Darbepoetin alfa protects podocytes from apoptosis *in vitro* and *in vivo*

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Detachment or apoptosis of podocytes leads to proteinuria and glomerulosclerosis. There are no current interventions for diabetic or non-diabetic glomerular diseases specifically preventing podocyte apoptosis. Binding of erythropoiesis stimulating proteins (ESPs) to receptors on non-hematopoietic cells has been shown to have anti-apoptotic effects *in vitro*, *in vivo*, and in preliminary human studies. Recently, erythropoietin receptors were identified on podocytes; therefore, we tested effects of darbepoetin alfa in preventing podocyte apoptosis. Cultured immortalized mouse podocytes were treated with low-dose ultraviolet-C (uv-C) irradiation to induce apoptosis in the absence or presence of darbepoetin alfa. Apoptosis was quantified by Hoechst staining and by caspase 3 cleavage assessed by Western blots. Pretreatment with darbepoetin alfa significantly reduced podocyte apoptosis with this effect involving intact Janus family protein kinase-2 (JAK2) and AKT signaling pathways. Additionally, darbepoetin alfa was found protective against transforming growth factor-β1 but not puromycin aminonucleoside induced apoptosis. Mice with anti-glomerular antibody induced glomerulonephritis had significantly less proteinuria, glomerulosclerosis, and podocyte apoptosis when treated with darbepoetin alfa. Our studies show that treatment of progressive renal diseases characterized by podocyte apoptosis with ESPs may be beneficial in slowing progression of chronic kidney disease.

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Primary function of erythropoietin (EPO) is to prevent apoptosis of developing erythroid precursor cells. Additionally, EPO works synergistically with multiple other growth factors to promote differentiation and proliferation of these erythroid progenitor cells.[1] Recently, the EPO receptor has been identified on non-hematopoietic tissues.[2,3] Similar to the effects on the developing erythroid cell, EPO has potent cell survival effects once it is bound to its tissue receptor. Toxic and ischemic models of central and peripheral neuronal injury have shown a profound anti-apoptotic effect for EPO in cell culture, animal models, and preliminary human studies.[4] EPO exhibits a prosurvival role in models of myocyte ischemia as well as toxic and hypoxic renal tubular injury.[5] In conjunction with other growth factors, EPO also plays a role in the proliferation and differentiation of non-erythroid cell lines such as astrocytes.[6] Other erythropoiesis stimulating proteins (ESPs) and non-hematopoietic derivatives of EPO, such as asialoEPO[7] and carbamylated EPO,[8,9] have demonstrated beneficial effects in non-hematopoietic tissues.

EPO exerts a hematopoietic function by triggering intracellular phosphorylation events that lead to activation of prosurvival pathways. The well-defined signaling mechanisms associated with erythroid precursor survival are multiple and redundant, culminating in the end point of being anti-apoptotic. After EPO binds to its receptor, there is activation of Janus family protein kinase-2 (JAK2), and downstream signaling cascades including STAT5, nuclear factor-κB, and phosphoinositide-3 kinase with subsequent phosphorylation of AKT.[4] The mechanisms by which EPO or ESPs are anti-apoptotic in non-hematopoietic tissues are thought to be similar in some aspects to the erythroid precursor cell-survival pathways but this has not been completely elucidated and is potentially cell specific.[10]

Owing to their quiescent phenotype, podocytes have a limited ability to perform a controlled re-entry into the mitotic cell cycle.[11] Therefore, when podocytes are reduced in number following injury, either by shedding of detached viable podocytes into the urine or apoptosis, they are typically not replaced. Podocyte loss, experimentally and in human disease, has been linked to the development of proteinuria and glomerulosclerosis,[12] and is predictive of the progression of diseases such as diabetes[13–16] and IgA nephropathy.[17] Although our group has shown recently that dexamethasone protects podocytes from apoptosis *in vitro*,[18]

there are no published therapies to prevent podocyte loss in diabetic or non-diabetic glomerular disease.

Recently, EPO receptors have been identified on podocytes in fixed tissue,[19] raising the possibility that podocytes *in vitro* and *in vivo* can respond to hematopoietic growth factor stimulation. The purpose of this study was to define the effects of ESPs on podocytes. Our hypothesis is that ESPs are prosurvival for podocytes both *in vitro* and *in vivo*. Our results show that the ESP darbepoetin alfa indeed protects podocytes from apoptosis and *in vivo* therapy with darbepoetin alfa results in less proteinuria and glomerulosclerosis in a mouse model of glomerulonephritis.

RESULTS

The erythropoietin receptor is expressed on cultured podocytes

We have reported previously on the characterization of immortalized heat-sensitive cultured mouse podocytes used in this study.[20–22] To ensure that these cells express the receptor for ESPs, we performed immunostaining and Western blot analysis. Both techniques demonstrated that podocytes used in the current study express the erythropoietin receptor, forming the rationale for further investigation (Figure 1).

Darbepoetin alfa protects cultured podocytes from apoptosis induced by low-dose uv-C, TGF-β1 but is not protective against puromycin aminonucleoside-induced apoptosis

EPO and other ESPs have been shown to be protective against neuronal cell apoptosis induced by a variety of stimuli.[4,8,23]

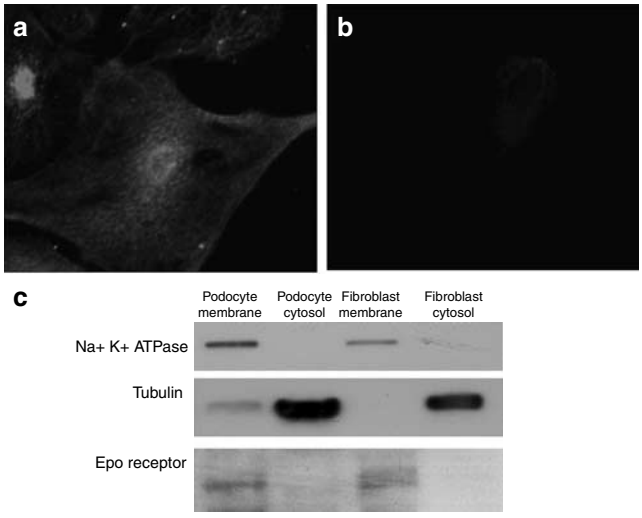

Figure 1 | The EPO receptor is expressed on cultured mouse podocytes. (**a**) Immunofluorescence staining performed on fixed cells demonstrated that podocytes in culture express the EPO receptor. (**b**) Negative control, omitting the primary antibody. (**c**) Western blot analysis of enriched membrane and cytosolic protein fractions. Lanes 1 and 3 are enriched membrane fractions as shown by Na^+/K^+-ATPase staining. Lanes 2 and 4 are cytosolic fractions (negative controls) as shown by tubulin staining. Probing the polyvinylidene difluoride membrane with an anti-EPO receptor antibody identified a band at approximately 55 kDa, the expected molecular weight of the EPO receptor in the membrane fractions of the podocytes (lane 2) and renal fibroblasts (lane 4, positive control).

