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ORIGINAL PAPER

Gas1 expression in parietal cells of Bowman's capsule in experimental diabetic nephropathy

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Abstract Gas1 (Growth Arrest-Specific 1) is a pleiotropic protein with novel functions including anti-proliferative and proapoptotic activities. In the kidney, the expression of Gas1 has been described in mesangial cells. In this study, we described that renal parietal cells of Bowman's capsule (BC) and the distal nephron cells also express Gas1. The role of Gas1 in the kidney is not yet known. There is a subpopulation of progenitor cells in Bowman's capsule with self-renewal properties which can eventually differentiate into podocytes as a possible mechanism of regeneration in the early stages of diabetic nephropathy. We analyzed the expression of Gas1 in the parietal cells of Bowman's capsule in murine experimental diabetes. We found that diabetes reduced the expression of Gas1 and increased the expression of progenitor markers like NCAM, CD24, and SIX1/2, and mesenchymal markers like PAX2 in the Bowman's capsule. We also analyzed the expression of WT1 (a podocyte-specific marker) on BC and observed an increase in the number of WT1 positive cells in diabetes. In contrast, nephrin, another podocyte-specific protein, decreases its expression in the first week of diabetes in the glomerular tuft, which is gradually restored during the second and third weeks of diabetes. These results suggest that in diabetes the decrease of Gas1 promotes the activation of parietal progenitor cells of Bowman's capsule that might differentiate into podocytes and compensate their loss observed in this pathology.

Keywords Gas1 · Kidney · Progenitor cells · CD24 · Diabetes · Podocyte regeneration

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Introduction

Diabetic nephropathy (DN) is a very common complication of diabetes and is considered the leading cause of end-stage renal disease (ESRD) (Caramori and Mauer 2003; Reutens and Atkins 2011). Diabetes induces glomerular alterations like podocyte loss, which significantly compromises kidney filtration process and in severe cases favors the evolution to chronic renal failure (CRF). The podocytes are highly differentiated epithelial cells that have a very low proliferation capacity under basal conditions (Lasagni et al. 2013; Vestra et al. 2003). The loss of podocytes is very important in the development of ND. The alterations that promote this loss and their effects on renal function have been widely studied; however, the mechanisms of podocyte regeneration involved in the early stages of kidney damage in diabetes are not fully established (Steffes et al. 2001; Wolf and Ziyadeh 2007; Shankland 2006).

A subpopulation of progenitor cells has been described in Bowman's capsule (BC). These cells have the capacity of self-renewal and eventually, under certain physiological conditions, might differentiate into podocytes (Benigni et al. 2011; Romagnani and Remuzzi 2013). It is possible that in the kidney damage induced by diabetes, these progenitor cells are activated and initiate their differentiation

to podocytes to compensate the loss of podocytes in early stages of kidney disease (Lasagni and Romagnani 2010).

Growth arrest-specific protein 1 (Gas1) is a glycosyl-phosphatidyl-inositol (GPI)-anchored protein. The mRNA encodes a 345 aa protein with a molecular mass of ≈37 kDa (Del Sal et al. 1994; Stebel et al. 2000). Gas1 is a pleiotropic protein with novel functions including antiproliferative and proapoptotic functions. The overexpression of Gas1 inhibits proliferation by blocking the transition of phase G0/S in cell cycle in human gliomas and lung adenocarcinoma and has a proapoptotic activity mainly in hippocampal neurons, neuroblastoma, and glioma cells (Dominguez-Monzon 2011; Segovia and Zarco 2014). Gas1 enhances Sonic Hedgehog (Shh) signaling, an important pathway in the development of nervous system, mainly during neural tube, vertebral, and craniofacial development (Allen et al. 2007; Martinelli and Fan 2007a). It has been observed that Gas1 has a potential role as a tumor suppressor in lung, bladder, thyroid, and gastric tumors and might be used as a marker of metastasis or benign tumors in prostate and colorectal cancer (Wang et al. 2012; Lapouge et al. 2005; Rizzi et al. 2008; Jiang et al. 2011). Recently it was described that Gas1 has an important role in folicular development, ovulation, and luteinization (Ren et al. 2016). Additionally, it is known that Gas1 is a negative modulator of the Glial cell-derived neurotrophic factor (GDNF) signaling, an important pathway in the development, differentiation, and maintenance of several neuronal cell types and in renal morphogenesis (Martinelli and Fan 2007b; Schueler-Furman et al. 2006; López-Ramírez et al. 2008; Zarco et al. 2012; Costantini and Shakya 2006).

In the kidney, Gas1 is expressed during nephrogenesis and its expression is modulated by the transcription factor WT1 (Spagnuolo et al. 2004; Kann et al. 2015). It has also been observed that Gas1 is expressed in mesangial cells of the glomerulus where it acts as an endogenous inhibitor of cell proliferation (Zhang et al. 2014). In experimental models of renal damage, a decrease in the expression of Gas1 mRNA has been found (van Roeyen et al. 2013). Because the expression and function of Gas1 in the kidney have been only partially studied, we decided to analyze the role of Gas1 in the parietal cells of Bowman's capsule in the early stages of diabetes. In addition, we explored whether Gas1 is expressed in other segments of the nephron, such as proximal tubules, podocytes, and distal tubules.

Materials and methods

Reagents and experimental design

The human anti-Gas1 antibody was produced by Pro-Sci (Poway, CA, USA) (López-Ramírez et al. 2008; López-Ornelas et al. 2014; Dominguez-Monzon et al. 2009). Rabbit anti-WT1, rabbit anti-acetylated histone H4, mouse anti-SIX1/2, goat anti-NCAM, goat anti AQP-2, and goat anti-Nephrin antibodies were purchased from Santa Cruz Biotech, Inc. (Dallas, TX, USA). Rabbit anti-PAX2, mouse anti-claudins-2, -8, rabbit anti-Alexa Fluor 594, goat anti- Alexa Fluor 488, and mouse anti- Alexa Fluor 488 antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-CD24 antibody was purchased from BD Pharmingen (San Diego, CA, USA). Mouse anti-dipeptidyl peptidase IV (DppD) antibody was purchased from AbD Serotec (Raleigh, NC, USA). Streptozotocin (STZ) was purchased from Sigma–Aldrich (St. Louis, Mo, USA).

Animal experiments were performed in accordance with the Mexican Official Norm NOM-062-ZOO-1999 and the Institutional Committee for the Care of Laboratory Animals (UPEAL). Wistar female rats (body weight, 200–250 g) were housed with 12/12-h light/dark cycles at 22 ± 1 °C and 50 ± 5% humidity. Animals had ad libitum access to food and water. Type 1 diabetes was induced by the intravenous administration of STZ (60 mg/kg body weight, single dose) diluted in citrate buffer, pH 6.0. Nondiabetic control rats were administered intravenously with only citrate buffer. After 72 h, blood glucose concentration was measured with the OneTouch® Ultra blood glucose meter (Milpitas, CA, USA) to confirm the induction of diabetes. Diabetic rats were sacrificed at the end of the first, second, and third week after the induction of diabetes.

Physiological and biochemical parameters

Glucose levels were monitored at the first, second, and third week of diabetes. 24 h prior to sacrifice, the rats were housed in metabolic cages. Urine samples were collected and volumes were measured. Rats were anesthetized with sodium pentobarbital (30 mg/kg IP) and blood was collected by cardiac puncture and serum was obtained by centrifugation at 300×*g* for 10 min. Total protein was measured by the Lowry method (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories, CA, USA) and creatinine clearance was measured by the modified Jaffe reaction as previously described (Bradford 1976; Hervey 1953; Molina-Jijón et al. 2014; Arreola-Mendoza et al. 2006). Serum samples and urine were stored at −70 °C until use. All tests were performed in triplicate.

Renal tissues and glomerular isolation

Kidneys from control and diabetic groups were perfused with isotonic saline solution (NaCl 0.9%), excised, and placed in ice-cold saline solution. Renal slices (0.3 cm thickness approx) were obtained. Slices were placed in cold 2-methylbutane for 1 min and then for 10 min in

liquid nitrogen for cryopreservation. Renal tissue slices were stored at −70°C until use. Once kidney tissue was obtained, the cortex was dissected and homogenized. The homogenized tissue was pushed through a stainless steel sieve with different pore sizes to isolate the glomeruli as previously described (Gauthier and Mannik 1988; Molina-Jijón et al. 2015).

