CD248$^+$ stromal cells are associated with progressive chronic kidney disease

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Stromal fibroblasts are the primary cells of the kidney that produce fibrotic matrix. CD248 is a stromal marker expressed on fibroblasts and pericytes within the human kidney. Here, we tested whether CD248 expression in the kidney colocalizes with fibrosis and if it is associated with known determinants of chronic kidney disease (CKD). CD248 expression was located and quantified *in situ* by immunohistochemistry in kidney biopsies from 93 patients with IgA nephropathy and compared with 22 archived biopsies encompassing normal kidney tissue as control. In normal kidney tissue, CD248 was expressed by resident pericytes, stromal fibroblasts, and was upregulated in human CKD. The expression was linked to known determinants of renal progression. This relationship was maintained in a multivariate analysis with CD248 expression linked to renal survival. CD248 was expressed by a population of α-smooth muscle actin (SMA)$^+$ myofibroblasts and α-SMA$^-$ stromal cells but not expressed on CD45$^+$ leukocytes. Thus, CD248 defines a subset of stromal cells, including but not limited to some myofibroblasts, linked to albuminuria and tubulointerstitial damage during tissue remodeling in CKD.

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Stromal fibroblasts are the primary fibrotic matrix-producing cells of the kidney. To date, there have been few systematic studies of stromal fibroblasts in human chronic kidney disease (CKD), reflecting the lack of cell-specific fibroblast markers.[1]

The most commonly used marker of an activated renal fibroblast, known as a myofibroblast, has been α-smooth muscle actin (α-SMA).[2] However, α-SMA may actually suppress and control myofibroblast activity,[3] and therefore does not represent a viable therapeutic target. Pioneering work by Strutz *et al.*[4–7] demonstrated that tubular epithelial cells can express fibroblast markers in various disease states, suggesting that epithelial–mesenchymal transformation is a potent source of myofibroblasts. Proximal tubular epithelial cells acquiring a fibroblast phenotype were accompanied by the *de novo* expression of fibroblast specific protein-1 (FSP-1)[8] indicating that FSP-1 may be a marker of epithelial–mesenchymal transformation. In an *ex vivo* study in human IgA nephropathy with preserved kidney function, there was a negative correlation between FSP-1 and renal survival.[9] However, these observations are confounded by FSP-1 expression on infiltrating leukocytes that by themselves are known determinants of progressive CKD.[10–12]

CD248 is a stromal cell marker[13] that may represent a more precise marker of fibrosis and a viable target for therapy. This type I transmembrane glycoprotein was originally identified using an antibody panel raised against fetal fibroblasts.[13] At nonrenal sites, CD248 definitively localizes to fibroblasts and pericytes found in inflammatory and cancer stroma, suggesting a pivotal role in tissue remodeling and repair.[14]

At basal levels in the adult kidney, CD248 is expressed in the glomerulus on specialized pericytes more commonly referred to as mesangial cells.[15] In renal cell carcinoma, the expression is significantly increased and large rafts of CD248-positive fibroblasts can be seen within the interstitium.[15] Similar patterns of CD248 expression are seen within other organs during malignant disease.[16]

In vitro studies have shown that fibronectin, collagen type I, and collagen type IV act as specific ligands for CD248 with adhesion and migration mediated through CD248.[17] CD248

regulates cell proliferation *in vivo*,[18] and this process is mediated through platelet-derived growth factor receptor signaling.[19] Murine models have demonstrated that CD248 is anatomically and temporally regulated with high levels of expression found in the embryo and newborn tissue,[20] and low levels in normal adult tissues. CD248 knockout mice develop normally, and have normal postnatal growth and wound healing.[21] However, if tumors are implanted in the abdominal cavity, there is impaired tumor growth, invasion, and metastasis. This indicates that CD248 may have a critical role in determining tumor behavior. Recently, Maia *et al.*[22] have demonstrated using a murine model of arthritis that CD248 contributes to synovial hyperplasia, fibrosis, and leukocyte accumulation in inflammatory arthritis and that CD248 may represent a target for the treatment of arthritis. Indeed, a monoclonal anti-human CD248 antibody (MOR-Ab-004) is currently undergoing phase 1 clinical trials for the treatment of solid tumors (ClinicalTrials.gov identifier: NCT008470544).

We therefore hypothesized that CD248 expression is upregulated in CKD and may be a potential therapeutic target. Initially, we reaffirmed earlier observations from the studies of other groups[13,14] concerning the expression of CD248 in normal renal tissue before characterizing CD248 expression in patients with progressive CKD. We related CD248 expression to known *in situ* determinants of renal progression to assess the relevance of this molecule in human kidney disease.

RESULTS

Characterization of CD248 expression in cultured human renal cells

We isolated renal fibroblasts from the nonaffected pole of human kidney samples removed for the treatment of malignant disease to examine CD248 expression *in vitro*. CD248 was expressed by renal fibroblasts ($n = 3$) but not by glomerular endothelial cells, podocytes, or human umbilical vein endothelial cells. Protein and RNA expression was confirmed by western blotting and reverse transcription-PCR, respectively (Figure 1). Explant culture may potentially lead to fibroblast activation, and thus expression of CD248 *in vivo* expression was also investigated.

Characterization of CD248 expression within normal kidney

To examine CD248 expression in human renal stromal cells *in vivo*, we performed immunohistochemistry on a control group of patients with archived tissue taken from the nonaffected pole of nephrectomies performed for the treatment of malignant disease ($n = 22$, 9 females and 11 males; median age 65, range 39–85). All control patients had normal renal function (estimated glomerular filtration rate (eGFR) > 90). These studies demonstrated CD248 expression within the glomerulus, localized to mesangial cells (Figure 2). Tubulointerstitial staining was scant, and when found present it was localized to the interstitial space.