We hypothesized that ESPs would be protective against podocyte apoptosis, and we induced apoptosis in cultured podocytes using ultraviolet-C (uv-C) irradiation, transforming growth factor-β1 (TGF-β1), and puromycin aminonucleoside (PA). Because protection against apoptosis in other cell types typically requires pre-exposure to ESPs, darbepoetin alfa was first applied from 0 to 6 h before the induction of apoptosis. Podocytes required a minimum of 3 h of pre-exposure to darbepoetin alfa before a statistically significant protective benefit was achieved (Figure 2a). Dose titration experiments showed that exposing podocytes to darbepoetin alfa 3 h before the induction of apoptosis significantly reduced uv-C-induced apoptosis in a dose-dependent manner (Figure 2b and c). Additionally, uv-C dose titration experiments were performed which showed that darbepoetin alfa protects against low-dose uv-C-induced apoptosis. In contrast, darbepoetin alfa did not reduce necrosis induced by high doses of uv-C (data not shown). Apoptosis was verified further by Western blot analysis of caspase cleavage, as uv-C irradiation is known to induce caspase cleavage (Figure 2d).[24] Densitometry analysis of Western blots for caspase and the caspase cleavage product showed that pre-exposure of podocytes to darbepoetin alfa before uv-C irradiation results in a substantial decrease in the ratio of total caspase to caspase cleavage fragments from 75 to 40%.

DNA damage is the primary method by which uv-C irradiation induces apoptosis.[24,25] To ensure that pre-exposure with darbepoetin alfa did not interfere with the generation of DNA damage by uv-C, single-cell gel electrophoresis (Comet assay) was performed to quantify DNA strand breaks. Our data showed that there was an equivalent percentage of comet tails in control podocytes and darbepoetin alfa exposed podocytes, indicating that the amount of DNA damage induced by uv-C was equivalent and that darbepoetin alfa did not interfere with the generation of DNA damage, the apoptotic trigger (Figure 2e).

TGF-β1[20,26–28] and PA[20,29] have been shown to induce podocyte apoptosis *in vitro* and *in vivo*. To determine if ESPs universally protect podocytes against apoptotic triggers, we induced apoptosis with TGF-β1 (5 ng/ml) and PA (10–50 μg/ml) after pre-exposing cells to darbepoetin alfa. Figure 3 shows that darbepoetin alfa was protective against TGF-β1-induced apoptosis. Without darbepoetin alfa, the rate of apoptosis was 3.35% after 38 h of exposure to TGF-β1 versus 1.5% with darbepoetin alfa pre-exposure ($P<0.001$) (Figure 3a). In contrast, darbepoetin alfa had no impact on the quantity of apoptosis induced by PA (Figure 3b).

Darbepoetin alfa does not induce proliferation of podocytes

Exposure of neuronal and endothelial cells *in vitro* to high concentrations of EPO results in activation of the cell cycle and proliferation.[6,30] In contrast to these cells and other glomerular cell types, podocytes typically do not proliferate in response to injury. Thus, to determine if high dose darbepoetin alfa exposure induces podocyte proliferation, we utilized two approaches. First, we performed the 3-[4,5]