Extraction of total protein

To extract total protein, isolated glomeruli were incubated for 30 min at 4 °C in 200 µl of lysis buffer (RIPA; 10mM): 40 Tris–HCl. pH 7.6, 150 NaCl, EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, and dodecyl sulfate (SDS). Samples were sonicated three times at 40 microns for 30 s in a high-intensity ultrasonic processor (Vibra-Cell; Sonics & Materials, Inc., CT, USA) and centrifuged at 4 °C for 20 min, and supernatants were collected. Total protein quantification was performed using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Western blot analysis

Western blot analysis was performed as previously described (Arreola-Mendoza et al. 2009). Samples were denatured by boiling for 12 min. Proteins were loaded on SDS–PAGE gels at 10%. Gels were run at 70 V for 3 h. Molecular weight standards (GE Healthcare, Piscataway, NJ, -USA) were run in parallel. Proteins were transferred to polyvinylidene fluoride membrane (GE Healthcare, Uppsala, Sweden). Nonspecific protein binding was blocked with casein 3X (VECTOR, sp-5020; Burlingame, CA, USA) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with the appropriate primary antibodies anti-Gas1, acetylated histone H4, dendrin, SIX1/2, nephrin, CD24, and PAX2 (dilution 1:500). Thereafter, membranes were incubated with peroxidase-conjugated antibodies (dilution 1:20,000) for 1h. Immunoblots were developed using the ECL Prime Western Blotting detection reagent (Amersham™, GE Healthcare, Buckinghamshire, UK). The EC3 Imaging System (UVP Biolmaging Systems, Cambridge, UK) was used to detect chemiluminescence. Protein density band was quantified by transmittance densitometry (UVP Biolmaging Systems Software). All tests were performed in triplicate.

Immunofluorescence

Kidney samples were cryopreserved with 2-methylbutane (Aldrich M3, 263-1; Milwaukee, WI, USA). Kidney slices (4–6 µm thickness) were obtained with a Leica CM150 cryostat (Wetzlar, Germany) and mounted on gelatin-coated slides that were kept frozen at −70 °C. Immunofluorescence was performed as previously described (Loup et al. 1998; Molina-Jijón et al. 2015). Renal tissue sections were fixed with 3% paraformaldehyde (Aldrich M3, 263-1; Milwaukee, WI, USA) and incubated first with sodium citrate (J. T. Baker, Xalostoc, Mexico) 10 mM for 20 min at 90 ºC and then incubated for 20 min at room temperature. Sections were washed with PBS and blocked for 1h with 1% (wt/vol) IgG-free albumin (1331-A, Research Organics, Cleveland, OH, USA) and incubated overnight at 4 °C with the appropriate primary antibodies: anti-NCAM, Gas1, nephrin, AQP-2, PAX2, WT1, claudin −2, and −8 (dilution 1:50), dipeptidyl peptidase IV (dilution 1:200). Secondary antibodies Alexa Fluor 594 anti-rabbit, Alexa Fluor 488 anti-goat, and Alexa Fluor 488 anti-mouse (dilution 1:100) were used. Immunofluorescence was evaluated using a confocal inverted microscope (TCS-SP2 Leica, Heidelberg, Germany) and with the software (LAS AF LITE Leica Microsystem).

Staining quantitation and data analysis

For the semiquantitation of Gas1, Cln2, and NCAM at Bowman's capsule, a threshold segmentation was performed and a binarization system was applied to all images (Schindelin et al. 2015). We analyzed 3–6 glomeruli per group (control, first, second, and third weeks of diabetes) in each experiment. We used the ImageJ software (Version 1.50). Quantification of positively stained cells at the Bowman's capsule was performed on individual animals in diabetic and control groups using a combination of bright-field and fluorescent microscopy as previously described (Zhang et al. 2012; Naito et al. 2003).

Results are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used for multiple comparisons among groups. The Dunnett post hoc test was performed. $p < 0.05$ was considered statistically significant.

Results

Induction of experimental diabetes with streptozotocin

To confirm the induction of experimental diabetes in rats, we analyzed the glycemic index in experimental groups and controls (Fig. 1a). Diabetic groups showed hyperglycemic indexes (1st week 376 mg/dl ± 40.9, 2nd week 376.33 mg/

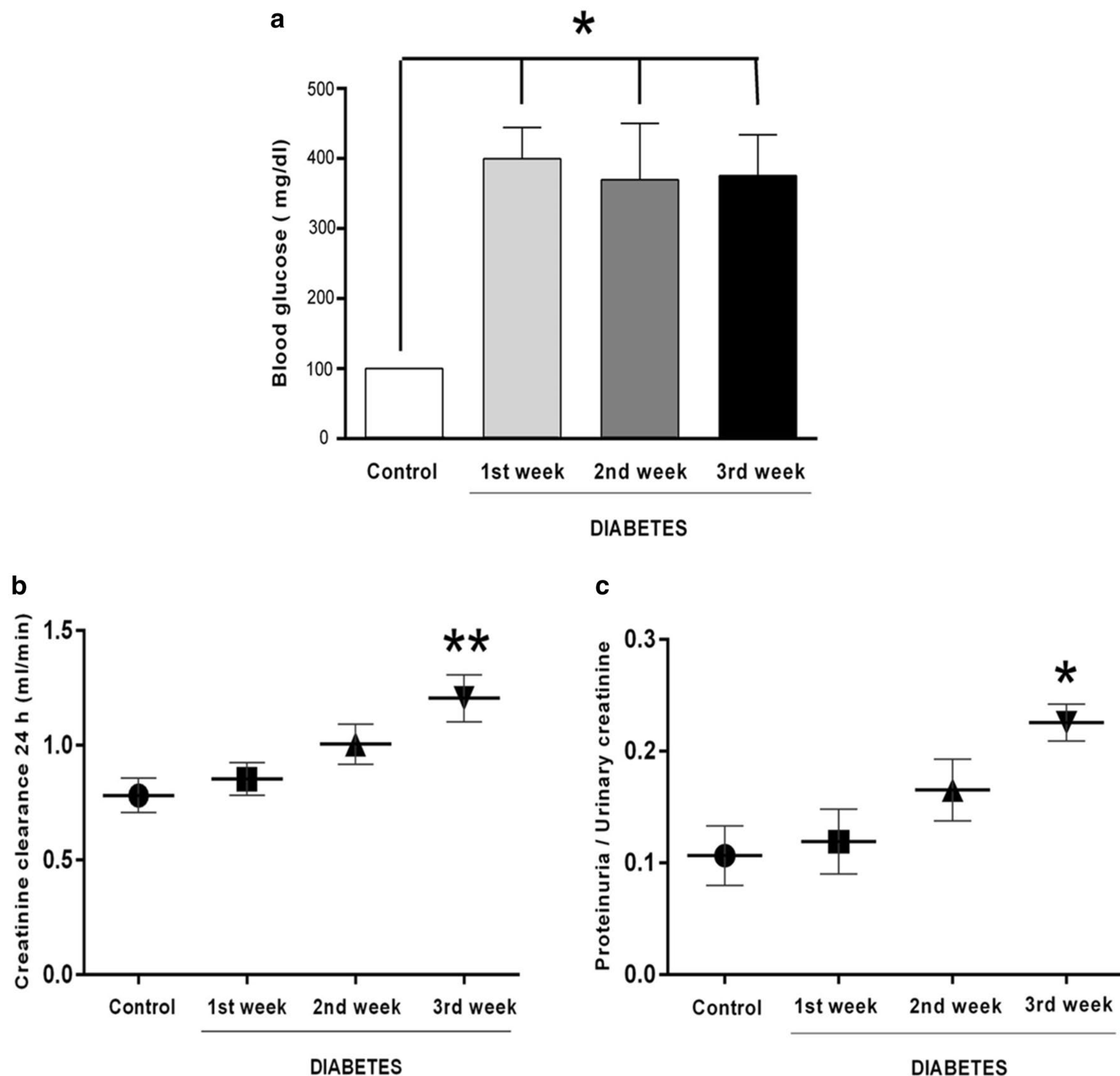

Fig. 1 Physiological and biochemical parameters in diabetic rats. **a** Diabetic rats treated with STZ showed hyperglycemic indexes (≈400 mg/dL) at the first three weeks of diabetes compared to controls (≈90 mg/dL). We also analyzed **b** creatinine clearance and **c** proteinuria. In both parameters, we observed a gradual increase at the first and second week of diabetes but it is until the third week of diabetes that we observed a significant increase. Values are means ± standard deviation (SD), *$p < 0.05$, **$p < 0.01$

dl ± 128.71, 3rd week 356.6 ± 88.5) compared to the control group (95.3 mg/dl ± 9.23).