To further identify renal cell populations expressing CD248 in normal kidney, we also examined kidney tissue

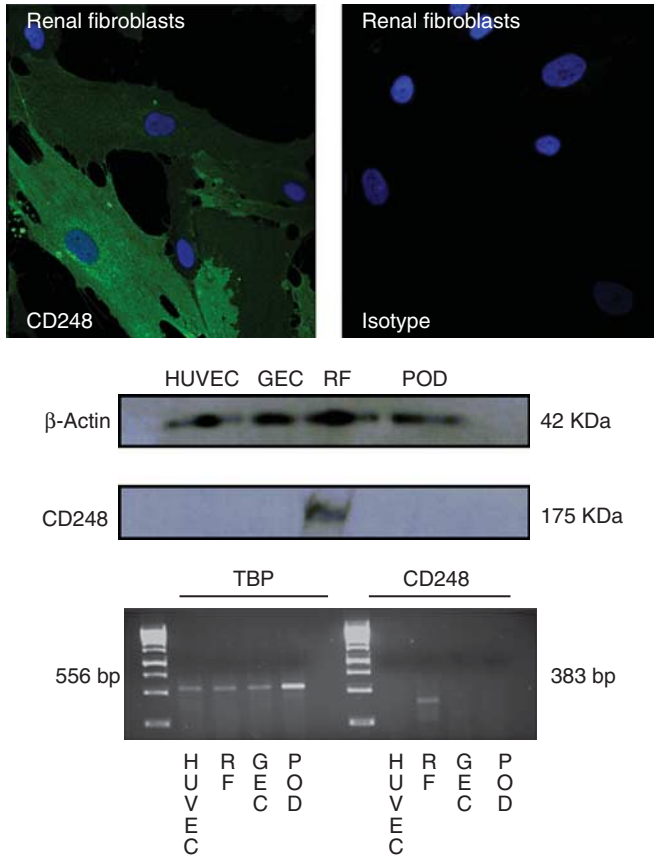

Figure 1 | CD248 expression *in vitro*. (Top) Confocal microscopy studies demonstrated CD248 expression by renal fibroblasts (RFs) ($n = 3$), but not by human umbilical vein endothelial cells (HUVECs), glomerular endothelial cells (GECs), or podocytes (POD) (data not shown). (Middle and bottom) Reverse transcription-PCR (RT-PCR) and western blotting corroborated the immunostaining pattern observed.

taken from healthy adult mice (Figure 3). CD248 shares similar homology in humans to mice. Murine tissue permitted more detailed confocal microscopy studies to be performed on noninflamed tissue. Again, CD248 localized to mesangial cells of the glomerulus, but it was also seen in pericytes wrapped around peritubular blood vessels (Figure 3) and on interstitial peritubular fibroblasts.

In summary, in healthy noninflamed kidney, CD248 is expressed by resident pericytes and on stromal fibroblasts.

Characterization of CD248 expression in relationship to known determinants of renal progression

To investigate the potential role that CD248 may play in tissue remodeling that accompanies progressive CKD, we performed a detailed histological analysis of a cohort of patients with IgA nephropathy. IgA nephropathy was chosen as progression in these patients is known to be tightly linked to albuminuria and interstitial fibrosis at diagnosis.[23]

A total of 100 patients with an adequate renal biopsy to make a diagnosis of IgA nephropathy were identified. Of these, 93 had archived tissue and clinical data suitable for analysis (60 males and 33 females). Table 1 summarizes the

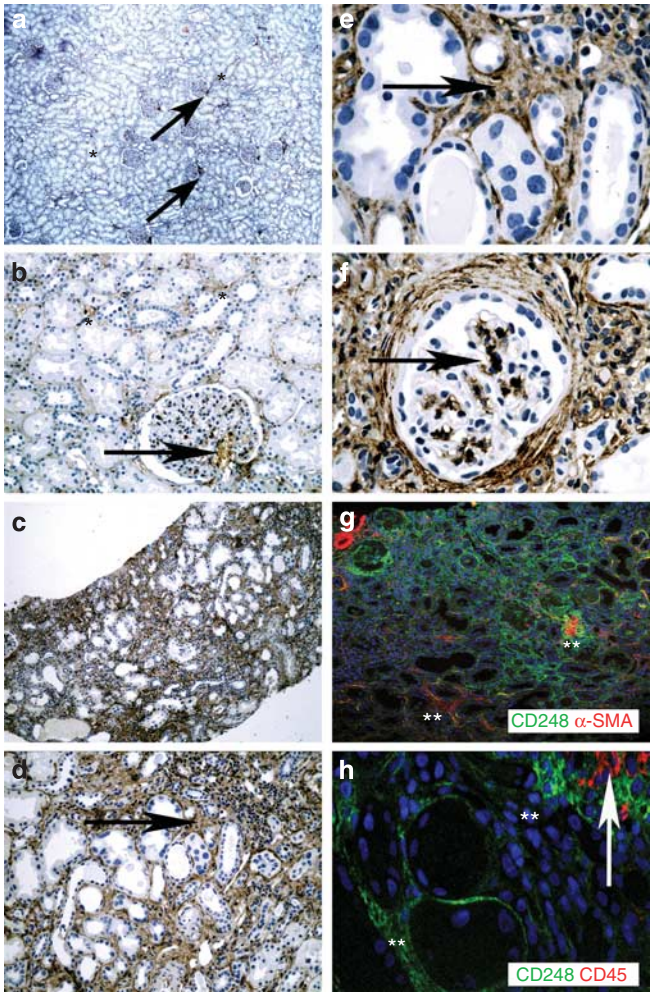

Figure 2 | Immunohistochemistry for CD248. Normal human kidney ($n = 22$). (**a**) CD248 (brown) localizes to the vascular pole (arrow) with weak expression seen in the mesangial cells of the glomerulus (original magnification $\times 100$). There is scant expression in the tubulointerstitium on peritubular fibroblasts (*). (**b**) Glomerular expression of CD248 (original magnification $\times 400$). (**c**) In progressive chronic kidney disease (CKD; $n = 93$) there is diffuse expression of CD248 throughout the tubulointerstitium. Shown at higher power in **d** and **e** (arrow to CD248 staining). (**f**) Glomerular expression of CD248 was also increased in the mesangial cells of inflamed kidney (original magnification $\times 630$). (**g**) Confocal microscopy to colocalize CD248 (green) and α-smooth muscle actin (α-SMA; red) (original magnification $\times 100$). Distinct sub-populations (**) can be seen and are described in more detail in Figure 5. (**h**) CD248 (green) was not expressed by CD45-positive cells (red; arrow).

clinical, pathological, and experimental characteristics analyzed. Histological analysis of the biopsy samples demonstrated diffuse tubulointerstitial staining for CD248 (Figure 2). CD248 expression was also seen to be increased in the mesangial cells of the glomerulus in inflamed kidney tissue (Figure 2f). Quantification of the interstitial staining using an interactive image analysis system showed expression to be greater in patients with more advanced disease. Previous studies by our group[12,24] have demonstrated the relationship between albuminuria, renal function, index of chronic damage, and progressive CKD. We therefore analyzed the relationship between these parameters and CD248 expression *in situ* in our IgA cohort.

