,17, [Fig. 2F–K,R]) and alteration of the glycocalyx as shown by downregulation of the wheat germ agglutinin (WGA) expression [Fig. 2L–Q,S] during the VEGF peak.18,19.

To specifically study the correlation between VEGF modulation and GEC damage, we generated an Alport mouse with fluorescently labeled GEC (Alport-Tek tdT), among other endothelial cells in other organs. We confirmed that glomeruli from WT-Tek tdT mice present with a strong tdTomato (tdT) signal (in red) in GEC [Suppl. Figure 2A–E, Fig. 3A,B]. Of note, we identified two distinct populations of tdT positive cells within the glomeruli, represented with bright and dim tdT expression [Suppl. Figure 2E,K]. The FACS sorted bright tdT-GEC cells were further confirmed for their endothelial phenotype for expression of CD31 by RT-PCR [Suppl. Figure 2F] for VE-Cadherin by flow cytometry [Suppl. Figure 3B] and for CD31 and Tie-2 by immunostaining [Suppl. Figure 3C]. The tdT-GEC were negative for markers like PDGFRβ, WT1 and Nephrin [Suppl. Figures 2G,H and 3A,D,E]; thus indicating that within the glomerulus, the tdT is expressed exclusively in GEC, allowing us to isolate GEC free from contamination of other glomerular cells. The dim population lacked strong expression of endothelial markers [Suppl. Figure 2I]. Therefore for the purpose of this study, we used the former in all the experiments described.

Isolated GEC from Alport-Tek tdT presented altered expression of VEGF signaling, including pAKT and pERK (downstream of VEGFR2). The levels of these downstream markers varied largely along disease progression with values close to normal at 2 months of age and almost absent at 4 months of age [Fig. 3C,D]; no significant variation in VEGFR1 expression was detected [Fig. 3E,F]. These variations of the VEGF signaling in GEC present a trend similar to that observed within the glomeruli specifically in relation to the turning down of the signaling after the VEGF peak. Functionally, these variations correspond to the onset of serum creatinine and proteinuria in AS mice as shown in Fig. 4I,J.
AFSC effects on intraglomerular regulation of VEGF signaling and on GEC biology. To study the modulation of VEGF signaling by AFSC, mice were treated with a single injection of CM-DiI or Q-Dot tagged AFSC (the same cell line as published12) prior to the onset of a high level of proteinuria [Suppl. Figure 4A,B]. AFSC localized predominantly within the kidney, specifically in glomeruli [Fig. 4A–C and Suppl. Figure 4C,D] in close association with GEC as shown by a co-staining with VE-cadherin (endothelial cells) and CD2AP (podocytes) [Suppl. Figure 4D] after 5 days of delivery. Cells were not found in other organs in any significant number [Suppl. Figure 4E,F], as previously reported12. After 2 weeks of AFSC injection, the pVEGFR2/VEGFR2 ratio and VEGFR1 expression within the isolated glomeruli was comparable with that of the WT [Fig. 4G]. Although no significant changes in the VEGFR1/VEGFR2 ratio was observed between AFSC treated and non-treated animals, AFSC were able to significantly reduce VEGFR2 phosphorylation when total VEGFR2 expression remained unchanged [Fig. 4D]. After 7 weeks of injection, VEGF level was unchanged and sVEGFR1 expression was unchanged. 