Diabetes induces proteinuria and alterations in renal filtration

We evaluated renal function in the first, second, and third weeks of diabetes. Proteinuria and creatinine clearance were measured. Both creatinine clearance and proteinuria progressively increased in the first and second weeks of diabetes, with a significant increase by the third week of diabetes compared to control (Fig. 1b, c).

Gas1 is expressed in the parietal cells of Bowman's capsule

In order to determine the expression of Gas1 in the parietal cells of Bowman's capsule, Gas1 expression and that of the tight junction protein, claudin-2 (Cln-2) (Kiuchi-Saishin et al. 2002; Ohse et al. 2008), were analyzed by immunofluorescence. As shown in (Fig. 2d), Gas1 co-localizes with claudin-2, indicating that it is expressed in the parietal cells of Bowman's capsule. The expression of Gas1 in the cells of Bowman's capsule is homogeneous in control rats. In contrast, in diabetic rats, the expression of Gas1 gradually

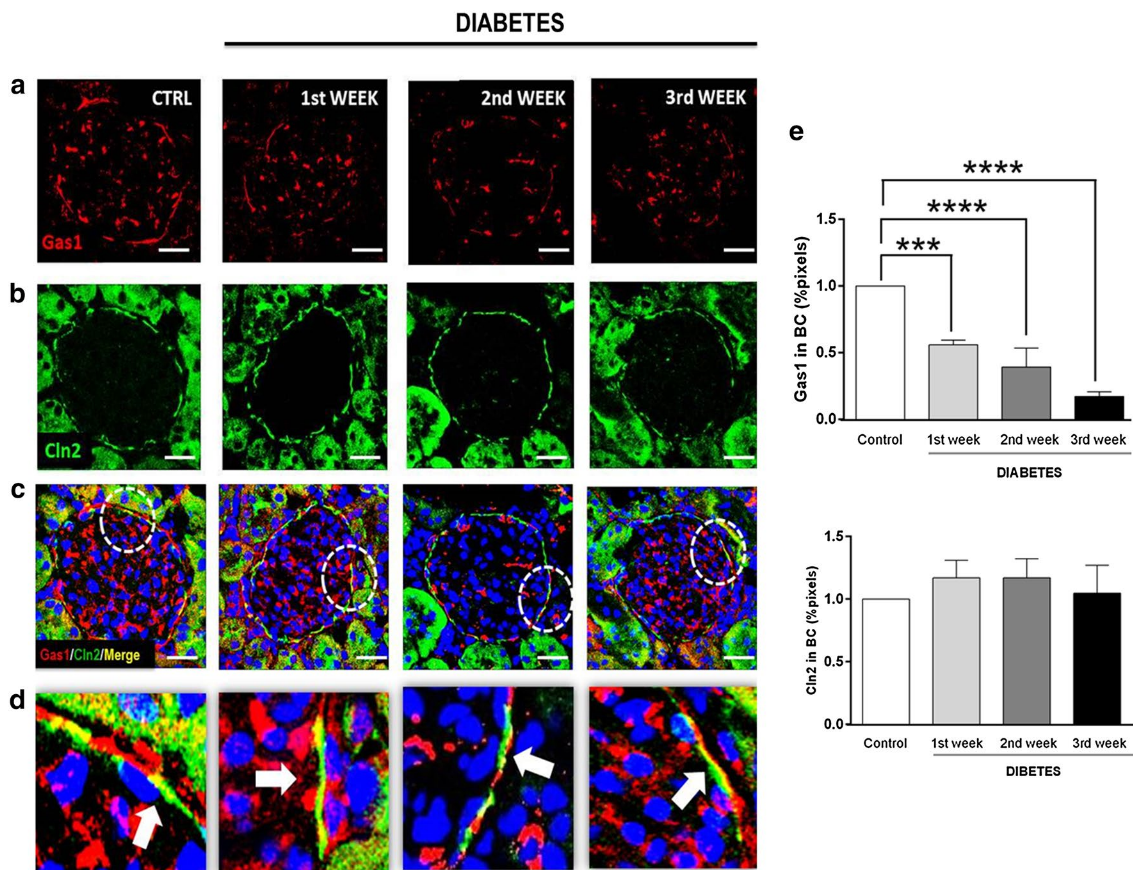

Fig. 2 Gas1 is expressed in the parietal cells of Bowman's capsule and diabetes decreases its expression. The expression of claudin-2 (Cln2) and Gas1 were analyzed by immunofluorescence in control and diabetic rats. **a** Gas1 is expressed homogeneously in Bowman's capsule of control rats. In contrast, in diabetic rats of 1, 2, and 3 weeks, the expression of Gas1 gradually decreases. The decrease is more evident by the third week of diabetes **b** Cln2 is a tight junction protein of Bowman's capsule cells. Cln2 expression defined Bowman's capsule in control and diabetic rats. **c, d** Gas1 and Cln2 co-localized (*white arrows, yellow* zone), demonstrating that Gas1 is expressed in the parietal cells of Bowman's capsule. **e** Semi-quantitative analysis of Gas1 and claudin-2 in Bowman's capsule. Gas1 is shown (*red*), Cln2 (*green*), and DAPI (*blue*). White circles focus a magnification of Bowman's capsule and white arrows indicate the colocalization of Gas1 and Cln2 (*yellow* areas). *Scale bar* 40 μm

decreases in the cells of Bowman's capsule in the first and second week of diabetes but the most notable decrement is observed at the third week (Fig. 2a, e). Interestingly, we found a different pattern of expression of Gas1 in the glomerular tuft, we also observed both by immunofluorescence and Elisa, a decrease in the expression of Gas1 in the first week of diabetes that it is restored by the second and third weeks of diabetes (Supplementary Figure 1).

Diabetes favors the decrease of Gas1 and increments progenitor cell markers in Bowman's capsule

It has been proposed that a subpopulation of progenitor cells exists in Bowman's capsule with properties of self-renewal. These cells can eventually differentiate into podocytes or proximal tubule cells (Benigni et al. 2010). To explore whether Gas1 is expressed in parietal progenitor cells of Bowman's capsule, the expression of NCAM (progenitor cell marker) and Gas1 were evaluated by immunofluorescence (Fig. 3a, b). Gas1 is homogeneously expressed in Bowman's capsule in control rats and decreases its expression in diabetic rats. In contrast, NCAM is expressed intermittently in cells of Bowman's capsule of control rats and gradually its expression is increased in this estructure in diabetic rats. The most notable increase of NCAM was observed by the third week of diabetes. It is evident in the high magnification that NCAM and Gas1 co-localize in controls. In diabetic rats, we observed a lower colocalization (Fig. 3a). Gas1 decreased in diabetes in Bowman's capsule and as expected the colocalization with NCAM decreased. In order to evaluate other markers of progenitor cells, we