By univariate analysis, urinary albumin–creatinine ratio (ACR) correlated with tubulointerstitial expression of CD248 (correlation 0.500; $P < 0.0000$; Table 2 and Figure 4). eGFR inversely correlated with urinary ACR but not as strongly as tubulointerstitial CD248 expression (correlation −0.360; $P = 0.0004$; Table 2 and Figure 4). Interstitial CD248 expression correlated with renal fibrosis as assessed by the index of chronic damage (correlation 0.539; $P < 0.0000$) and eGFR (correlation −0.679; $P < 0.0000$; Table 2 and Figure 4).

Importantly, these univariate correlations were maintained in a multivariate linear regression analysis of the variables. Tubulointerstitial CD248 expression, urinary ACR, index of chronic damage, and eGFR were all interdependent variables (Table 3). Notably, tubulointerstitial CD248 expression independently associated with urinary ACR (0.299; $P = 0.002$). Again, as we have previously published[12,24] using other disease cohorts, classical determinants of CKD progression, for example, urinary ACR and eGFR, were interdependent.

Does CD248 colocalize with α-SMA in advanced CKD?

Depletion of α-SMA has previously proven detrimental to the progression of renal disease.[3] We therefore wished to identify if CD248 was expressed by α-SMA$^+$ myofibroblasts to help determine if CD248 may represent a viable target for the treatment of CKD. Confocal microscopy was used to localize α-SMA and CD248 in advanced IgA nephropathy ($n = 3$). Again, CD248 was found to be localized to the tubulointerstitium. Immunofluorescence demonstrated three subsets of CD248$^+$ stromal cells: CD248$^+$αSMA$^-$, CD248$^+$αSMA$^+$, and CD248$^-$αSMA$^+$ (Figure 5). CD248 did not colocalize with the leukocyte marker CD45 (Figure 2).

Renal survival

To assess if CD248 expression is an independent predictor of renal survival, we reviewed the renal outcomes of our cohort. Data on renal outcome were available for all 93 patients studied. Patients were followed for up to 1095 days following renal biopsy. A total of 19 patients reached a renal end point (7 patients doubled their serum creatinine, and 12 patients reached end-stage renal failure) after a mean period of 285 days (range 1–975 days; one patient commenced dialysis at day 1).

Kaplan–Meier survival analysis demonstrates that patients with extensive tubulointerstitial staining for CD248, particularly tubulointerstitial CD248 $>9.8\%$, is predictive of poorer renal outcome (log rank testing for equality of survivor function statistic $\chi^2 = 18.28$, $P = 0.0001$). Similar predictive outcomes were seen for albuminuria ($\chi^2 = 25.72$, $P = 0.0001$) and index of chronic damage ($\chi^2 = 31.74$, $P = 0.0001$; Figure 4). Therefore, univariate and multivariate analyses of these variables were performed. This demonstrated CD248 to be an independent predictor of renal survival in our cohort (Table 4).