Figure 3. VEGF signaling is altered in GEC isolated from Alport mice. VEGF signaling changes in GEC were studied using tdTomato-labeled GEC (tdT GEC) isolated by FACS from Alport-Tek
tdT glomeruli (A, 10x). tdT signal (red) is strongly present in all cells as shown in (B) (10x, two passages in culture) (n = 3). Graphs representing immunoblot data of pAKT/AKT (C, 60kDa/60kDa), pERK/ERK (D, 42kDa/42kDa), VEGFR1 expression (E, 151 kDa) in glomeruli of tdT GEC derived from WT mice and Alport-Tek
tdT mice at different ages. Imbalanced VEGF signaling is evident during disease progression in GEC, identified by a strong alteration in pAKT/AKT and pERK/ERK signaling downstream of VEGFR2. (F) Immunoblots of all the experimental groups used to generate the graphs presented in Figures C–E. Immunoblots were quantified by densitometry (VEGFR1 measurements were normalized against corresponding housekeeping gene, β-actin, 42kDa). These data were obtained using GEC derived from n = 4 WT at 5 months of age, n = 4 AS mice at 2 months of age, n = 6 AS mice at 3 month of age, n = 4 AS mice at 6 months of age. One-way ANOVA with Tukey’s post hoc test was used to analyze the data and scatter plot values are presented as mean ± SD (*p < 0.05).
AFSC homing to the kidney and modulation of VEGF, pVEGFR2, VEGFR1 and sVEGFR1. Alport mice were injected at 3 months of age to study homing of AFSC and modulation of VEGF signaling. Injected labeled-AFSC (CM-DiI, red) were traceable after 5 days and could be detected by fluorescence within Alport glomeruli (A, 20x). Representative image of isolated glomeruli from Alport mice after 5 days of injection showing CM-DiI labeled AFSC in red (B, 10x; C, higher magnification 40x). Injection of AFSC modulated expression of pVEGFR2/VEGFR2 ratio (D, 230 KDa/150 kDa), VEGFR1 (E, 151 KDa) and VEGFR1/VEGFR2 ratio (F) within the glomeruli of Alport mice (n = 3 AS mice) compared to non-injected siblings (n = 3 AS mice) after 2 weeks of injection, restoring the activity of these VEGFRs almost at normal levels. Immunoblots were quantified by densitometry (VEGFR1, VEGFR2 and pVEGFR2 measurements were normalized against corresponding housekeeping gene, β-actin, 42 KDa; pVEGFR2/VEGFR2 ratio was assessed after β-actin normalization). As shown in graph G–H, injection of AFSC modulated levels of VEGF and sVEGFR1 as measured by ELISA in Alport (n = 3 AS mice) versus non-treated mice (n = 3 AS mice) at 7 weeks after injection. Injection of AFSC also ameliorated serum creatinine (I) and proteinuria (J) in Alport mice (n = 6) compared to non-treated Alport mice (n = 6) and WT (n = 5) measured over 12 weeks period after AFSC injection. *Significant difference between WT and AS+AFSC; †Significant difference between WT and AS. *Significant difference between AS and AS+AFSC. One-way ANOVA with Tukey’s post hoc test was used to analyze the data in Fig. (D–H); Two-way ANOVA with Tukey’s post hoc test was used to analyze the data in Fig. (I,J); values in (I) and (J) are presented as mean ± SEM. All scatter plot values are presented as mean ± SD; (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 4. AFSC homing to the kidney and modulation of VEGF, pVEGFR2, VEGFR1 and sVEGFR1. Alport mice were injected at 3 months of age to study homing of AFSC and modulation of VEGF signaling. Injected labeled-AFSC (CM-DiI, red) were traceable after 5 days and could be detected by fluorescence within Alport glomeruli (A, 20x). Representative image of isolated glomeruli from Alport mice after 5 days of injection showing CM-DiI labeled AFSC in red (B, 10x; C, higher magnification 40x). Injection of AFSC modulated expression of pVEGFR2/VEGFR2 ratio (D, 230 KDa/150 kDa), VEGFR1 (E, 151 KDa) and VEGFR1/VEGFR2 ratio (F) within the glomeruli of Alport mice (n = 3 AS mice) compared to non-injected siblings (n = 3 AS mice) after 2 weeks of injection, restoring the activity of these VEGFRs almost at normal levels. Immunoblots were quantified by densitometry (VEGFR1, VEGFR2 and pVEGFR2 measurements were normalized against corresponding housekeeping gene, β-actin, 42 KDa; pVEGFR2/VEGFR2 ratio was assessed after β-actin normalization). As shown in graph G–H, injection of AFSC modulated levels of VEGF and sVEGFR1 as measured by ELISA in Alport (n = 3 AS mice) versus non-treated mice (n = 3 AS mice) at 7 weeks after injection. Injection of AFSC also ameliorated serum creatinine (I) and proteinuria (J) in Alport mice (n = 6) compared to non-treated Alport mice (n = 6) and WT (n = 5) measured over 12 weeks period after AFSC injection. *Significant difference between WT and AS+AFSC; †Significant difference between WT and AS. *Significant difference between AS and AS+AFSC. One-way ANOVA with Tukey’s post hoc test was used to analyze the data in Fig. (D–H); Two-way ANOVA with Tukey’s post hoc test was used to analyze the data in Fig. (I,J); values in (I) and (J) are presented as mean ± SEM. All scatter plot values are presented as mean ± SD; (*p < 0.05, **p < 0.01, ***p < 0.001).
Characterization of AFSC-derived EVs. Since EVs have been established as an important mechanism of cell-to-cell communication\(^{13-15}\) we hypothesized that secretion of EVs from AFSC could be one of their mechanisms of action. In order to test our hypothesis, EVs from culture supernatants of AFSC were isolated by sequential ultracentrifugation. By Nanosight analysis, EVs were characterized as a heterogeneous population ranging from 100 nm to 400 nm in size, and their production in basal culture conditions appeared as roughly \(2 \times 10^{10}\) EVs released by million cells in 24 hours [Fig. 5A]. AFSC-derived EVs expressed surface markers typical of the cell of origin (CD73 and CD29) and CD24, a marker of amniotic fluid-derived exosomes\(^20\) [Fig. 5B,C]. EVs also expressed VEGFR1 and VEGFR2 [Fig. 5B,C] but did not contain VEGF [Fig. 5D]. EVs contained miRNAs that specifically modulate VEGF/VEGFRs signaling\(^21\) including miR-16.1, miR-23a, miR-27a, miR-93, miR-221, miR-145 and miR-322 [Suppl. Figure 6A].