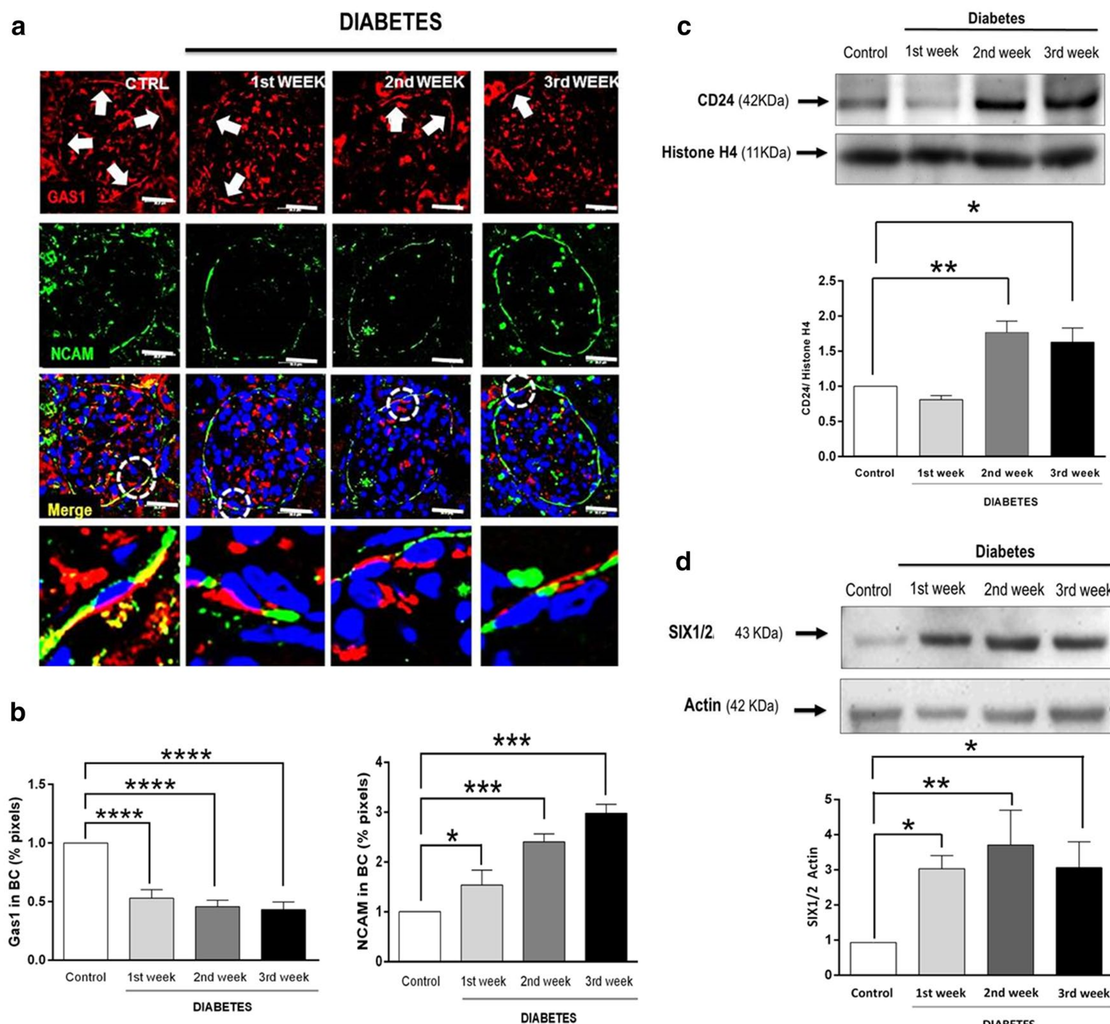

Fig. 3 Diabetes favors the decrease of Gas1 and increases the expression of NCAM in Bowman's capsule. **a** Gas1 and NCAM (progenitor cell marker) expressions were analyzed by immunofluorescence in Bowman's capsule. Gas1 (*red*) is expressed homogeneously in control rats and its expression is decreased in diabetic rats in Bowman's capsule cells. NCAM (*green*) is expressed heterogeneously in control rats and is progressively upregulated in diabetic rats in the cells of Bowman's capsule. **b** Semi-quantitative analysis of Gas1 and NCAM in Bowman's capsule. **c** We also analyzed the expression of CD24 (kidney progenitor cells marker) by Western blot analysis and we found significant increases in CD24 expression compared to control in the second and third week of diabetes. **d** We analyzed other progenitor marker, SIX1/2 by Western blot analysis. We found that SIX1/2 increased its expression in diabetic groups in comparison to controls. Densitometric analysis of Western blot is shown. Data for control and diabetic groups are expressed as relative density from six rats/group normalized to histone H4 as loading control. Values are means ± standard deviation (SD), $*p < 0.05$, $**p < 0.01$. White circles show a magnification of the colocalization of Gas1 and NCAM at the Bowman's capsule (*yellow* areas). *Scale bar* 50 µm. *AU* arbitrary units

analyzed the expression of CD24 and SIX1/2 (kidney progenitor cell markers) in a glomerular enriched fraction by Western blot analysis. CD24 is expressed in control rats and in the second and third weeks of diabetes, its expression is significantly increased when compared to control (Fig. 3c). We found an enhanced expression of SIX1/2 in the diabetic groups in comparison to controls (Fig. 3d). These findings suggest that diabetes promotes a decrease in the expression of Gas1 and may favor progenitor cells activation and/or proliferation.

Diabetes favors the increase of PAX2 in Bowman's capsule

Renal progenitor cells suffer a process of embryonic reprogramming acquiring mesenchymal markers when they are activated. To determine if the decrease of Gas1 favors an increase of mesenchymal markers, we analyzed the expression of PAX2 in Bowman's capsule by immunofluorescence and Western blot analysis. As determined both by immunofluorescence and Western blot analysis (Fig. 4a–c),

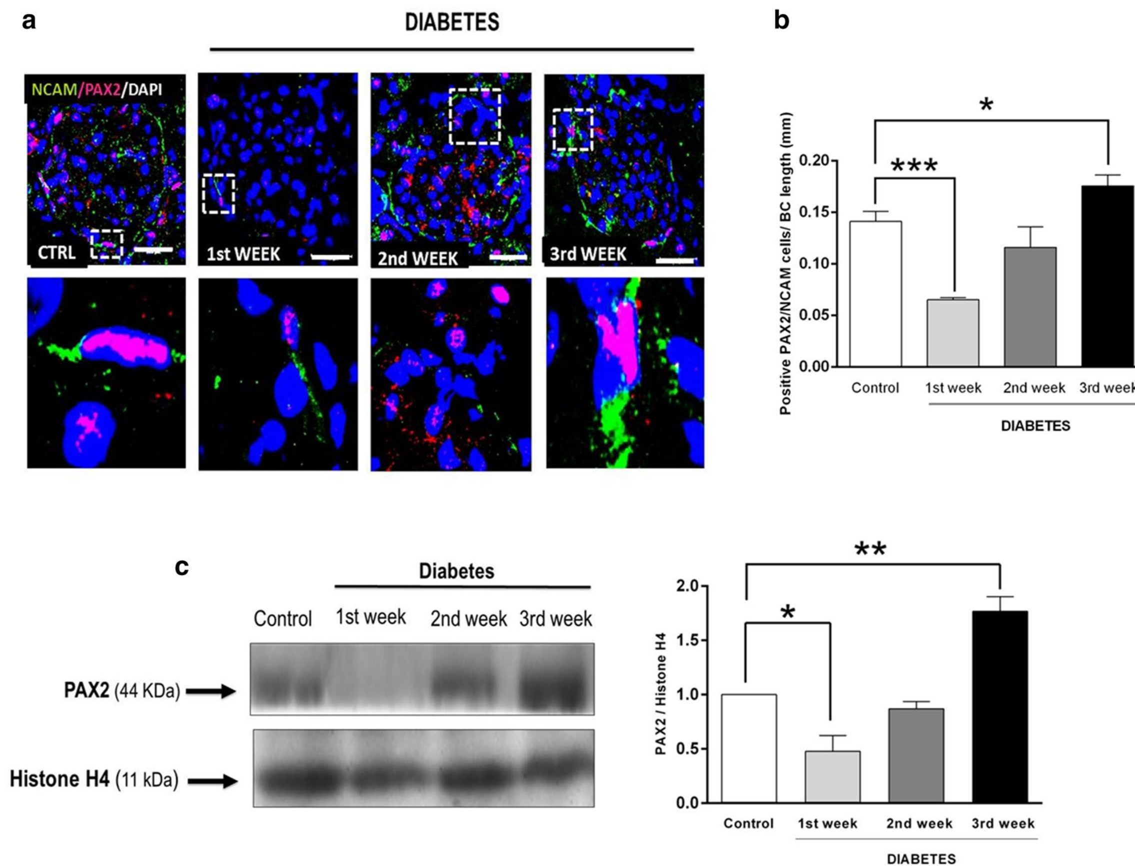

Fig. 4 Diabetes favors the expression of PAX2 in cells of Bowman's capsule. **a** We analyzed by immunofluorescence the expression of the transcription factor PAX2, which is a marker of progenitor mesenchymal cells. We observed that in controls there are some PAX2+cells (*pink*) but in the first week of diabetes its expression decreases and is restored in the second and third weeks of diabetes. In the approach, we observed cells of Bowman's capsule PAX2 positive. **b** Number of PAX2/NCAM positive cells/mm Bowman's capsule length in controls and in diabetic rats. **c** We also analyzed PAX2 by Western blot analysis and we found that in the first week of diabetes the expression of PAX2 decreases significantly. In the second week of diabetes, the PAX2 expression is restored and it increases significantly in the third week. Densitometric analysis of Western blot is shown. Data for control and diabetic groups are expressed as relative density from six rats/group normalized to histone H4 as loading control. Values are means ± standard deviation (SD), $*p<0.05$, $**p<0.01$. *Scale bar* 50 µm. *AU* arbitrary units. *White box* indicates approach