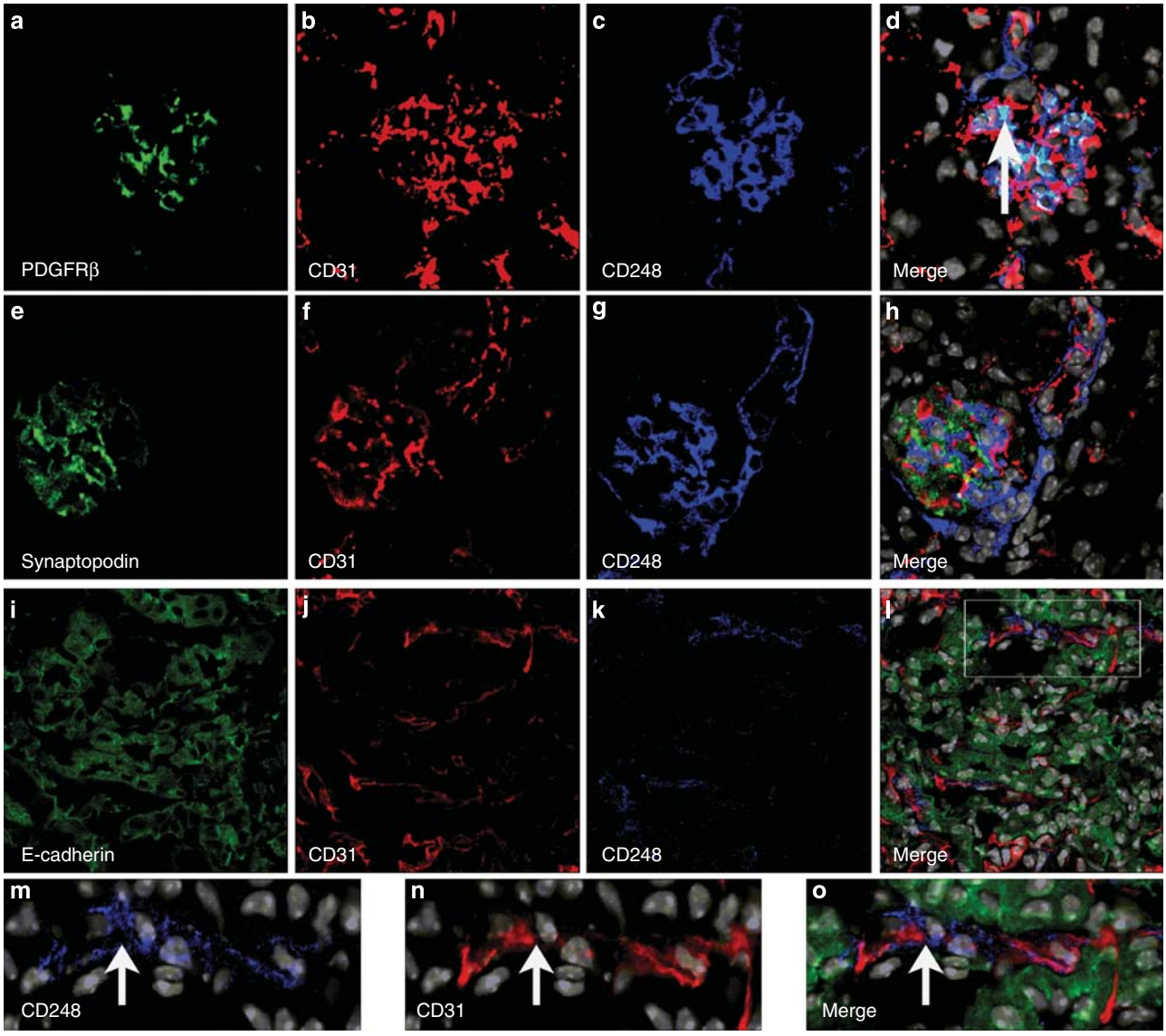

Figure 3 | In normal, noninflamed kidney, CD248 is expressed by resident renal pericytes. Murine tissue was analyzed. Glomerular expression of CD248. CD248 (**c**) localizes to mesangial cells of the glomerulus identified by platelet-derived growth factor receptor-β (PDGFRβ; panel **a** green and arrow panel **d** cyan) but does not colocalize with CD31-positive endothelial cells (red; **b**, **f**). CD248 (**g;** blue) does not colocalize with podocytes identified with synaptopodin (**e**; green). (**d**, **h**) Composite images. (**i**–**k**) Tubulointerstitial expression of CD248. CD248 is expressed by pericytes wrapped around peritubular blood vessels. E-cadherin (green) identifies tubular epithelial cells. CD31 (red; arrow, panel **n**)-positive endothelial cells within the interstitium are supported by CD248-positive pericytes (blue; arrow, panel **m**). Merged image shown in **l** and zoomed images in **m**–**o**.

Table 1 | Study population: clinical, pathological, and experimental characteristics (*n*=93)

	Median	Range
Age (years)	42	18–82
eGFR (ml/min per 1.73 m^2)	56	6–99
ACR (mg/mmol)	60	0.8–1097
Index of chronic damage (%)	17	0–94
Tubulointerstitial CD248 (%)	6.8	1.42–31.57

Abbreviations: ACR, albumin–creatinine ratio; eGFR, estimated glomerular filtration rate.

DISCUSSION

This study characterizes the expression of the stromal cell marker CD248 in normal kidney and in the kidney tissue of a cohort of patients with progressive renal disease. IgA nephropathy was chosen as a model of progressive human CKD because classical determinants of renal progression are recognized to be important in determining long-term renal survival in these patients.[9,12] CD248 has previously been demonstrated to be expressed on fibroblasts and pericytes found within inflammatory stroma.[15]

The in-house anti-CD248 antibodies used in this study have been extensively characterized within the literature, both *in vitro*[13,25] and *in vivo*,[14,26] to recognize fibroblasts and pericytes, and the studies presented here are further confirmation of this specificity. Importantly, they do not recognize endothelial cells or leukocytes.[26] Although in humans a

Table 2 | Univariate analysis of correlations between urinary ACR, tubulointerstitial CD248, index of chronic damage, and serum creatinine (*n*=93)

	Urinary ACR Correlation; *P*-value	Tubulointerstitial CD248 Correlation; *P*-value	Index of chronic damage Correlation; *P*-value
Urinary ACR	NA		
Tubulointerstitial CD248	0.500; 0.0000*	NA	
Index of chronic damage	0.512; 0.0000*	0.539; 0.0000*	NA
eGFR	−0.360; 0.0004*	−0.515; 0.0000*	−0.679; 0.0000*

Abbreviations: ACR, albumin–creatinine ratio; eGFR, estimated glomerular filtration rate; NA, not applicable.
*$P<0.05$.