To demonstrate that AFSC-derived EVs can be transferred to GEC, AFSC were transduced with LentiBrite GFP packaged lentiviral particles to constitutively express GFP and additionally tagged with CM-DiI. After overnight incubation with GECs, red/green EVs were found within the cytoplasm of GEC in proximity to the perinuclear zone [Suppl. Figure 6B–I].

AFSC-derived EVs contribute to VEGF signaling modulation in GEC. To demonstrate EV ability to modulate VEGF activity and confirm the potential role of VEGFR1 and sVEGFR1 in GEC damage/repair processes we generated knockout (KO) EVs for Flt1, EVFR1\(^{-/-}\) (Flt1 gene codifies for both the VEGFR1 and sVEGFR1) by transfecting AFSC using a shRNA Lentiviral Particles Transduction system. The clone with the highest efficiency of transduction was used to derive and collect EVFR1\(^{-/-}\). Both, KO AFSC and derived EVs showed reduced VEGFR1 expression [Fig. 6A–D]. After generation of the EVFR1\(^{-/-}\) we exposed GEC to VEGF and treated them with normal EVs, KO EVs and neutralizing VEGF antibody (used as a positive control). pVEGFR2/VEGFR1 ratio and VEGFR1 expression were restored to baseline in the presence of EVs [Fig. 6E,G,I,K] but not with KO EVs, thus confirming that sVEGFR1 plays a role in regulating VEGFR1 expression and VEGFR2 activity. Importantly, EVs seem to have more efficiently downregulated VEGFR2 phosphorylation when compared with neutralizing antibody [Fig. 6E,F], and they both showed a similar effect when assessed against total VEGFR2 [Fig. 6G,H]. Interestingly, EVs but not anti-VEGF neutralizing antibody downregulated VEGFR1 expression in...
GEC [Fig. 6I,J]. We speculate that EVs might present multiple mechanisms of action (due to their cargo) that are lacking in the control antibody experiment. Of note, slight differences in VEGFR1 level between in vitro and in vivo data can be attributed to the fact that in vivo data shows expression of all the glomerular cell types, including GEC, whereas in vitro data only reflects GEC response.