we observed that PAX2 is expressed in control rats while in the first week of diabetes its expression is significantly decreased compared to control. In the second week of diabetes, the expression of PAX2 recovers and increases significantly by the third week. These results suggest that diabetes promotes the decrease of Gas1 expression in Bowman's capsule and induces the activation and reprogramming in progenitor cells.

Diabetes increases the number of WT1 positive cells in Bowman's capsule

To explore whether the progenitor cells of Bowman's capsule that are activated in diabetes, differentiate to podocytes, we analyzed the expression of Wilms tumor protein (WT1), a specific podocyte marker, by immunofluorescence in Bowman's capsule. We first analyzed the expression of WT1 and Cln2 (a tight junction protein of parietal cells) to delimit the Bowman's capsule (Ohse et al. 2009). In control rats, some WT1 positive cells are observed in the Bowman's capsule. In contrast, in diabetic rats, the number of WT1 positive cells in Bowman's capsule increased (Fig. 5a, b). We then analyzed the expression of NCAM (progenitor cell marker) and WT1 in Bowman's capsule (Fig. 5c, d). In controls, the expression of NCAM is heterogeneous in Bowman's capsule and we observed scant WT1 positive cells. On the first, second, and third weeks of diabetes, we found an increase in the number of WT1 positive cells in the Bowman's capsule and observed that the expression of NCAM is increased. Interestingly, we noticed that WT1 positive cells also express NCAM. These results suggest that diabetes promotes the activation and differentiation of progenitor cells in Bowman's capsule to podocytes

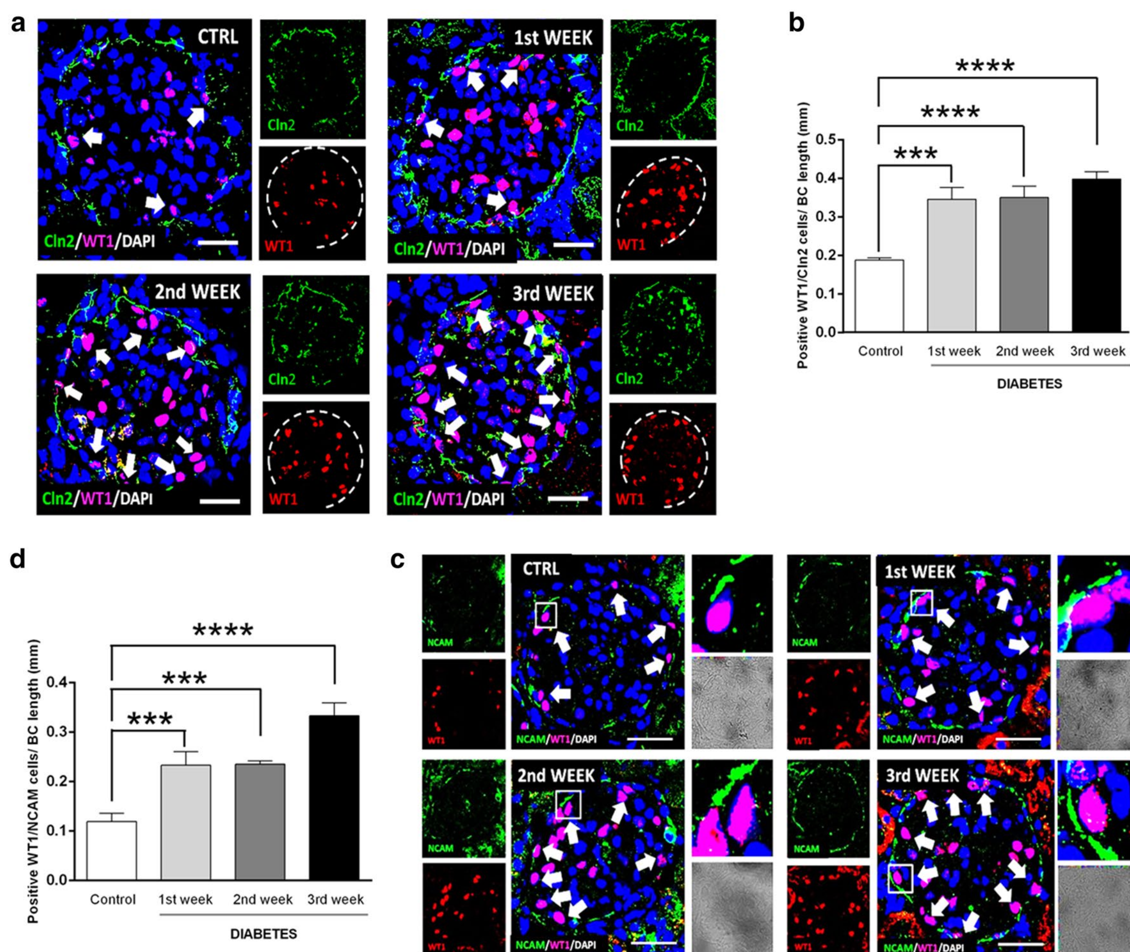

Fig. 5 Diabetes favors the increase of WT1 positive cells in Bowman's capsule. **a** We analyzed by confocal microscopy the expression of WT1 (specific marker of podocytes) at the Bowman's capsule and we used cln-2 to delimit this structure. In controls, we observed few WT1 positive cells (*pink*) in Bowman's capsule. In the first, second, and third weeks of diabetes, we found an increase in the number of WT1 positive cells at the Bowman's capsule. **b** Number of WT1/Cln2 positive cells/mm Bowman's capsule length in controls and in diabetic rats. **c** We analyzed by immunofluorescence the expression of NCAM (progenitor cell marker) and WT1 (specific marker of podocytes) at the Bowman's capsule. In controls, the expression of NCAM is heterogeneous at Bowman's Capsule and we observed few WT1 positive cells. In the first, second, and third weeks of diabetes, we found a increase in the number of WT1 positive cells at the Bowman's capsule and observed that the expression of NCAM increases and is most homogeneous in this estructure. Interestingly, we noticed that the WT1 positive cells express NCAM (*white boxes*). This suggests that progenitor cells are activated and their differentiation to podocytes in diabetes is initiated. **b** Number of WT1/NCAM positive cells/mm Bowman's capsule length in controls and in diabetic rats. WT1 (*red*), NCAM (*green*), and DAPI (*blue*). *White boxes* indicated magnification of positive WT1/ NCAM cells in Bowman's capsule. *White arrows* indicate WT1 positive cells (*pink*) in Bowman's capsule. *Scale bar* 40 µm

and support the hypothesis that Gas1 participates in this process.

Expression of nephrin and dendrin in the first weeks of diabetes

In order to determine the conditions of podocytes during the first week of diabetes, the expressions of nephrin and dendrin, both important proteins in the slit diaphragm of podocytes, were analyzed by immunofluorescence and Western blot analysis (Fig. 6). It was found that diabetes decreased the expression of nephrin in the first week. Interestingly, we observed that the expression of nephrin recovers by the second and third weeks of diabetes (Fig. 6a, b). Dendrin expression was analyzed by Western blot analysis (Fig. 6c), its expression decreases in the first week of diabetes, starts to recovery by the second week, and is completely restored by the third week of diabetes.