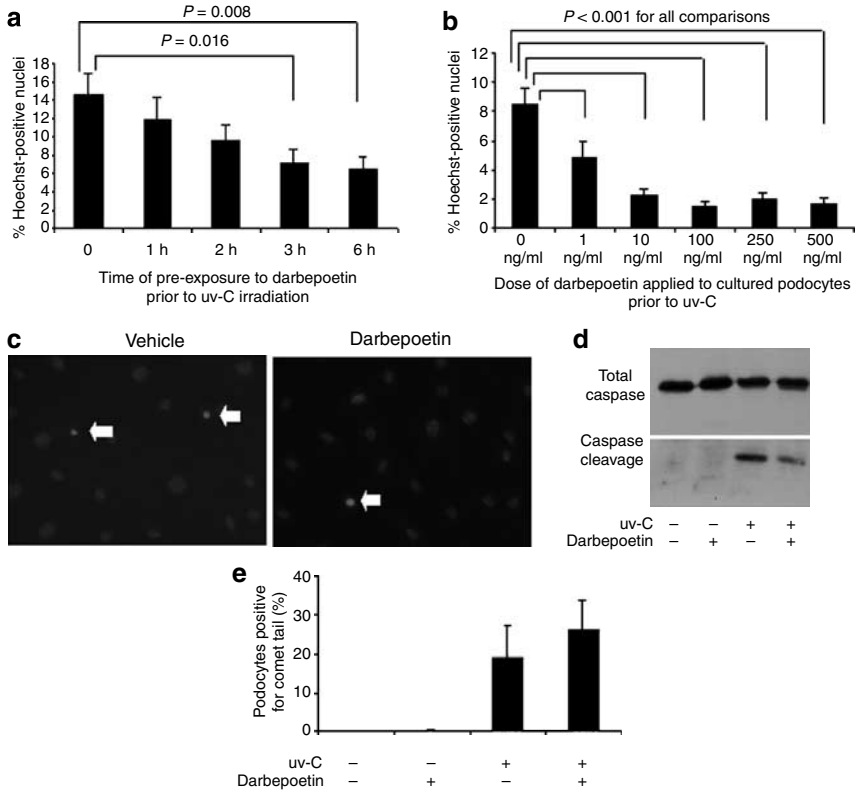

Figure 2 | Darbepoetin alfa protects podocytes from apoptosis induced by uv-C. (**a**) Darbepoetin alfa is protective against apoptosis induced by uv-C irradiation after 3 h of pre-exposure. Heat-sensitive mouse podocytes were pretreated for 0–6 h with darbepoetin alfa (100 ng/ml) and then exposed to uv-C irradiation (10 J/m^2). Apoptosis was measured at 6 h by Hoechst 33342 staining. When darbepoetin alfa is applied immediately after uv-C, at this dose of uv-C, there was 14.6% apoptosis which decreased to 11.8% with 1 h of pre-exposure, 9.58% after 2 h of pre-exposure, 7.2% after 3 h, and 6.6% after 6 h of pre-exposure to darbepoetin alfa. Statistically significant anti-apoptotic effects were seen after 3 h of pre-exposure ($P = 0.016$ for 3 h pre-exposure compared with no pre-exposure and $P = 0.008$ for 6 h of pre-exposure, $P =$ not significant (NS) for other time points). (**b**) Darbepoetin alfa is protective against apoptosis induced by uv-C irradiation in a dose-dependent manner. Cultured podocytes are pre-exposed for 3 h to darbepoetin alfa (varying doses 0–500 ng/ml) and then exposed to uv-C irradiation (5 J/m^2). Apoptosis was measured at 6 h by Hoechst staining. ($P < 0.001$ for each dose compared to 0 ng/ml). (**c**) Representative Hoechst 33342 staining for vehicle and darbepoetin alfa (100 ng/ml) pre-exposed podocytes 6 h after uv-C irradiation showing more apoptotic nuclei (arrows) in the vehicle-treated cells. (**d**) Uv-C irradiation primarily induces caspase-dependent apoptosis. Western blot analysis of total caspase 3 and caspase 3 cleavage fragments shows decrease in caspase activation due to uv-C irradiation in darbepoetin alfa-exposed podocytes compared to vehicle-exposed cells. (**e**) Darbepoetin alfa does not interfere with the generation of DNA damage by uv-C irradiation. Single-cell gel electrophoresis (Comet assay) was performed to measure induction of DNA damage by uv-C irradiation. As expected, control podocytes (column 1) and podocytes exposed to darbepoetin alfa, but not uv-C irradiation (column 2), have almost no detectable DNA damage at baseline. After DNA damage is induced by uv-C, regardless of exposure to darbepoetin alfa (columns 3 and 4), podocytes develop equivalent amounts of DNA damage. ($P = 0.53$ for comparison of uv-C and uv-C + darbepoetin alfa-treated cells).

dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay, which is an assay of cell viability and cell number. Figure 4 shows that darbepoetin alfa had no effect on proliferation of cultured podocytes grown under growth permissive (i.e., undifferentiated and proliferating) and restrictive (i.e., differentiated and quiescent) conditions. Second, 5-bromo-2-deoxyuridine (BrdU) staining was utilized as a marker for cell proliferation in proliferating cultured podocytes after exposure to a wide range of darbepoetin alfa doses (0–500 ng/ml). Immunostaining for BrdU in podocytes did not show an increase in DNA incorporation (data not shown). Taken together, these results demonstrate that darbepoetin alfa does not alter or induce podocyte proliferation in the mature, differentiated podocyte in culture nor affect the rate of proliferation of the undifferentiated cultured podocyte, and that any benefit on survival were unlikely due to alterations in cell cycle.

Antiapoptotic effect of darbepoetin alfa requires intact JAK2 and AKT pathways

After binding of ESPs to the EPO receptor, studies in non-renal cells have shown that there is activation of a variety of intracellular signaling pathways that result in a prosurvival effect. Activation of JAK2/STAT pathway[31] and the downstream of phosphoinositide-3 kinase/AKT pathway have been shown to be critical to the anti-apoptotic effects of ESPs in neuronal cells.[4,32] We investigated the role of these crucial pathways in podocytes by incubating cultured podocytes with the JAK2 inhibitor, AG490, and with AKT inhibitors before induction of apoptosis with uv-C. Our results show

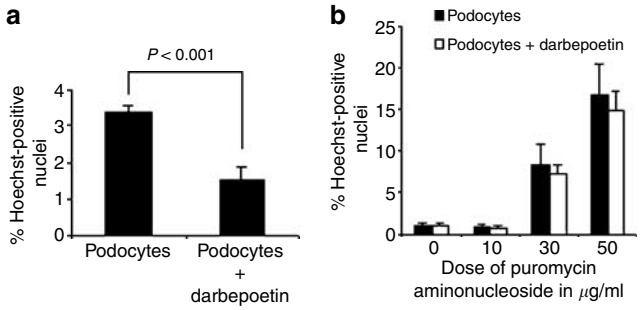

Figure 3 | Darbepoetin alfa protects podocytes from apoptosis induced by TGF-β1 but not puromycin aminonucleoside. (**a**) Cultured podocytes are protected from apoptosis induced by TGF-β1 by darbepoetin alfa. TGF-β1 (5 ng/ml) induced apoptosis in podocytes was measured by Hoechst staining at 38 h. Apoptosis was decreased from 3.3 to 1.5% (*$P<0.001$) with darbepoetin alfa pre-exposure. (**b**) Darbepoetin alfa does not protect podocytes from apoptosis induced by PA. PA (10, 30, and 50 μg/ml) induced cultured podocyte apoptosis, measured by Hoechst staining at 48 h. Apoptosis was not significantly reduced by pre-exposure to darbepoetin alfa.

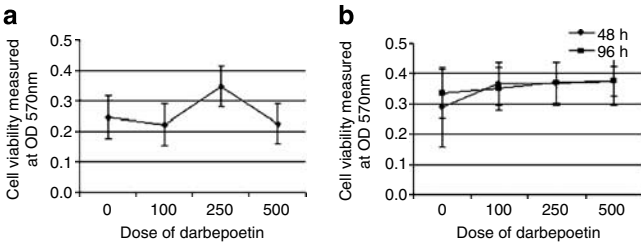

Figure 4 | Darbepoetin alfa does not induce proliferation of cultured podocytes. (**a**) Undifferentiated and proliferating podocytes. After 48 h of exposure to a range of doses of darbepoetin alfa (0–500 ng/ml), the MTT assay was performed. Podocyte viability and therefore cell number is equivalent in the exposed versus unexposed, cultured, undifferentiated, and proliferating podocytes, ($P>0.2$ for every concentration compared to 0 ng/ml) demonstrating that darbepoetin alfa exposure does not accelerate podocyte proliferation. (**b**) Differentiated and quiescent podocytes. The MTT assay was performed after 48 and 96 h of exposure to darbepoetin alfa. These results demonstrate that darbepoetin alfa does not induce quiescent podocytes to proliferate ($P>0.2$ for every dose compared to 0 ng/ml).