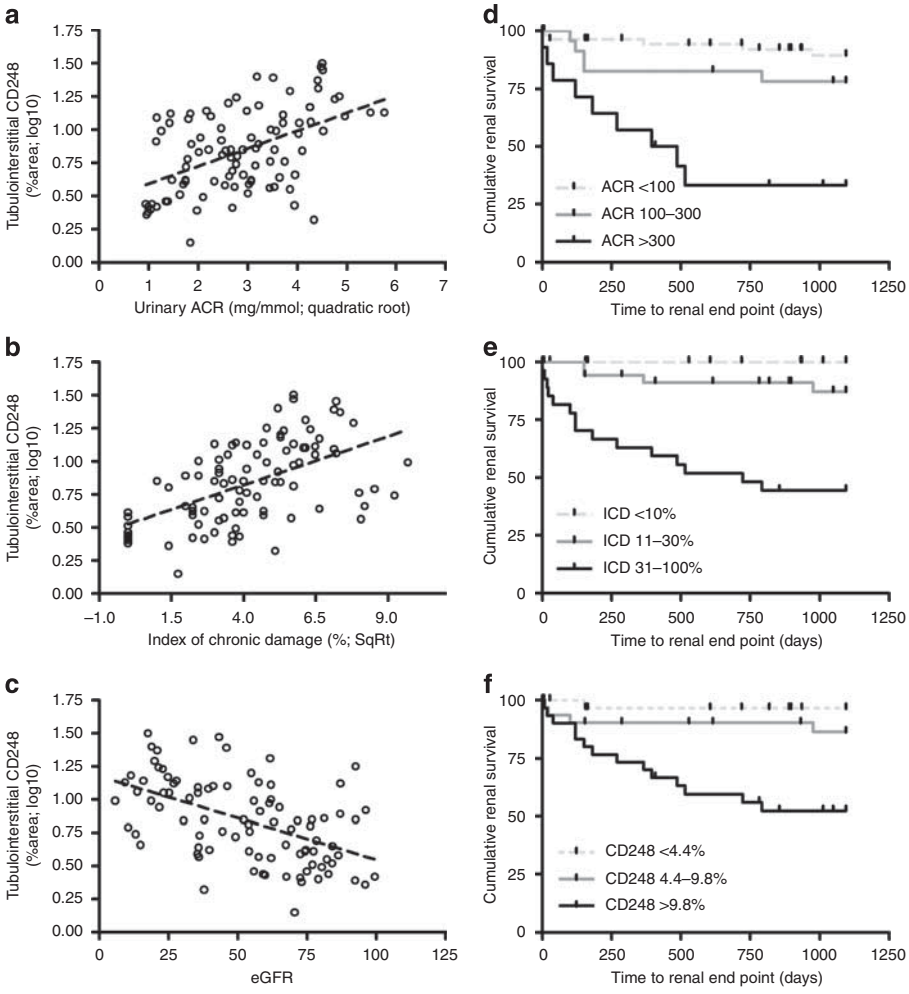

Figure 4 | Plots of correlation (correlation; *P*-value). Plots of correlation between (**a**) albumin–creatinine ratio (ACR) and tubulointerstitial CD248 staining (0.500; $P<0.0000$), (**b**) index of chronic damage and tubulointerstitial CD248 (0.539; $P<0.0000$), and (**c**) estimated glomerular filtration rate (eGFR) and tubulointerstitial CD248 (−0.679; $P<0.0000$). Kaplan–Meier analysis of (**d**) urinary ACR on renal outcome ($\chi^2=25.72$, $P=0.0001$); (**e**) index of chronic damage (ICD) on renal outcome ($\chi^2=31.74$, $P=0.0001$), and (**f**) tubulointerstitial expression of CD248 on renal outcome ($\chi^2=18.28$, $P=0.0001$).

population of CD8$^+$CD45$^+$ T cells and also a population of vascular leukocytes have been reported,[27,28] importantly no CD248$^+$CD45$^+$ cells were seen in our study.

Our *in vitro* and *in vivo* studies have shown in healthy noninflamed kidney that CD248 is expressed by resident pericytes and on stromal fibroblasts. In injured fibrotic kidney tissue, CD248 is increased and expressed on a subpopulation of myofibroblasts (CD248$^+$αSMA$^+$) in addition to a population of CD248$^+$αSMA$^-$ stromal fibroblasts. CD248$^-$αSMA$^+$ myofibroblasts were also seen. We were unable to phenotype these cells further using the tissue samples that we had available; however, this is the first description of a novel myofibroblast population that is CD248$^+$αSMA$^+$ and also of CD248$^+$αSMA$^-$ fibroblasts.

The origin and heterogeneity of renal stromal myofibroblasts is diverse and recruitment may occur either

Table 3 | Multivariate linear regression analysis between urinary ACR, tubulointerstitial CD248, index of chronic damage, and serum creatinine (*n*=93)

	Dependent variable			
	Urinary ACR β; *P*-value	Tubulointerstitial CD248 β; *P*-value	Index of chronic damage β; *P*-value	eGFR β; *P*-value
Urinary ACR	NA	0.299; 0.002*	0.253; 0.003*	0.517; 0.577
Tubulointerstitial CD248	0.329; 0.002*	NA	0.150; 0.097	−0.225; 0.019*
Index of chronic damage	0.380; 0.003*	0.204; 0.097	NA	−0.584; 0.000*
eGFR	0.067; 0.577	−0.268; 0.019*	−0.510; 0.000*	NA

Abbreviations: ACR, albumin–creatinine ratio; eGFR, estimated glomerular filtration rate; NA, not applicable.
*$P<0.05$.