Gas1 expression in the proximal and distal nephron cells

The renal expression of Gas1 has been described in glomerulus (van Roeyen et al. 2013; Zhang et al. 2014). We

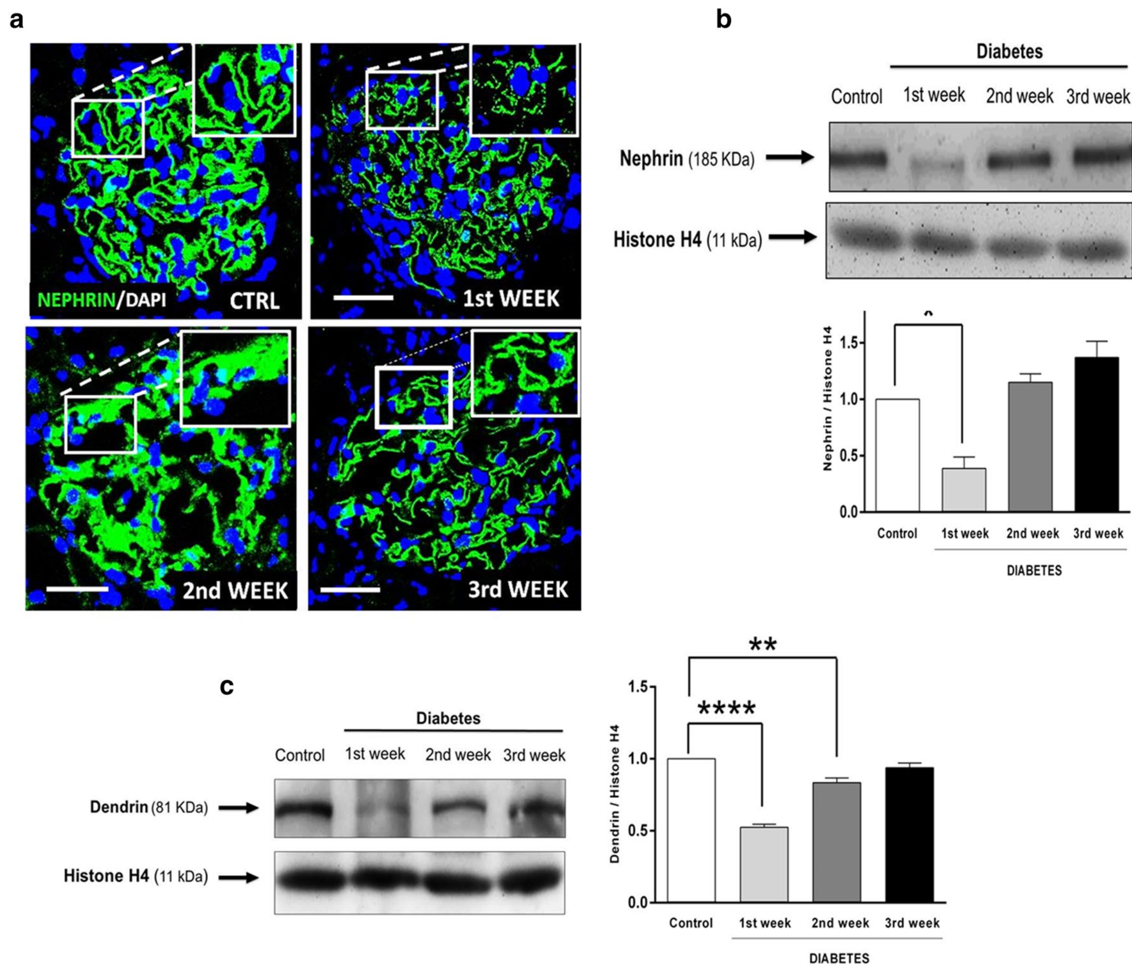

Fig. 6 Expression of dendrin and nephrin in the first, second, and third weeks of diabetes. **a, b** Nephrin expression was analyzed by immunofluorescence and Western blot analysis. In both, we observed a decrease in nephrin expression in the first week of diabetes. In the second and third weeks of diabetes, the expression of nephrin is restored. **c** Dendrin expression was analyzed by Western blot analysis. In the first week of diabetes, the expression of dendrin decreases significantly, and it is restored in the second and third weeks of diabetes. Isolated glomeruli were used for Western blot analysis. Densitometric analysis of Western blot is shown. Data for control and diabetic groups are expressed as relative density from six rats/group normalized to histone H4 as loading control. Values are means ± standard deviation (SD), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Scale bar* 50 µm. *AU* arbitrary units. It is shown as nephrin (*green*) and DAPI (*blue*). *White box* indicates approach

explored its expression in other nephron segments. Gas1 expression was analyzed by immunofluorescence in the proximal tubule cells (PT), in the distal nephron (DN), and in podocytes. To examine if Gas1 is expressed in proximal tubular cells, we used as a specific marker of this cell type, dipeptidyl peptidase IV (DPPD) (Molina-Jijón et al. 2014). DPPD does not co-localize neither in the diabetic nor in the control condition with Gas1, demonstrating that Gas1 is not expressed in proximal tubular cells (Fig. 7b). To analyze the expression of Gas1 in slit diaphragm, we used nephrin as specific marker (Ruotsalainen et al. 1999; Welsh and Saleem 2010). Gas1 and nephrin did not co-localize neither in the control nor in the diabetic condition (Fig. 7a). These results suggest that Gas1 is not expressed in the slit diaphragm. To assess if Gas1 is expressed in the distal nephron, particularly in the collecting duct, we used as a specific marker, the tight junction protein claudin-8 (Fig. 8a) (Gonzalez-Mariscal et al. 2006; Kiuchi-Saishin et al. 2002). It was found that both proteins co-localized in both diabetic and control conditions consistently. In both conditions, it is observed that Gas1 is expressed at the level of the cell membrane and cytoplasm and heterogeneously between cells (Fig. 8b). The collecting duct is characterized by possessing principal and intercalated cells. Aquaporin 2 is a protein expressed in principal cells and has an important role in the regulation of water reabsorption (Pearce et al. 2015; Hasler et al. 2009; Jung and Kwon 2016; Kim et al. 2016). To explore if Gas1 is expressed in principal cells of collecting duct, we analyzed the expression of Gas1 and Aquaporin 2 (AQP2) by immunofluorescence (Fig. 9a,