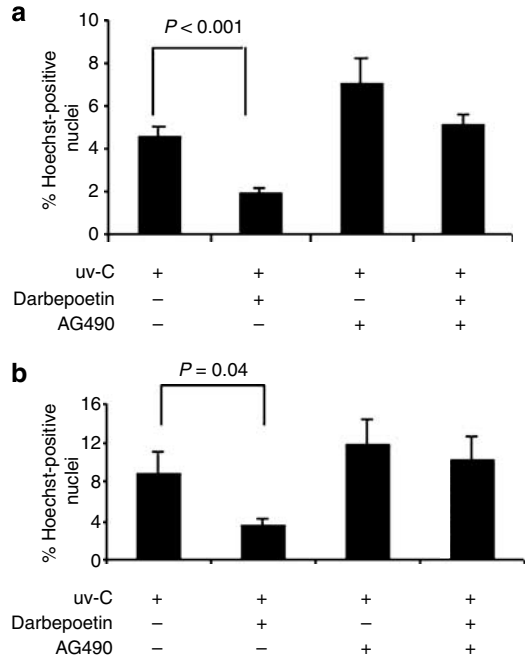

Figure 5 | Inhibition of JAK2 or AKT phosphorylation eliminates the protective effects of darbepoetin alfa on podocyte apoptosis induced by uv-C. (**a**) Podocytes were incubated for 24 h with the JAK2 inhibitor AG490 (20 μM) and then apoptosis was induced with uv-C irradiation with or without darbepoetin alfa pre-exposure. In the presence of AG490, the anti-apoptotic effect of darbepoetin alfa is abolished with 6.92% apoptosis in the AG490 + uv-C-exposed podocytes versus 5.07% in the AG490 + darbepoetin alfa + uv-C-exposed cells ($P=0.17$). (**b**) Podocytes were incubated for 24–36 h with the AKT inhibitor, AKT IV, and then apoptosis was induced by uv-C irradiation. In the presence of the AKT inhibitor, there was similar amount of apoptosis in the darbepoetin alfa exposed (10.2%) and vehicle-exposed podocytes (11.8%) ($P=0.66$). Controls for these experiments included podocytes incubated with vehicle or inhibitor alone. Incubation with AKT IV but not AG490 or AKT VII induces an increase in podocyte apoptosis at 32 h of 3.9%, likely accounting for the higher rate of apoptosis in AKT IV exposed podocytes.

that the protective effect of darbepoetin alfa was abolished after incubation with either AG490 (Figure 5a) or AKT IV (Figure 5b) and AKT VII inhibitor (data not shown), indicating that these pathways were critical in the regulation of apoptosis in cultured podocytes and that the survival benefits of darbepoetin alfa on cultured podocytes were mediated in part through these pathways.

In vivo studies

The results shown above were performed in cultured immortalized mouse podocytes. To validate these findings *in vivo*, we utilized a mouse model of acute nephrotoxic glomerulonephritis induced by intraperitoneal injection of sheep anti-rabbit glomerular antibody, which is characterized by podocyte apoptosis, dedifferentiation, and proliferation, to test the hypothesis that darbepoetin alfa protects podocytes from apoptosis *in vivo*. In this model, nephritic mice develop azotemia, proteinuria, glomerulosclerosis, and crescents composed of proliferating podocytes.[22,33,34] Similar to the *in vitro* study design, the mice in the experimental groups were pretreated with vehicle (group 2, $n=6$) or darbepoetin alfa (group 3, $n=13$) 24 h before induction of the animal model. To insure that darbepoetin alfa itself had no effect on podocytes *in vivo*, a control group (group 1, $n=9$) included animals injected with darbepoetin alfa in the absence of the disease-inducing anti-glomerular antibody.

Mice were injected daily with darbepoetin alfa (25 ng/g) or vehicle for 10 days. Nephritis was induced in the experimental groups on days 2 and 3 with injection of the anti-glomerular antibody. Immunostaining for sheep and mouse IgG in darbepoetin alfa and vehicle-treated animals demonstrated that anti-glomerular antibody deposition and the autologous phase immune response were similar in both groups, indicating that pretreatment with darbepoetin alfa did not affect disease induction (data not shown). As shown

in Table 1, all mice treated with darbepoetin alfa had a significant increase in hematocrit. Control animals from group 1, which received darbepoetin alfa only, had an increase in serum blood urea nitrogen (BUN) from 35 mg/dl at baseline to 61.7 mg/dl at day 10 ($P<0.001$). Both of the anti-glomerular antibody-treated experimental groups had their BUN increase to 52 mg/dl. Although this was significantly above the baseline BUN of 35 mg/dl ($P<0.04$), the difference between nephritic mice given darbepoetin alfa or vehicle was not statistically significant at day 10. Therefore treatment with high-dose darbepoetin alfa did not improve azotemia in this model.

Table 1 | Baseline characteristics of control and treatment groups

Experimental group	1	2	3
Antiglomerular antibody	–	+	+
Darbepoetin	+	–	+
Day 0 HCT	49.8	52.8	53.1
Day 10 HCT	68.7	46.2	66.1
Day 10 BUN	61.7	52.3	52.7

BUN, blood urea nitrogen; HCT, hematocrit.
The first group of animals was injected with darbepoetin alfa alone. This group had a significant increase in their HCT from 49.8 to 68.7 at day 10 as well as a significant increase in BUN from baseline 43 to 61.7 mg/dl at day 10 ($P<0.001$) probably due to induction of renal vasoconstriction and a decrease in renal blood flow after 10 days of treatment with high-dose darbepoetin alfa. Group 2 received the antiglomerular antibody disease induction and vehicle treatment intraperitoneal. As expected, this group had a decrease in HCT from baseline and an increase in BUN from 43 to 52.3 mg/dl. Group 3 received the antiglomerular antibody disease induction with darbepoetin alfa treatment. These animals had an increase in HCT from 53 to 66.1 and an increase in BUN from 43 to 52.7 mg/dl. Although the increases in BUN from baseline to day 10 was significant in both groups 2 and 3 ($P<0.04$ for both), there was no significant difference in BUN between these two groups at the end of the study.