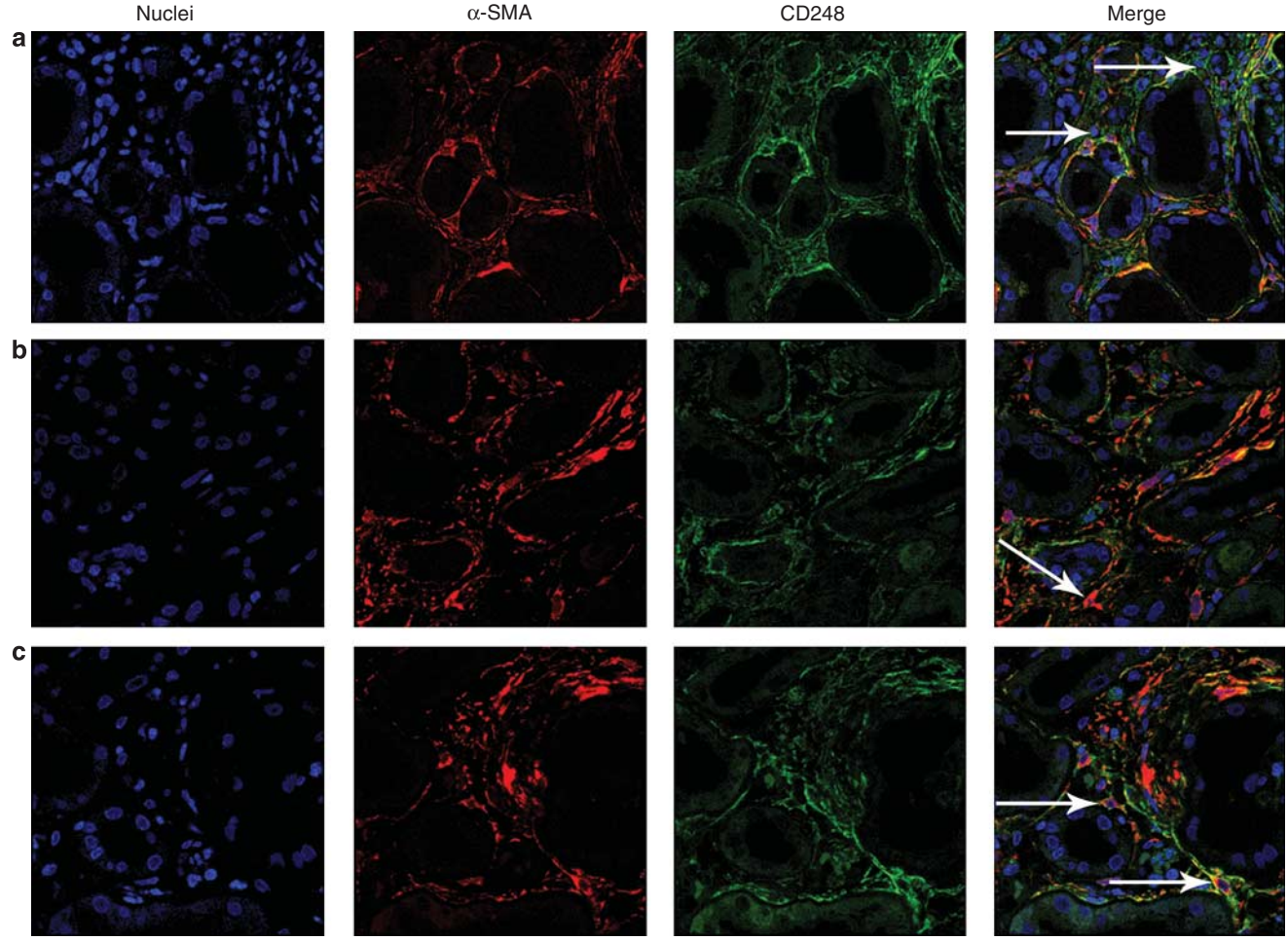

Figure 5 | Confocal microscopy. Human renal biopsy samples from patients with chronic kidney disease (CKD) 3 and 4 ($n=3$) were stained to colocalize α-smooth muscle actin (α-SMA; red) and CD248 (green). Nuclei are shown in blue. $CD248^+\alpha SMA^-$, $CD248^+\alpha SMA^+$, and $CD248^-\alpha SMA^+$ sub-populations can be seen in all biopsy samples and specific cell populations are highlighted in each panel. (**a**) $CD248^+\alpha SMA^-$ (arrows). (**b**) $CD248^-\alpha SMA^+$ (arrow). (**c**) $CD248^+\alpha SMA^+$ (arrows).

Table 4 | Univariate and multivariate analyses of the impact of studied variables on renal survival

	Urinary ACR Exp(B); *P*-value; 95% CI	Tubulointerstitial CD248 Exp(B); *P*-value; 95% CI	Index of chronic damage Exp(B); *P*-value; 95% CI	Age Exp(B); *P*-value; 95% CI	Sex Exp(B); *P*-value; 95% CI	eGFR Exp(B); *P*-value; 95% CI
Univariate	2.120; <0.0001 1.428–3.146	44.997; <0.0001 7.649–264.712	1.666; <0.0001 1.324–2.096	1.008; 0.561 0.982–1.034	1.113; 0.821 0.438–2.82	0.935; <0.0001 0.908–0.963
Multivariate	NS	12.09; 0.008; 1.89–77.39	NS	0.968; 0.008 0.942–0.995	NS	0.934; <0.0001 0.905–0.964

Abbreviations: ACR, albumin–creatinine ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate; NS, not statistically significant.

from the resident cell populations, from circulating bone marrow-derived precursors, or through the process of epithelial–mesenchymal transformation.[8] Iwano *et al.*[29] have attempted to describe, in animal models, the relative importance of the various potential sources of fibroblasts. Using bone marrow chimeras and transgenic reporter mice, the authors estimate that resident fibroblasts, epithelial–mesenchymal transformation, and circulating precursors contribute 52, 38, and 9%, respectively.

More recently, Zeisberg *et al.*[30] have demonstrated, using lineage tracing techniques in mice, that endothelial–mesenchymal transformation may also contribute to the fibroblast populations observed in renal disease. They demonstrated that 30–50% of fibroblasts seen in an animal model of renal fibrosis coexpressed the endothelial cell marker CD31 and both fibroblast and myofibroblast surface markers.[30] Here, we describe a novel subset of CD248$^+$ αSMA$^+$ myofibroblasts and also a population of CD248$^+$ αSMA$^-$ fibroblasts, thus emphasizing the heterogeneity of the fibroblast and myofibroblast populations found in CKD. αSMA$^-$ fibroblasts are described in the renal literature and are known to contain and express interstitial collagens *in vivo*.[31] Further fibroblasts isolated from α-SMA-null mice produce more type collagen 1 compared with wild-type controls.[3]

Elegant studies by Lin *et al.*[32] have highlighted the importance of the renal pericyte and of injury to the vasculature in driving fibrosis. Using a transgenic reporter mouse, they demonstrated that activation of pericytes and peritubular fibroblasts contributed significantly to interstitial myofibroblast populations in experimental renal fibrosis. Their observations from murine models of renal injury are supported further by our identification of CD248 expression by resident renal pericytes in close approximation to the vasculature and the accompanying upregulation of CD248 in human disease. However, our studies fall short of demonstrating categorically that in progressive human renal disease, CD248$^+$ cells, or a sub-population thereof, are derived from pericytes.

The extent of tubulointerstitial fibrosis seen in kidney biopsy has been repeatedly demonstrated to be a rigorous predictor of renal progression,[12,33] and our data are the latest confirmation of this long-known association. This study does not, however, demonstrate that a causal role is played by CD248 in progressive renal scarring, but the close association with albuminuria and renal scarring, which are known determinants of renal disease progression, and also the association between CD248 and renal survival outcomes is intriguing. Importantly, this relationship is maintained in our multivariate analysis.

In conclusion, we have demonstrated that the stromal fibroblast and pericyte marker CD248 is upregulated in chronic kidney disease and that this is linked to known determinants of renal progression. The expression of CD248 on stromal fibroblasts and pericytes is already robustly established in the literature. However, here we report the additional observation that CD248 defines a sub-population of myofibroblasts and also a sub-population of CD248$^+$ αSMA$^-$ fibroblasts that are linked to albuminuria and tubulointerstitial damage, suggesting that CD248 may be implicated in the tissue remodeling seen in CKD. Immunostaining for CD248-positive cells may not only provide a valuable histological guide to predict renal progression, but also raise the possibility that CD248-positive stromal cells may be a potential target for the modulation of renal disease using novel antiangiogenic drugs such as the anti-human CD248 monoclonal antibody MORAb-004.

MATERIALS AND METHODS

Anti-human CD248 antibody

The generation of the human anti-CD248 monoclonal antibody (B1/35) has been described previously.[13]

Cell culture

Immortalized glomerular endothelial cells and podocytes were a kind gift from Dr Simon Satchell (University of Bristol, Bristol, UK). Following NRES (National Research Ethics Service) ethical approval (Research Ethics Committee approval number 08/H1203/8) and under the regulations described within the Human Tissue Act 2004, human renal fibroblasts were isolated using the explant method of Grimwood *et al.*[34] from the normal pole of nephrectomy samples removed for the treatment of malignant disease. Fibroblasts stained negative for CD31, cytokeratin, and desmin, but positive for vimentin. Passages 2–8 were used for all *in vitro* studies.