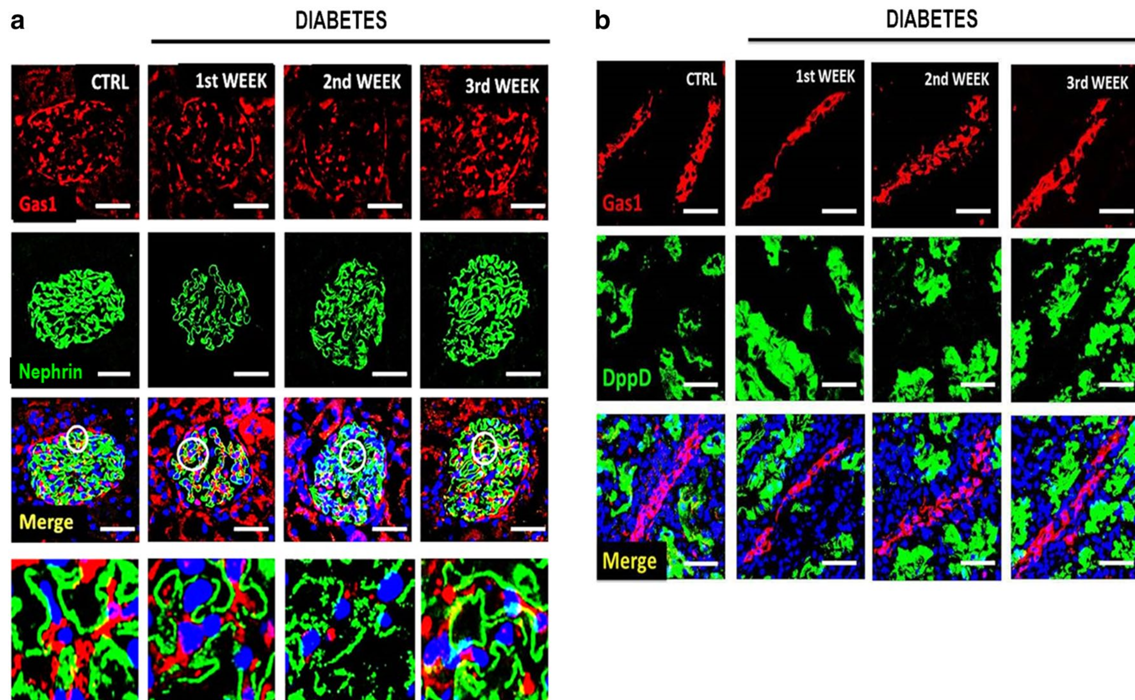

Fig. 7 Gas1 is not expressed in podocytes and in proximal tubule cells. **a** Gas1 expression in proximal tubule cells was analyzed by immunofluorescence. We used as a specific proximal tubule marker, dipeptidyl peptidase IV (DPPD). Gas1 and DppD did not co-localize in control and diabetic conditions. It is shown as Gas1 (*red*), DPPD (*green*), DAPI (*blue*). **b** We also analyzed the Gas1 expression in the slit diaphragm of podocytes by immunofluorescence. We used as a specific marker of podocytes, nephrin. Gas1 and nephrin did not co-localize in control and diabetic conditions. It is shown as Gas1 (*red*), DPPD (*green*), DAPI (*blue*). *Scale bar* 50 µm. *White circles* indicate augmented zone

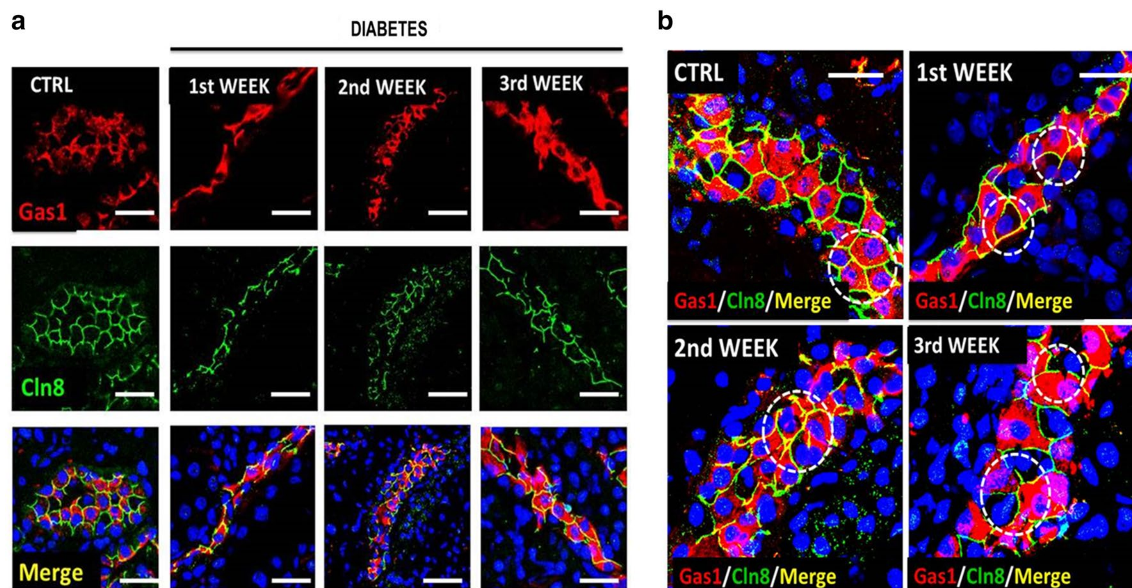

Fig. 8 Gas1 is expressed in the distal nephron. **a** We analyzed by immunofluorescence the expression of Gas1 in the collecting duct. We used as specific marker claudin-8 (Cln-8). Gas1 and Cln8 co-localized in diabetic and control conditions (*yellow* zones). **b** We observed Gas1 expression in the collecting duct cells in both cytoplasm and the cellular membrane (*White circles*). An interesting finding is that its expression is intermittent between cells in diabetic rats and controls. It is shown as Gas1 (*red*), Cln8 (*green*), DAPI (*blue*). *Scale bar* 50 µm

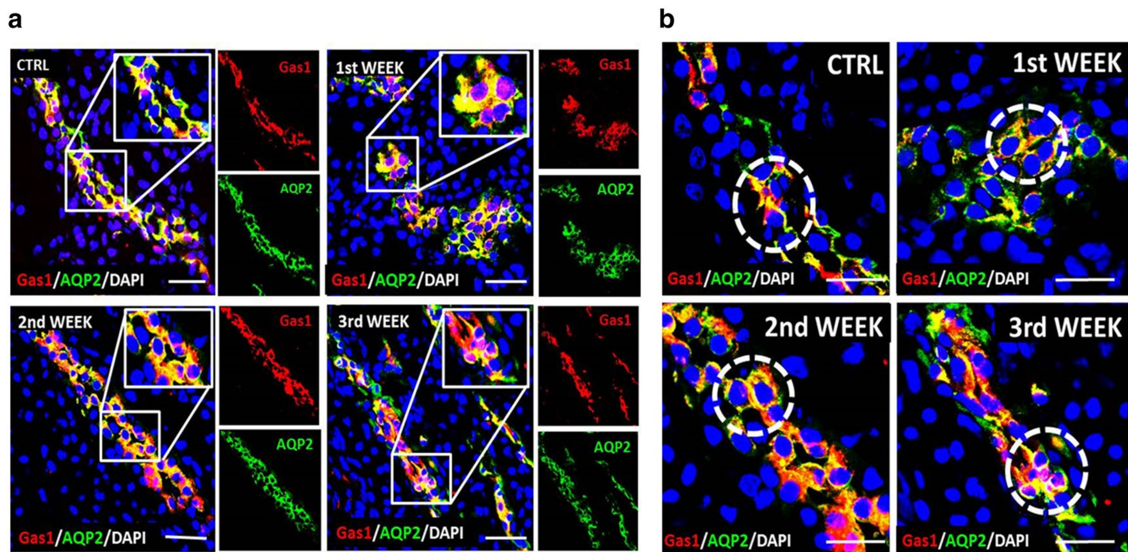

Fig. 9 Principal cells of collecting duct express Gas1. **a, b** We analyzed by confocal microscopy the expression of Gas1 in the principal cells of collecting duct. We used as specific marker aquaporin-2 (AQP-2). Gas1 and AQP-2 co-localized in diabetic and control conditions (*yellow* zones). It is shown as Gas1 (*red*), AQP-2 (*green*), DAPI (*blue*). *Scale bar* 50 µm. *White boxes* indicate approaches. *White circles* indicate colocalization of Gas1 with AQP2

b). We observed colocalization of Gas1 and AQP2 in diabetic and control rats, confirming the expression of Gas1 in the collecting duct and suggesting that it is mainly in the principal cells.

Discussion

In this study, we explored the expression of Gas1 in the cells of Bowman's capsule. The main findings of this study are (1) Gas1 is expressed in the parietal cells of Bowman's capsule and in collecting ducts of the distal nephron; (2) diabetes induces the decrease of Gas1 expression and increased the levels of the progenitor cell marker NCAM in Bowman's capsule. To our knowledge, this is the first study where Gas1 expression is explored in the parietal cells of Bowman's capsule and wherein the expression of progenitor markers was analyzed in murine experimental diabetes.