Interestingly, GEC exposed to EVs showed a greater retention of VEGF within the media [Fig. 7A] compared to GEC exposed to KO EV. These data suggest that EVs (that do not contain VEGF as reported above) could trap excess VEGF contained in the media, thus preventing its binding to VEGFRs on GEC and internalization subsequently regulating VEGFR2 and VEGFR1 activity. To prove our hypothesis we performed a co-immunoprecipitation assay, using anti-VEGFR1 antibody to pull down VEGFR1 on the same culture media and probed for VEGF. Indeed, Fig. 7B shows a lower level of VEGF in the experimental group with KO EV.
compared to normal EVs. This data was confirmed independently using a different assay [Fig. 7C], where EVs and KO EVs were exposed to VEGF without the presence of GEC. Both these experiments confirmed that EVs can trap VEGF through VEGFR1, thus balancing VEGF signaling.

Direct administration of EVs into AS mice at 8 weeks of age and before the onset of a high level of proteinuria improved renal physiological parameters, including proteinuria and serum creatinine [Fig. 7D,E]. In particular, proteinuria level was significantly ameliorated in treated animals over many weeks as compared to non-treated mice [Fig. 7D].

Discussion
Various molecular signaling pathways contribute to the cell-cell communication between podocytes and GEC1,22 but VEGF expression plays a key role in the maintenance of the structure and function of glomerular capillaries including permeability, while its alteration plays a major role in CKD5. In normal animals, the blockade of VEGF correlates with GEC damage and an increase of proteinuria23. On the contrary, in diabetic mice, VEGF is upregulated, and its blockade ameliorates diabetic albuminuria24. Therefore, both deficiency and excess of VEGF appear to be detrimental to the physiological integrity of glomerular capillaries. An imbalance between VEGF and sVEGFR1 has been reported in many diseases, including the kidney25, as inducing widespread endothelial dysfunction, proteinuria, and hypertension.

Figure 7. VEGF modulation by AFSC-EVs in GEC and AFSC-EV effect on renal function. (A) Graph showing level of VEGF (24 KDa, monomer) within the media collected from GEC (basal condition, control), GEC stimulated with VEGF (100 ng/ml) and treated with EVs and KO EVs for 24 hr. VEGF level was higher in GEC stimulated with normal EVs compared to GEC stimulated with KO EV. Experiments were repeated in triplicate and data were quantified (measurements were normalized against VEGF basal level in GEC only group since Western Blot was performed in collected media; Western Blot bands are presented below the graph). (B) Representative immunoblot of VEGF after co-immunoprecipitation with VEGFR1 on the supernatant collected from experimental groups described in (A), showing the inefficiency of the KO EV to trap VEGF when compared to that of normal EVs. (C) Representative immunoblot of VEGF after co-immunoprecipitation with VEGFR1 on samples of PBS solution containing high VEGF dose (100 ng/ml) and treated with EVs and KO EVs for 24 hr further confirming the inability of the KO EV to effectively trap VEGF compared to normal EVs. The weak band detected in the PBS/VEGF only group represent a VEGF carryover due to incomplete removal of VEGF during the washing steps. Immunoblots were quantified by densitometry. (D) Injection of EVs ameliorated proteinuria in Alport mice (n = 8) compared to non-treated Alport mice (n = 8) and WT (n = 5) measured over 28 weeks period after EVs injection. (E) EV treatment also improved serum creatinine level within the same treated animals. Note: toward the end of the study at 28 weeks few mice were lost due to advanced disease. *Significant difference between AS and AS+AFSC. One-way ANOVA with Tukey’s post hoc test was used to analyze the data. All scatter plot values are presented as mean ± SD, (*) p < 0.05, (**) p < 0.01, (***) p < 0.001).
Important, accumulation of VEGF in human glomeruli was reported in various kidney diseases\textsuperscript{25-26}, including AS\textsuperscript{27}, thus supporting its importance in kidney disease. However, the biological role of VEGFR1/sVEGFR1 in the context of glomerular endothelial damage and its correlation with increased proteinuria is still unknown. Because GEC express an abundance of both VEGFR2 and VEGFR1, it is plausible that VEGFR1 and sVEGFR1 might have key importance in the regulation of the VEGF signaling activity in these cells.