Immunocytochemistry

Cells were seeded onto glass chamber slides (Becton Dickinson Falcon, San Diego, CA) at 10,000 cells per well and were allowed to grow to confluence at 37 °C. They were then fixed with methanol at −20 °C and stained with either anti-human CD248 or mouse IgG1 (Dako, Cambridge, UK) overnight at 4 °C. Goat anti-mouse IgG1 conjugated to Alexa 488 (Invitrogen, Paisley, UK) was then applied and the slides were visualized using a Zeiss confocal LSM 510 microscope (Zeiss, Gottingen, Germany) and processed using Zeiss LSM Image Examiner software (Zeiss).

Reverse transcription-PCR

Cells were grown to confluence in six-well plates. A Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate RNA according to the manufacturer's protocol. Complementary DNA was generated using TaqMan Reverse Transcription (Applied Biosystems, Carlsbad, CA). PCR was performed using the following primers: CD248 forward 5′-TTTGGCTTCGAGGGCGCCTG-3′, CD248 reverse 5′-TCACACTGCTGCTCGCACGG-3′; TBP forward 5′-AACTTCGCTTC CGCTGGCCC-3′, TBP reverse 5′-GCTGTGGTGCCTGGCCTGAG-3′ (Alta Bioscience, Birmingham, UK).

Western blotting

Cells were lysed in CellLyticMT (Sigma, York, UK) sample buffer and 22.5 μg of protein per sample was run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Gels were transferred onto nitrocellulose membrane, blocked with 5% milk, and incubated with the primary antibodies: B1/35 and β-actin (Sigma), followed by horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK). Immunodetection was carried out using the ECL Kit (Amersham) followed by exposure to X-ray film for 15 min.

Mice
Wild-type 129SvEv mice were obtained from Harlan UK (Oxford, UK). All experiments were performed in accordance with UK laws and with the approval of the local ethics committees. Kidney tissue samples were removed from adult mice ($n=6$) and snap frozen in liquid nitrogen. Frozen 6 μm sections were cut for staining.

Patient samples
Following local ethical approval (NRES 07/Q2602/42), we identified patients who underwent a percutaneous renal biopsy at the University Hospital Birmingham NHS Foundation Trust for the investigation of renal disease between June 1998 and November 2009. A randomly selected prevalent cohort of 100 patients with a histological diagnosis of IgA nephropathy, who also had archived formal-saline fixed paraffin tissue, was used for the study. Clinical and demographic data were collected from patient records. Normal tissue sections were taken from nephrectomy samples and also purchased commercially (Abcam, Cambridge, UK).

Immunohistochemistry for CD248
The immunohistochemical detection of CD248 was performed using established methods. Briefly, 4 μm tissue sections were cut onto glass slides from paraffin blocks. Sections were dewaxed in xylene and rehydrated through a series of graded alcohols to water. Antigen retrieval was performed using Dako Target Antigen Retrieval solution in a water bath at 95 °C for 30 min. Sections were stained according to the Dako mouse Envision-HRP kit protocol with monoclonal mouse anti-human CD248 (Clone B1/35, in-house antibody) at a concentration of 0.2 μg/ml overnight at 4 °C. Sections were then either counterstained with Mayer's hematoxylin for CD248 localization or left unstained for quantitative analysis. Mouse IgG1 (Dako) was used as an isotype control and substituted for primary antibody on serial sections.

Quantification of interstitial CD248 expression
An interactive image analysis system was used for blinded assessment of interstitial CD248 fibroblast numbers. We have previously found this to be a reliable method in the analysis of renal tissue sections.[12,24]

The sections were blinded to the operator and stained for CD248. They were visualized at ×200 magnification and images were captured using a Nikon Eclipse E400 microscope with a digital imaging system controlled by NIS Elements Version 3.0 (Nikon, Kingston Upon Thames, UK). Images were stored as TIFF files and imported into Aequitas IA image analysis software (DynamicData Links, Cambridge, UK) where the digitalized image is converted to a two-color scale image. Using the threshold function, the image was processed so that positive staining was represented by yellow pixels measured as a percentage of the area of total image analyzed. Sections with excessive background staining that made it impossible to digitally differentiate specific staining were excluded from analysis. For each patient, the mean measurement of interstitial CD248 staining of five randomly selected nonconfluent microscopic fields was determined. Blood vessel and glomerular staining was identified by the operator and excluded from the analysis using the computer software.

Quantification of chronic damage
The extent of chronic tissue damage within each biopsy specimen was assessed using the index of chronic damage, an established and rigorous predictor of renal outcome previously developed by our group.[35] For each biopsy, one section was stained by periodic acid-methenamine silver and assessed morphometrically using this method.

Confocal microscopy
Confocal microscopy was performed on 4 μm tissue sections cut from paraffin blocks using the methods of Robertson *et al.*[36] The following antibodies were used: monoclonal mouse IgG1 anti-human CD248 and monoclonal mouse IgG2 anti-human α-SMA (R&D Research Systems, Oxford, UK). Goat anti-mouse IgG1 conjugated to Alexa 488 or goat anti-mouse IgG2a conjugated to Alexa 633 (Invitrogen) was then applied and the slides were visualized using a Zeiss confocal LSM 510 microscope (Zeiss) and processed using Zeiss LSM Image Examiner software (Zeiss).

Frozen murine tissue was stained with: polyclonal rabbit anti-mouse CD248 (in-house), hamster anti-mouse CD31, rat anti-mouse platelet-derived growth factor receptor-β, rat anti-mouse synaptopodin, and rat anti-mouse E-cadherin (eBioscience, San Diego, CA). For staining of platelet-derived growth factor receptor-β, 0.5% Tween was added to the antibody buffer.

Statistics
Linear regression analyses were performed to determine correlations between normally distributed data variables. Variables determined to not follow a Gaussian distribution by normality testing for skewness were normalized by transformation before analysis. Correlations are presented by expressing the β-correlation coefficient along with the *P*-value. Linear regression multivariate analysis of these correlations with a dependent variable was also performed. Renal outcome was assessed by Kaplan–Meier survival analysis with log rank testing after categorization of tubulointerstitial CD248 expression into tertiles. End points were either doubling of serum creatinine concentration or end-stage renal failure, defined by requirement of dialysis. All statistical tests were performed using SPSS (IBM, Middlesex, UK). Graphs were created using Prism 4 (GraphPad, San Diego, CA). The level of significance was set at $P<0.05$.