Darbepoetin alfa improves glomerulosclerosis and reduces proteinuria

All animals injected with anti-glomerular antibody developed acute glomerulonephritis as determined by histological examination by periodic acid-Schiff staining. Glomeruli were analyzed for the presence of mesangial expansion, focal and global glomerulosclerosis, and crescent formation by a semiquantitative score based on percentage of glomerular tuft involvement in the sclerotic process and the glomerulosclerosis index was calculated.[21,35] Characteristic glomeruli from each group of animals are shown in Figure 6a–c. The glomeruli from nephritic vehicle-treated animals (group 2) had significantly more of the characteristic segmental sclerosis than the darbepoetin alfa-treated animals (group 3), and this was shown quantitatively by the higher glomerulosclerotic index ($P=0.016$) (Figure 6d). Darbepoetin alfa treatment alone did not cause any significant glomerulosclerosis in group 1 animals.

As shown in Figure 7, darbepoetin alfa treatment alone (group 1) resulted in a minimal change in proteinuria from baseline over the 10 days ($11\pm24\%$). In contrast, nephritic animals had a significant increase in the percentage change in urine protein excretion from baseline ($446\pm72\%$). Nephritic animals treated with darbepoetin alfa had a significant

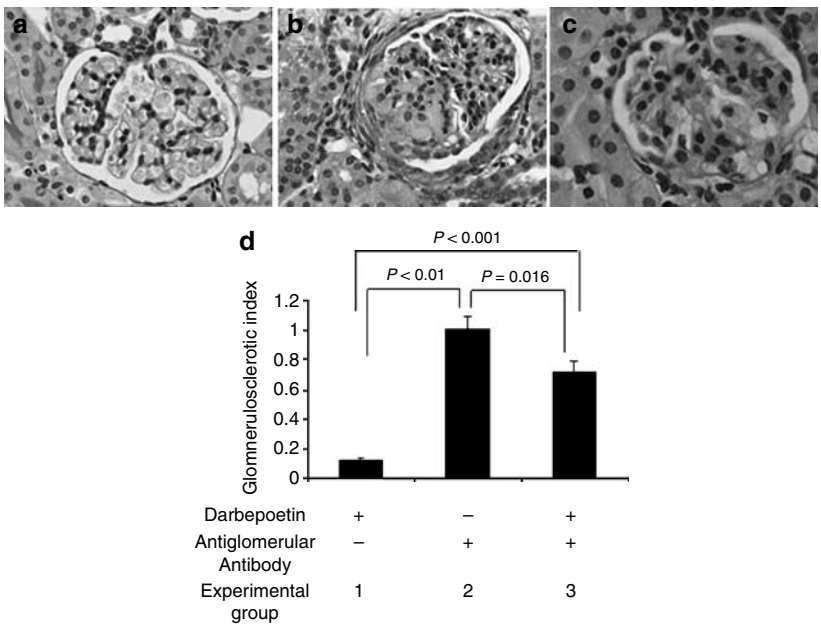

Figure 6 | Periodic Acid-Schiff (PAS) staining in mice with anti-glomerular antibody acute glomerulonephritis at day 10. (**a**) Representative glomerulus from group 1 mouse treated with darbepoetin alfa alone showing normal mouse phenotype. (**b**) A glomerulus characteristic of a nephritic, vehicle-treated mouse (group 2) showing segmental sclerosis typical of this model. (**c**) Mesangial expansion and glomerular sclerosis is seen in both vehicle and darbepoetin alfa-treated animals but glomerular architecture is better preserved and there is less sclerosis in the darbepoetin alfa-treated nephritic animals (group 3). (**d**) Glomerulosclerosis index. Kidneys from mice receiving darbepoetin alfa alone had a very low degree of glomerular sclerosis as expected. Kidneys from nephritic, vehicle-treated mice (group 2) exhibited a significantly greater disease severity than darbepoetin alfa-treated nephritic mice (group 3) as quantified by the glomerulosclerosis score decreasing from 1.01 to 0.715 with darbepoetin alfa treatment ($P=0.016$).

blunting in their development of proteinuria with a change in protein excretion of only 213±53% ($P=0.017$).

Darbepoetin alfa reduces apoptosis *in vivo*

To determine if ESPs have a similar anti-apoptotic effect *in vivo* that we have demonstrated *in vitro*, we assessed podocyte apoptosis following acute experimental glomerular injury by terminal deoxy transferase uridin triphosphate nick end labeling (TUNEL) staining. Characteristic glomeruli from nephritic animals treated with vehicle or darbepoetin alfa are shown in Figure 8. There was a significant decrease in podocyte apoptosis in nephritic animals treatment with darbepoetin alfa, with the number of TUNEL-positive cells per glomerular cross-sectional area displayed in a podocyte distribution decreasing from 0.1 to 0.05 with darbepoetin alfa therapy ($P=0.0045$). Additionally, WT-1 staining was performed to quantify podocyte number. At this early time point, we were not able to detect a significant change in podocyte number by WT-1 staining, with 7.12±0.12 WT-1-positive cells on average per glomerulus in group 1 mice compared with 7.06±0.4 WT-1-positive cells per glomerulus in group 2 and 7.14±0.3 WT-1-positive cells per glomerulus in group 3 mice. ($P=$ not significant (NS) for comparisons between all groups.)

DISCUSSION

There is mounting evidence demonstrating that podocyte apoptosis is an important determinant of proteinuria and glomerulosclerosis in diabetic[14–16] and non-diabetic renal disease.[17] Experimental and clinical studies have shown that a reduction in podocyte number due to apoptosis or detachment correlates with, and leads to, proteinuria and glomerulosclerosis. Therefore, preventing or reducing podocyte apoptosis should become an important therapeutic target in the care of patients with glomerular disease. Despite this, little progress has been made in augmenting existing therapies with new prosurvival interventions. The current study shows that darbepoetin alfa may serve to limit podocyte loss through apoptosis, as our results demonstrate that darbepoetin alfa reduced podocyte apoptosis *in vitro* and *in vivo*.