Indeed, in our CKD animal model, we found that VEGF is elevated particularly early in the disease, reaching the maximum expression at 3 months of age, and then decreasing in more advanced stages. Interestingly, the level of sVEGFR1 within AS glomeruli also decreased after the peak of VEGF expression, thus possibly indicating that sVEGFR1 fails to effectively counter-balance the VEGF increase gradually causing glomerular damage. In an elegant study, Quaggin’s group\textsuperscript{28} identified that podocytes produce sVEGFR1 and that its elimination in mice (as evidenced by the regulation of proteinuria and serum creatinine), similar to that injected with AFSC. Of AFSC, we clearly demonstrated that EVs are capable of changing the course of disease progression in Alport mice, by triggering mechanisms of repair/regeneration at the transcriptional level. We focused on the VEGF signaling because of its importance in maintenance of the glomerular capillary network. Moreover, the presence of EVs can provide a functional benefit, similar to that of injection of AFSC. This effect could be due to EVs functioning as a “trap” for excess VEGF by binding to VEGFR1 presented on the EVs surface. We, therefore, speculate that modulation of VEGF within the glomeruli involves trapping of VEGF by VEGFR1 on the surface of AFSC-derived EVs. In this context, the EVs presenting VEGFR1 on their surface might counterbalance the decrease in sVEGFR1 observed during AS progression. It is also known that VEGF signaling disruption is a major occurrence in diabetic nephropathy, especially during the early phase of the disease\textsuperscript{3}. Therefore, we speculate that EVs might also regulate VEGF expression in diabetic nephropathy as well as in other forms of CKD with altered glomerular VEGF signaling. Importantly, despite the higher level of VEGFR2 over VEGFR1 on EVs, the affinity of the VEGF-VEGFR1 interaction is ten times stronger to that between VEGF-VEGFR2\textsuperscript{35}. This mechanism of action could potentially be expanded to other molecules (such as TGF\textbeta\textsuperscript{3} or ang II), since EVs present many different receptors. Downregulation of these molecules through “trapping” could thus be beneficial to resolving glomerular damage.

Recently, it was shown that EVs derived from AFSC contain specific miRNAs as cargo\textsuperscript{36,37}. Indeed, we also found that EVs contain miRs known to act in the modulation of VEGF levels (miR-16.1, -93), VEGF receptors (miR-16.1), as well as both positive and negative regulators of the VEGF signal transduction cascade (miR-23a, -27a, -221, -318). This angiomodulatory cargo could involve potential new mechanisms of VEGF regulation, by triggering mechanisms of repair/regeneration at the transcriptional level. We focused on the VEGF signaling because of its importance in maintenance of the glomerular capillary network. Moreover, the presence of a wide variety of surface markers as well as regulatory miRNAs within the EV, likely indicates potential of the AFSC-derived EVs to be able to regulate other important mechanisms essential to the glomerular homeostasis, and especially to the endothelium (such as the oxidative stress pathway, iNOS, TGF\textbeta\textsuperscript{3}, and Ang II). However this tantalizing finding requires further investigation and is beyond the scope of the current study.