Functional alterations induced by experimental diabetes in early stages were determined. Gradual increases in proteinuria and serum creatinine were observed, during the first and second weeks of diabetes and in the third week both parameters attained the highest alterations. These results are in agreement with previous studies describing that diabetic nephropathy induces urinary protein excretion and development of hyperfiltration, two important factors in early stages of kidney damage (Campbell et al. 2003; Meeme and Kasozi 2009).

van Roeyen et al. (2013) described the presence of Gas1 in the kidney in a model of mesangioproliferative glomerulonephritis, in anti-Thy 1.1-treated rats and in human and murine mesangial-cultured cells. In addition, in this study, we describe the presence of Gas1 in Bowman's capsule and in the collecting duct cells of the distal nephron in a model of experimental diabetes induced by streptozotocin. Gas1 is expressed in the parietal cells of Bowman's capsule in control rats which gradually decreases in diabetic rats; the decrease is more evident by the third week of diabetes. The decrease of Gas1 expression may be associated with the expression of microRNA 34a (miR- 34a) and platelet-derived growth factor (PDGF). It has been described that glomerular mesangial cells overexpress miR-34a and PDGF in hyperglycemic conditions and favor the decrease of Gas1 expression (Zhang et al. 2014; van Roeyen et al. 2013). We also observed the expression of Gas1 in the glomerular tuft; these findings might be associated with the expression of mesangial cells and podocytes as described by van Roeyen et al. (2013).

Interestingly, the expression of NCAM increases gradually during the first 3 weeks of diabetes in Bowman's capsule. NCAM is a protein expressed during nephrogenesis (Abbate et al. 1999). Recent studies show that renal progenitor cells express NCAM and also increase the expression of mesenchymal progenitor cells markers, as SALL1, and SIX1/2 (Buzhor et al. 2013; Kobayashi et al. 2008). These findings are in agreement with our results where we observed an increment in the expression of NCAM and SIX1/2 in the diabetic groups. We also analyzed the expression of CD24, a marker of renal progenitor cells (Challen et al. 2004), and found that it increases its expression

in diabetes with a pattern similar to NCAM. These results suggest that the decrement in Gas1 plays an important role in the activation and/or proliferation of progenitor cells of Bowman's capsule in diabetes.

It has been proposed that active progenitor cells in Bowman's capsule are reprogrammed to a similar condition to that observed during embryogenesis (Benigni et al. 2010), thus expressing mesenchymal markers observed during renal development. For this reason, we evaluated the expression of PAX2, a nuclear protein (Dressler and Woolf 1999) of the parietal cells of Bowman's capsule which is expressed during nephrogenesis (Vainio and Lin 2002). By Western blot analysis and immunofluorescence, we observed that the expression of PAX2 is significantly decreased in the first week of diabetes. In contrast, in the second and third weeks of diabetes, PAX2 expression is restored and progressively increases. Interestingly, we observed that the cells of Bowman's capsule PAX2 positive also express NCAM (Buzhor et al. 2013), suggesting that in diabetes the expression of NCAM favors the maintenance of the progenitor cell phenotype, as well as the acquisition of mesenchymal markers. These findings suggest that Gas1 maintains the progenitor cells of Bowman's capsule in a quiescent state. When the expression of Gas1 decreases in diabetes, it is possible that these progenitor cells are activated and express NCAM, which is not only a progenitor cell marker, but can also induce the proliferation of progenitor cells and promote their differentiation into podocytes.

There is a loss of podocytes in diabetic nephropathy (Li et al. 2007) which is a key point in the evolution of kidney damage. Under normal conditions, podocytes have a very limited capacity to divide in situ (Griffin et al. 2003). A question that has arisen is how podocytes can be replaced. A potential mechanism of podocyte regeneration is the migration of progenitor cells from the bone marrow through the blood stream into the kidney (Vigneau et al. 2006). In this study, we propose that the subpopulation of progenitor cells of Bowman's capsule is a compensatory mechanism induced by the loss of podocytes in diabetes. Therefore, we evaluated the expression of WT1 (a podocyte-specific protein) in Bowman's capsule. We found that under normal conditions there are few cells in Bowman's capsule that are WT1 positive, but interestingly in the diabetic condition at 1, 2, and 3 weeks, the number of WT1 positive cells in this structure increases. This suggests that diabetes, favors the decrease of Gas1 expression promoting differentiation of progenitor cells in Bowman's capsule to podocytes, to compensate the loss of podocytes that occurs in the diabetic condition. All these results are in agreement with previous studies showing that parietal epithelial cells express podocyte markers (e.g., synaptopodin, podocalyxin, podocin, WT1) under physiological conditions and in kidney damage (Berger et al. 2014; Bariety 2006). A recent study showed that in the early stages of diabetes in human renal biopsies, there is a progressive decrease of podocytes and they observed in the Bowman's capsule an increase of podocyte markers, suggesting that parietal cells of Bowman's capsule might, to a limited extent, regenerate podocytes and proposed that it could be a potential process to ameliorate the evolution of the diabetic nephropathy, via podocyte restoration (Andeen et al. 2015).

Nephrin and dendrin are podocyte-specific proteins, which are part of the slit diaphragm, an important structure in the kidney filtration process (Shankland 2006). In order to explore the conditions of podocytes in the first weeks of diabetes, we analyzed their expression by immunofluorescence and Western blot analysis. We noticed that the expression of nephrin and dendrin decreased during the first week of diabetes, whereas in the second and third week of diabetes the expression of both proteins was restored. These findings suggest that the decrease in the expression of nephrin and dendrin in the first week of diabetes is associated with the loss of podocytes induced by hyperglycemia. Restoring the expression of both proteins in the second and third weeks of diabetes suggests that progenitor cells that have differentiated into podocytes migrate from Bowman's capsule into the visceral part of the glomerulus to compensate for the loss of podocytes. This process is induced in the early stages of the disease. On the other hand, it has been described that during podocyte damage, a state of "adaptation" or conditioning develops that subsequently ends in a state of decompensation, characterized by the loss of these cells in the glomerulus (Wiggins 2007). The hypothesis that both processes are occurring has not been discarded; perhaps, in diabetes the loss of podocytes could be compensated by progenitor renal cells of the BC as a regeneration mechanism and some podocytes develop an adaptive state in diabetic conditions. This is reflected in the restored expression of dendrin and nephrin during the second and third week of diabetes.

We found that Gas1 is not expressed in the cells of the proximal tubule. These findings are interesting because Gas1 is a protein that arrests cell proliferation. The cells of the proximal tubule have a high proliferation rate in basal conditions (Vogetseder et al. 2008) and this is augmented in renal damage, e.g., experimental diabetes and ischemia (Humphreys et al. 2011; Vallon 2011). Perhaps the expression of Gas 1 is not necessary in the proximal tubule cells due to the high rate of proliferation they possess and to the role of Gas1 as a inhibitor of the cellular proliferation. van Roeyen et al. (2013) described the expression of Gas1 in podocytes in healthy rats and in a model of anti-Thy1.1 mesangioproliferative glomerulonephritis. We analyzed, by confocal microscopy, the expression of Gas1 and nephrin, and these proteins did not co-localize. Nephrin is a specific protein of the slit diaphragm of the podocytes (Patrakka

and Tryggvason 2007); therefore, the intracellular location of this protein might correspond to the label observed in glomeruli.

Other novel finding in this study is the expression of Gas1 in the collecting duct of the distal nephron. Our results suggest that its expression is specific in the principal cells. We observed that Gas1 is not only found in the cell membrane but also in the cytoplasm. These results are interesting since Gas1 is a GPI-anchored-protein (Ruaro et al. 2000; Stebel et al. 2000). The cytoplasmic expression of Gas1 might be associated with the co-translational modifications that Gas1 suffers before reaching its mature form (Segovia and Zarco 2014) or with the soluble form that has been previously described (López-Ornelas et al. 2014; Jiménez et al. 2014; van Roeyen et al. 2013). We described for the first time the expression of Gas1 in the principal cells of the collecting duct. The function of Gas1 in this segment of the nephron is unknown and requires further studies.

In this study we report, for the first time, to our knowledge, the expression of Gas1 in Bowman's capsule cells and observed that diabetes favors the decrease of Gas1 expression and increased progenitor cell markers (NCAM, CD24, SIX1/2) as well as the expression of WT1 (podocyte marker) in Bowman's capsule cells. We also found Gas1 expression in the distal nephron. These results suggest that the loss of Gas1 in renal damage in the early stages of diabetes favors the activation and/or proliferation of parietal progenitor cells of Bowman's capsule and they possibly would differentiate to podocytes, acquiring specific markers (WT1) of these glomerular cells, suggesting it as a possible mechanism of renal regeneration in diabetes.

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Compliance with ethical standards

Conflict of interest All authors declare that research was done without any potential conflict of interest. No competing financial interests exist.

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