Although the EPO receptor has been reported recently to be expressed by podocytes *in vivo*,[19] no reported data exists showing its expression in cultured podocytes. Our data indicates that cultured mouse podocytes do express the EPO receptor, providing the rationale that ESPs might have pleiotropic effects on this non-hematopoietic cell type. The first major finding in the current study was that darbepoetin alfa significantly reduced apoptosis in cultured podocytes in response to certain forms of injury. Previous studies have shown that darbepoetin alfa and other ESPs reduce apoptosis in other renal and non-renal cells such as neurons and

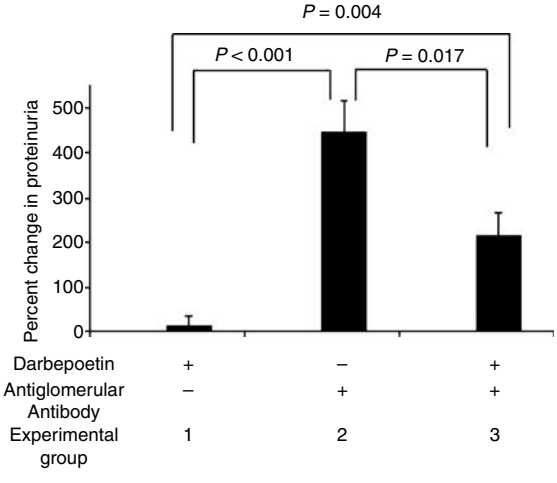

Figure 7 | Darbepoetin alfa treatment decreases urine proteinuria. Urine was collected for 12 h on day 10. The change in protein excretion was calculated for each group of animals, compared to baseline urine protein excretion. Darbepoetin alfa treatment significantly decreased the rise in protein excretion that occurs with acute glomerulonephritis in this model, from a 446±72% increase in vehicle-treated animals to 213±52% increase in darbepoetin alfa-treated animals ($P=0.017$). Control animals treated with 10 injections of darbepoetin alfa alone had a minimal change in protein excretion of 11±24% over the course of the study.

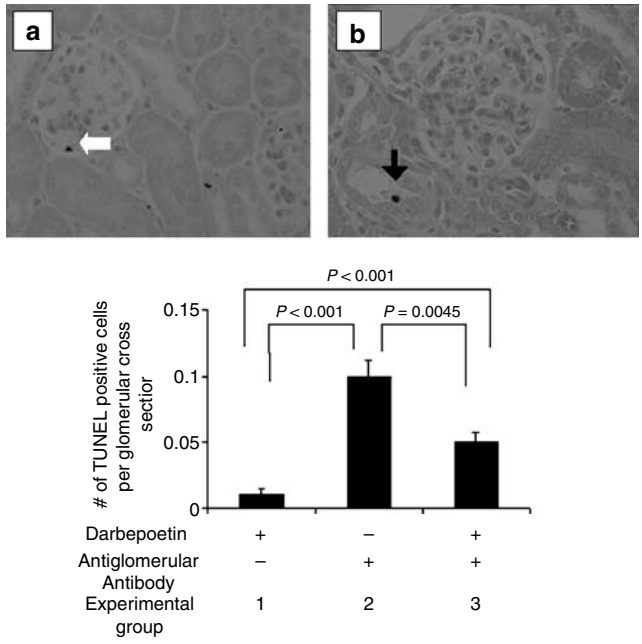

Figure 8 | Darbepoetin alfa treatment decreases podocyte apoptosis. (**a**) A representative glomerulus from a nephritic vehicle-treated mouse (group 2) with a TUNEL-positive apoptotic body in a podocyte distribution (white arrow) (**b**) A glomerulus from a nephritic darbepoetin alfa-treated mouse (group 3) does not show any TUNEL-positive cells in the glomerulus. A TUNEL-positive tubular cell (black arrow) is a positive internal control. (**c**) There were significantly fewer glomeruli with TUNEL-positive cells in the darbepoetin alfa-treated animals. The number of glomerular cross-sections with TUNEL-positive cells was decreased 50% from 0.1 to 0.05 with darbepoetin alfa treatment ($P=0.0045$). Control animals treated with darbepoetin alfa alone had minimal evidence of apoptosis with 0.01 TUNEL-positive cells in a podocyte distribution per glomerular cross-section.

cardiomyocytes.[4–8,10] Thus, the current study adds another cell type to the non-hematopoietic benefits of ESPs.

The protective effect of ESPs requires pre-exposure in non-renal cells, and this was also true in cultured podocytes. Our data shows that darbepoetin alfa did not alter the induction of apoptotic triggers as the quantity of DNA damage following uv-C injury was similar in vehicle or darbepoetin alfa-treated podocytes, suggesting that the primary effect of ESPs is directly reducing apoptosis. This led to the hypothesis that ESP treatment may improve the podocyte's ability to respond to apoptotic stimuli. Although many of the hematopoietic pathways of cell survival may be activated in podocytes, it is probably that there are podocyte-specific pathways that are activated. One clue to potential mechanisms is the fact that the prosurvival effects of ESPs on podocytes are not universal. Our results showed that darbepoetin alfa reduced apoptosis in response to low-dose uv irradiation and TGF-β. Both uv-C[24] and TGF-β[26–28] cause caspase-3-dependent apoptosis. In contrast, darbepoetin alfa had no effect on podocyte apoptosis induced by puromycin aminonucleoside, which we have shown previously to cause apoptosis in a p53-dependent and in a caspase-3-independent manner in podocytes.[18]

We next explored potential mechanisms that darbepoetin alfa might afford a survival benefit in podocytes. Studies in renal and non-renal cells suggested multiple signaling pathways upstream of caspases are potentially activated by ESPs, including the activation of JAK2, STAT5, nuclear factor-κB, BclXL, PI3K, and then downstream phosphorylation of AKT.[4] Our initial studies have shown that JAK2 and AKT phosphorylation are critical and required for the protective effects of ESPs in podocytes. Further studies are underway to explore the redundancy of these pathways and the activation of other survival pathways in podocytes in response to stimulation from hematopoietic growth factors.