Although in depth analyses would be required to determine if EVs account for all of the renoprotective effects of AFSC, we clearly demonstrated that EVs are capable of changing the course of disease progression in Alport mice (as evidenced by the regulation of proteinuria and serum creatinine), similar to that injected with AFSC. In conclusion, we demonstrated that 1. VEGF signaling plays an important role in the disruption of glomerular homeostasis and that injection of AFSC can restore the activity of this signaling pathway, possibly preventing...
further loss of kidney function. 2. Alterations in VEGF signaling affect the glomerular endothelium during early AS and before massive loss of podocytes and elevated proteinuria. 3. AFSC home within glomeruli in proximity to GEC and release "angiomodulatory" EVs. 4. AFSC-derived EVs can modulate VEGF signaling by podocytes and other glomerular cells (such as mesangial cells) that can contribute to the variation of VEGF during disease progression by trapping excess VEGF through VEGFR1-binding. Since AFSC localize within the glomeruli in our animal model, we speculate that the delivery of EVs by AFSC can specifically target VEGF signaling in this key renal compartment, thus favoring a correctly dosed, local therapeutic action of VEGF exactly where it is needed. Potential strategies to modulate glomerular cell crosstalk, specifically for preventing initiation and progression of GEC damage will strengthen the rationale for novel therapeutic approaches directed towards homeostatic regulation of glomerular function.

**Methods**

**AFSC culture, GFP infection and Flt1 knockout experiments.** Clonal lines of AFSC were derived as previously described12. For GFP transfection, AFSC were seeded at a density of 2.5 × 10^5/cm^2 and transduced with LentirBrite GFP packaged lentiviral particles (Millipore, #17-10387) at 35MOI. Transduction efficiency was assessed by inverted fluorescence microscope (Leica DMI6000 B). AFSC^GFP^ were selected by FACSD (BD, FACSAria III) and used for EV isolation.

AFSC^Flt1^−/− were generated by using a shRNA Lentiviral Particles Transduction system (Santa Cruz Biotechnology, sc-35395-V) following manufacturers’ instructions. Seeded AFSC (10,000/cm^2) were transduced at 37 °C. Cells were then passed through a 100 μm strainer and GEC were flow sorted using FACSAria III (Becton Dickinson).

Animal models (WT mice, Alport mice, WT- Alport-Tek^tdT^, Alport-Tek^tdT^), AFSC and EV injections and AFSC in vivo tracking, serum creatinine, and proteinuria measurements. All animals were purchased from the Jackson Laboratory12. The Alport-Tek^tdT^ mice were generated by breeding Alport mice (B6.Cg-Col4a5tm1Yseg/J) with an endothelial specific Cre-driver mouse (B6.Cg-Tg(Tek-cre)1Ywa/J); these mice express tdTomato (tdT) in all endothelial cells including GEC. To study disease progression n = 24 WT and n = 24 Alport mice were sacrificed at 1, 2, 3, 4, 5 and 6 months of age (n = 4/time point) to isolate glomeruli. For TEM analysis n = 3 of WT and n = 3 of Alport mice were used. For in vivo experiments n = 10 of WT, n = 10 of Alport mice non-injected and n = 10 Alport mice injected with AFSC were used. Mice were injected with 1 × 10^5 AFSC through the left ventricle at 3 months of age, before an onset of proteinuria as published12. Mice were sacrificed at 2 and 7 weeks post injections. In addition, Alport mice were injected with the equivalent number of EV (2.0 × 10^11) as produced by 1 × 10^9 AFSC (n = 8). Non-injected, aged matched Alport mice (n = 8) and WT mice (n = 5) were served as control. 5 WT-Tek^tdT^ were used for flow cytometry data.