The use of immortalized mouse podocytes in culture has substantially enhanced our understanding of podocyte biology. However, to validate that the findings in culture can be replicated *in vivo*, we used a well-described mouse model of podocyte apoptosis. The second major finding of the current study was that darbepoetin alfa significantly reduces proteinuria, glomerulosclerosis, and podocyte apoptosis. Although dexamethasone,[18] insulin-like growth factor,[36] exogenous vascular endothelial growth factor,[37] and all-trans retinoic acid[38] have been shown to reduce podocyte apoptosis *in vitro*, only chronic apocynin therapy which reduces nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species production in the db/db mouse model of diabetic nephropathy has been shown to reduce podocyte apoptosis *in vivo*.[39]

We acknowledge that the exact identity of the cells undergoing apoptosis in the experimental model was not defined based on molecular markers, but rather on histologic location. The rationale for this is that following apoptotic forms of injury, podocytes *in vivo* have a marked reduction in their characteristic markers such as nephrin, podocin, and WT-1. Additionally, there is no known expression *de novo* of a novel protein that can be used for double staining purposes to identify that the cells undergoing apoptosis (TUNEL-positive) are indeed podocytes.

We were not able to detect any improvement in azotemia by the treatment with darbepoetin alfa. One explanation might be that high-dose EPO therapy may have contributed to a rise in BUN in the darbepoetin alfa-treated animals as this has been shown to occur with EPO therapy due to induction of renal vasoconstriction and a decline in renal blood flow.[40] This was clearly demonstrated in the control animals receiving darbepoetin alfa alone (without the induction of nephritis) who displayed significant increases in BUN. Further studies evaluating the benefits of lower doses of ESPs will be necessary to identify the minimum dose required to provide an anti-apoptotic benefit without the side effect of polycythemia.

Despite the beneficial effects of darbepoetin alfa on preventing podocyte apoptosis, there were no differences in podocyte number as quantified by WT-1 staining in the experimental and control groups. One explanation is that while the benefits of darbepoetin alfa are clearly on apoptosis, other causes of reduced podocyte number, such as detachment, may not have been affected. Second, WT-1 staining which was used to identify podocytes is reduced following podocyte de-differentiation that occurs in the animal model used for this study. Finally, this model does have some podocyte proliferation in response to injury, and this may have masked the overall effect of podocyte apoptosis on podocyte number. Importantly, our data shows that darbepoetin alfa alone does not increase podocyte proliferation *in vivo*.

This study was terminated after 10 injections of darbepoetin alfa due to polycythemia, and therefore the long-term benefits of darbepoetin alfa, especially at lower doses, on glomerular disease has yet to be determined. As recently reviewed,[41] the results of clinical studies evaluating the effects of ESP treatment on progression of chronic kidney disease have been mixed, but overall suggest a beneficial effect of ESP therapy on stabilizing or slowing progression of renal decline in diabetics and non-diabetics, especially when treated early in their disease process.[42–44] These studies have primarily focused on the beneficial effects of improved oxygen delivery to the interstitium and decreased reactive oxygen species production combined with the anti-apoptotic effects of ESPs on the tubular cells to slow progression. However, our results suggest another potential beneficial mechanism at the glomerular level with prevention of podocyte apoptosis. The requirement for ESP pretreatment does not decrease clinical utility, as most diabetic and non-diabetic glomerular diseases are not acute phenomenon and are characterized by continuous injury. Additionally, the recent development of non-hematopoietic forms of ESPs, such as carbamylated EPO,[40] have shown that the tissue-specific effect can be maintained without excessive adverse hematopoiesis.

In summary, this study shows that darbepoetin alfa reduces apoptosis in podocytes *in vitro* and in an

experimental animal model. These effects are mediated in part by JAK2/STAT and AKT pathways. Further studies are ongoing to identify a dose-specific effect and the applicability of this finding to human glomerular disease.

MATERIALS AND METHODS

Immortalized mouse podocytes

Experiments were performed with passage 10-22 growth restricted, conditionally immortalized heat-sensitive mouse podocytes. To generate these cells, H-2Kb-tsA58 transgenic mice were utilized. These mice harbor interferon-γ inducible promotor for expression of the thermosensitive SV40 large T antigen. Glomeruli were isolated from kidneys, and podocytes were isolated from glomerular outgrowths by dilution cloning.[20,22,45] Podocytes were identified by confirming the presence of podocyte-specific proteins by immunostaining.[20,46]

Cells are initially cultured under growth permission conditions on collagen I-coated plates at 33°C in the presence of interferon-γ (50 U/ml). To induce the development of the differentiated and quiescent phenotype, mirroring the *in vivo* podocyte phenotype, cells were switched to 37°C without interferon-γ for longer than 12 days, which results in expression of the podocyte-specific proteins.[20] Cells were grown in Roswell Park Memorial Institute 1640 media containing 9% fetal bovine serum (Summit Biotechnology, Ft Collins, CO, USA), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mmol/l), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (10 mmol/l), and sodium bicarbonate (0.075%).

Immunostaining, membrane protein extraction, and Western blotting

Immunoflourescent staining and Western blot analysis was performed on differentiated podocytes. Podocytes were plated onto collagen-coated glass coverslips, fixed in 2% paraformaldehyde and 4% sucrose, and then permeabilized in 0.3% Triton X-100 before incubation overnight at 4°C with rabbit polyclonal EPO receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a secondary biotinylated mouse anti-rabbit antibody and Alexa-Fluor 594-conjugated streptavidin (Molecular Probes, Eugene, OR, USA). Omitting the primary antibody was used as a negative control.

Isolation of enriched membranous protein fractions was performed by subcellular fractionation. Cells were lysed in 10 mM Tris buffer (pH 7.5) containing 300 mM sucrose, 1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, St Louis, MO, USA), protease inhibitors (Roche, Indianapolis, IN, USA), 50 mM NaF (Sigma), 1 mM Na-orthovanadate (Sigma) and homogenized by sonication. The cell homogenate was centrifuged for 5 min at 800 $\times$ *g*, 5 min at 2000 $\times$ *g*, and then 20 min at 10 000 $\times$ *g*. The supernatant was further centrifuged for 60 min at 100 000 $\times$ *g* resulting in a cytosolic protein fraction and a membranous enriched fraction. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Reduced protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting. Membranes were incubated overnight at 4°C with the following primary antibodies: EPO receptor antibody (Abcam, Cambridge, MA, USA), caspase 3 (Cell Signaling, Danvers, MA, USA) Na^+/K^+-ATPase (Abcam), and tubulin (Neomarkers, Fremont, CA, USA) then incubated with enhanced chemiluminescence anti-rabbit IgG horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA) before detection with enhanced chemiluminescence Western blotting detection system.