For cell tracking, 6 Alport mice were injected with AFSC pre-labeled with Qdot (or CM-DiI) immediately before the procedure (Invitrogen). The animals were killed at 5 days (n = 3), and 10 weeks (n = 3) after treatment, and the heart, kidney, liver, and lung were processed for FACS analysis as previously described12. A non-injected littermate served as the negative control throughout the analysis for each time point.

Renal function was assessed as previously described12. Of note, we refer to early stage AS when the level of proteinuria is < 3 g/g (up to 3 months of age), to middle stage AS when proteinuria is ~ 10-13 g/g (around 4 months of age) and to late stage AS when proteinuria is > 20 g/g (5 months of age and beyond). Experiments were performed in adherence to the NIH Guidelines for the Care and Use of Laboratory Animals and with Children’s Hospital Los Angeles Institutional Animal Care and Use Committee (IACUC) approval. Total number of mice used in experiments is 177: WT mice (n = 33), Alport mice (n = 24), WT-Tek^tdT^ (n = 24), Alport-Tek^tdT^ (n = 23).

Glomeruli and GEC isolation. Glomerulus isolation was performed as previously described12 and GEC from WT-Tek^tdT^ (n = 7) and Alport-Tek^tdT^ (n = 20) were isolated by further digesting the glomeruli with 0.25% trypsin (Gibco, ThermoFisher Scientific) / 0.6% collagenase IV (Worthington) solution in media supplemented with phosphatase inhibitors (ThermoFisher Scientific) for 20' at 37 °C. Cells were then passed through a 100μm strainer and GEC were flow sorted using FACSAria III (Becton Dickinson).

EVs isolation, characterization and labeling. EVs were isolated from supernatants of AFSC cultured overnight in RPMI-1640 (Lonza) without serum13. Supernatants were centrifuged at 6,000 × g for 20' and ultracentrifuged at 100,000 × g (Optima L-100K ultracentrifuge; Beckman Coulter) for 2 hours at 4 °C; pellets were resuspended in serum-free RPMI-1640 containing 1% DMSO and stored at ~ 80 °C until use. EVs were characterized by cytfluorometric analysis14, using fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated rat antibodies. AFSC-EVs (1 × 10^10 particles) were incubated for 15' at 4 °C and immediately acquired by FACS analysis using a Guava easyCyte™ Flow Cytometer (Millipore) and analyzed with Incyte software (See list in Supplementary Material and Methods). Fluorochrome conjugated rat non-immune isotypic immunoglobulin G (Miltenyi Biotec) was used as a control. The size and distribution of the AFSC-EVs were analyzed using a NanoSight LM10 instrument (NanoSight Ltd.) equipped with the nanoparticle tracking analysis (NTA) 2.0 analytic software.

RNA isolated using the mirVana RNA isolation kit (Ambion), was analyzed using miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (both from Qiagen). Fold change in miRNA expression was calculated as 2^-ΔΔCt using the snoRNA RNU6b as normalizer18. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a 96-well StepOne™ Real-Time System (Applied Biosystems) in order to analyze the EVs miRNA content. The sequence-specific oligonucleotide primers were all obtained from MWG-Biotech (www.mwg-biotech.com, see list in Supplementary Materials and Methods).
Western blotting, immunostaining, TEM, GEC quantification, VEGF and sVEGFR1 ELISA, PCR, PCR array, flow cytometry. Western blotting, immunostaining, PCR and PCR array, flow cytometry were performed as previously published. Details are described in the Supplementary Materials and Methods. TEM analysis was performed as published and expression for VEGF and sVEGFR1 in glomerular extracts, serum and supernatants were assessed by ELISA according to manufacturers’ instructions (RayBiotech, #ELM-VEGF and #ELM-VEGFR1). GEC fenestration size was evaluated in 20 randomly selected TEM images per sample (n = 3) with a field of view at 28,000x. The surface areas of the fenestrations were quantified by ImageJ software (NIH). All measurements were done in a double-blinded fashion.

Alport (n = 3) and WT (n = 3) mice were administered with FITC-WGA lectin (from Triticum vulgaris; 6.25 mg/kg body wt; Ab20528; Abcam, MA) via an intracardiac injection and sacrificed after 30 minutes. Kidneys were snap frozen and 5μm sections were processed for confocal microscopy. Images were taken with Leica Zeiss 710 microscope and analyzed using the ZEN10 software.

Co-immunoprecipitation assay. To evaluate VEGF/VEGFR1 interactions between VEGFR1 expressed by EVs and the excess of VEGF described in the in-vitro experiments, immunoprecipitation for VEGFR1 using VEGFR1 antibody and Protein G-agarose conjugate (Santa Cruz) was applied overnight at 4 °C. Immunoprecipitates were collected by centrifugation at 1,000 × g for 5′ at 4 °C, washed with PBS and resuspended in electrophoresis sample buffer, denatured and ran under reducing conditions as previously described. The immunoblots were then probed for VEGF detection using VeriBlot-HRP conjugated secondary antibody (Abcam) and standard Western blotting techniques as previously described.

In vitro co-culture of GEC-AFSC and GEC-EVs. GEC were seeded at 1 × 10^4 cells/cm^2 at 37 °C and 5% CO2, in medium supplemented with 0.1% VEGF, 0.1% ECGS, 0.1% Heparin, 0.1% EGF, 0.1% Hydrocortisone, 1% L-Glutamine, 1% antibiotic-antimycotic solution, 5% FBS (CellBiologics). GEC were overstimulated with recombinant VEGF (Gibco, ThermoFisher Scientific) at 100ng/ml. Simultaneously, AFSC (ration 1:1, on transwell inserts, Corning) or EVs and EV*Flt1−/− (10,000:1 ratio, on culture media) were co-cultured with GEC under the same growth conditions. Experiments were terminated at 24 hours and culture media and cells were collected for analysis. Integration of EVs into GEC was performed by applying EVs with green and red fluorescence tags derived from AFSCGFp co-labeled with CM-Dil as previously reported to GEC overnight. Integration of EV into GEC was assessed by inverted fluorescence microscope (Leica DMI6000 B). Experiments were repeated in triplicate.

Statistical analysis. All test populations were assumed to have Gaussian distribution and equal variance. Data shown in bar graphs are expressed as means ± SEM. Two-tailed Student’s t-test was used for comparisons between two groups. One-way ANOVA with Tukey’s post hoc test was applied for comparison of three or more groups for the same time point. Two-way ANOVA with Tukey’s post hoc test was applied for comparison of three or more groups between different time points (Fig. 4LJ). All statistical analysis was done with graphPad Prism 7.0a (GraphPad Software, Inc.). A p-value less than 0.05 was considered statistically significant.

Data availability statement. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions
Sargs Sedrakyan designed and executed the in vitro studies, histological and functional studies. He performed statistical analysis, interpretation of results, prepared the Manuscript and all the Figures. Valentina Villani contributed to generating of the Western Blot data in Figs 1, 3, 4, 6, 7 and Suppl. Figure 1. Stefano Da Sacco provided Figure editing support and reviewed the Manuscript. Nikita Tripuraneni and Maria Lavarreda-Pearce generated and maintained the experimental animals. Nikita Tripuraneni contributed to Figs 1, 4 and 7. Stefano Porta performed EV characterization and contributed to Fig. 5 and Suppl. Figure 6A. Andrea Achenaa contributed to Suppl. Figure 6B–I. Astigik Petroyan and Hasmik Soloray contributed to Fig. 4A–C and Suppl. Figure 5. Roger E De Filippo provided editing and reviewing of the Manuscript. Benedetta Bussolati provided the characterization of EVs, contributed to the study design and writing and reviewing of the Manuscript. Laura Perin contributed to the study design, study coordination, interpretation of the data and writing of the Manuscript. All authors reviewed the Manuscript.

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