In vitro experimental design

Apoptosis was induced in podocytes by three methods.

(i) uv-C irradiation. Uv-C irradiation (5–20 J/m^2) was applied to podocytes to induce apoptosis. Control cells were treated with vehicle, and experimental cells were pre-exposed to darbepoetin alfa (1–500 ng/ml) (Amgen, Thousand Oaks, CA, USA). These experiments were repeated in three separate cell lines. For inhibitor studies, podocytes were exposed for 24 h to the following inhibitors, 20 μM AG490 (Toronto Research Chemicals, North York, ON, USA), 1.25 μM AKT IV (Calbiochem, San Diego, CA, USA), 10–25 μM AKT VII (Calbiochem), or vehicle (dimethyl sulfoxide) and then darbepoetin alfa for 3 h before uv-C irradiation.

(ii) PA. Apoptosis was induced by exposing podocytes to PA (10–50 μg/ml) (Sigma) for 48 h, as we have reported previously.[20]

(iii) TGF-β1. Podocytes were exposed to 5 ng/ml of human recombinant TGF-β1 (R&D Systems Inc, Minneapolis, MN, USA) for 38 h.

Measuring apoptosis

Podocyte apoptosis was measured by staining with 0.0056% Hoechst 33342. Propidium Iodide staining was performed initially to confirm that time points and doses used for the study were not associated with significant necrosis. Apoptosis was defined by the presence of chromatin condensation and formation of apoptotic bodies. Morphologic analysis was performed on a minimum of 900 cells per experimental condition using inverted fluorescent microscopy.[20,47] All experiments were performed a minimum of three times.

Quantification of DNA damage

The Comet Flare assay (Trevigen, Gaithersburg, MD, USA) was performed to quantify cleaved DNA fragments. Fifteen minutes after uv-C irradiation, the cells were trypsinized and single-cell gel electrophoresis (Comet assay)[48,49] was performed according to the manufacture's instructions.

Assessment of cell proliferation

MTT assay. Both proliferating growth-permissive and growth-restricted podocytes were utilized separately for this assay. Equivalent numbers of cells were plated at 30% confluence and then exposed to darbepoetin alfa (1–500 ng/ml). After 48 and 96 h, the MTT assay was performed according to the manufacturer's instructions.

BrdU incorporation. Growth-permissive podocytes were plated at 30% confluence and darbepoetin alfa added to the media (1–500 ng/ml). After 48 h, the cells were labeled with BrdU labeling media (GE Healthcare). After 4 h, the labeling media was removed and the cells fixed in methanol/acetone. BrdU immunostaining was performed using the cell proliferation labeling kit (GE Healthcare) with diaminobenzidine (Fisher, Santa Clara, CA, USA) as the chromogen.

In vivo studies: animal study and experimental design

An animal model of passive nephrotoxic glomerulonephritis was induced in C57Bl/6 mice as reported previously by our group.[22,33,34,50] This model is characterized by podocyte injury, de-differentiation, proliferation, apoptosis, and the development of

proteinuria and glomerulosclerosis. This model is complement independent and is not characterized by any significant amount of neutrophil, T cell, or macrophage infiltration of the glomeruli. Mice used for this experiment were housed according to the standardized specific pathogen-free conditions in the University of Washington Animal facility. The Animal Care Committee of the University of Washington, Seattle reviewed and approved the experimental protocol. Mice were divided into two groups: an experimental group administered daily intraperitoneal darbepoetin alfa (25 ng/g) ($n = 13$), or the control group administered vehicle (2.5% human serum albumin, sodium phosphate monobasic monohydrate, sodium phosphate dibasic monohydrate, and sodium chloride, pH 6.0) ($n = 6$), starting 24 h before the induction of podocyte injury by intraperitoneal injection of sheep anti-rabbit glomerular antibody (0.5 ml/20 g) on two consecutive days. Darbepoetin alfa or vehicle was administered daily for a total of 10 doses. An additional control group of animals was injected daily with darbepoetin alfa only (25 ng/g, $n = 9$) for a total of 10 days.

Blood was collected at days 0 and 10 by retro-orbital bleed, for hematocrit and BUN measurement.[22] Before killing, mice were placed in metabolic urine collection cages for 12 h. The urine was analyzed for protein concentration by the sulfosalicylic acid method.[51] To perform immunostaining, renal biopsies were fixed in 10% neutral-buffered formalin, methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) or snap-frozen and embedded in 22-oxacalcitriol.

Immunostaining

To ensure that the ESP treatment did not interfere with the glomerular deposition of the anti-glomerular antibody used to induce glomerulonephritis, frozen sections were fixed in methanol at -20°C and then stained with fluorescein isothiocyanate-conjugated antibody to sheep IgG (Cappel, Durham, NC, USA). The autologous phase of the disease was assessed by immunostaining with the fluorescein isothiocyanate-conjugated mouse IgG (Cappel). Apoptosis was measured using the TUNEL assay.[52] Over 75 glomeruli per cross-section were evaluated by a second masked observer and determined to be TUNEL-positive if there was typical apoptotic morphology and positive staining of the nuclei black. Indirect immunoperoxidase staining was performed on methyl-carnoy fixed tissue with antibody to WT-1 (Santa Cruz). Periodic acid-Schiff staining was performed for the semiquantitative assessment of glomerulosclerosis. Over 50 randomly selected glomeruli per section were graded based on the percentage of glomerular tuft involved in mesangial thickening or glomerular sclerosis as follows: $0 =$ normal glomerulus, $1 \leqslant 25\%$, $2 = 25$–50%, $3 = 50$–75%, and $4 \geqslant 75\%$. Sclerotic index was calculated as described by Saito *et al.*[35]

Statistical analysis

All results are expressed as mean $\pm$ s.e. Student *t*-test analyses were performed using Microsoft Excel. Statistical significance was defined as $P < 0.05$.

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