DISCLOSURE
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REFERENCES
1. Strutz F, Zeisberg M. Renal fibroblasts and myofibroblasts in chronic kidney disease. *J Am Soc Nephrol* 2006; **17**: 2992–2998.
2. Goumenos DS, Brown CB, Shortland J *et al.* Myofibroblasts, predictors of progression of mesangial IgA nephropathy? *Nephrol Dial Transplant* 1994; **9**: 1418–1425.
3. Takeji M, Moriyama T, Oseto S *et al.* Smooth muscle α-actin deficiency in myofibroblasts leads to enhanced renal tissue fibrosis. *J Biol Chem* 2006; **281**: 40193–40200.
4. Okada H, Inoue T, Suzuki H *et al.* Epithelial-mesenchymal transformation of renal tubular epithelial cells in vitro and in vivo. *Nephrol Dial Transplant* 2000; **15**(Suppl 6): 44–46.
5. Strutz FM. EMT and proteinuria as progression factors. *Kidney Int* 2009; **75**: 475–481.
6. Strutz F, Okada H, Lo CW *et al.* Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol* 1995; **130**: 393–405.

7. Okada H, Danoff TM, Kalluri R *et al.* Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 1997; **273**: F563–F574.
8. Hewitson TD. Renal tubulointerstitial fibrosis: common but never simple. *Am J Physiol Renal Physiol* 2009; **296**: F1239–F1244.
9. Nishitani Y, Iwano M, Yamaguchi Y *et al.* Fibroblast-specific protein 1 is a specific prognostic marker for renal survival in patients with IgAN. *Kidney Int* 2005; **68**: 1078–1085.
10. Ohradanova A, Gradin K, Barathova M *et al.* Hypoxia upregulates expression of human endosialin gene via hypoxia-inducible factor 2. *Br J Cancer* 2008; **99**: 1348–1356.
11. Inoue T, Plieth D, Venkov CD *et al.* Antibodies against macrophages that overlap in specificity with fibroblasts. *Kidney Int* 2005; **67**: 2488–2493.
12. Eardley KS, Zehnder D, Quinkler M *et al.* The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int* 2006; **69**: 1189–1197.
13. MacFadyen JR, Haworth O, Roberston D *et al.* Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium. *FEBS Lett* 2005; **579**: 2569–2575.
14. Simonavicius N, Robertson D, Bax DA *et al.* Endosialin (CD248) is a marker of tumor-associated pericytes in high-grade glioma. *Mod Pathol* 2008; **21**: 308–315.
15. Christian S, Winkler R, Helfrich I *et al.* Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. *Am J Pathol* 2008; **172**: 486–494.
16. Rouleau C, Curiel M, Weber W *et al.* Endosialin protein expression and therapeutic target potential in human solid tumors: sarcoma versus carcinoma. *Clin Cancer Res* 2008; **14**: 7223–7236.
17. Tomkowicz B, Rybinski K, Foley B *et al.* Interaction of endosialin/TEM1 with extracellular matrix proteins mediates cell adhesion and migration. *Proc Natl Acad Sci USA* 2007; **104**: 17965–17970.
18. Lax S, Hardie DL, Wilson A *et al.* The pericyte and stromal cell marker CD248 (endosialin) is required for efficient lymph node expansion. *Eur J Immunol* 2010; **40**: 1884–1889.
19. Tomkowicz B, Rybinski K, Sebeck D *et al.* Endosialin/TEM-1/CD248 regulates pericyte proliferation through PDGF receptor signaling. *Cancer Biol Ther* 2010; **9**: 908–915.
20. Rupp C, Dolznig H, Puri C *et al.* Mouse endosialin, a C-type lectin-like cell surface receptor: expression during embryonic development and induction in experimental cancer neoangiogenesis. *Cancer Immun* 2006; **6**: 10.
21. Nanda A, Karim B, Peng Z *et al.* Tumor endothelial marker 1 (Tem1) functions in the growth and progression of abdominal tumors. *Proc Natl Acad Sci USA* 2006; **103**: 3351–3356.
22. Maia M, de Vriese A, Janssens T *et al.* CD248 and its cytoplasmic domain: a therapeutic target for arthritis. *Arthritis Rheum* 2010; **62**: 3595–3606.
23. Donadio JV, Grande JP. IgA nephropathy. *N Engl J Med* 2002; **347**: 738–748.
24. Eardley KS, Kubal C, Zehnder D *et al.* The role of capillary density, macrophage infiltration and interstitial scarring in the pathogenesis of human chronic kidney disease. *Kidney Int* 2008; **74**: 495–504.
25. Lax S, Hardie DL, Wilson A *et al.* The pericyte and stromal cell marker CD248 (endosialin) is required for efficient lymph node expansion. *Eur J Immunol* 2010; **40**: 1884–1889.
26. Lax S, Hou TZ, Jenkinson E *et al.* CD248/Endosialin is dynamically expressed on a subset of stromal cells during lymphoid tissue development, splenic remodeling and repair. *FEBS Lett* 2007; **581**: 3550–3556.
27. Watkins NA, Gusnanto A, de Bono B *et al.* A HaemAtlas: characterizing gene expression in differentiated human blood cells. *Blood* 2009; **113**: e1–e9.
28. Conejo-Garcia JR, Buckanovich RJ, Benencia F *et al.* Vascular leukocytes contribute to tumor vascularization. *Blood* 2005; **105**: 679–681.
29. Iwano M, Plieth D, Danoff TM *et al.* Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002; **110**: 341–350.
30. Zeisberg EM, Potenta SE, Sugimoto H *et al.* Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. *J Am Soc Nephrol* 2008; **19**: 2282–2287.
31. Okada H, Inoue T, Kanno Y *et al.* Renal fibroblast-like cells in Goodpasture syndrome rats. *Kidney Int* 2001; **60**: 597–606.
32. Lin SL, Kisseleva T, Brenner DA *et al.* Pericytes and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney. *Am J Pathol* 2008; **173**: 1617–1627.
33. Bohle A, Mackensen-Haen S, von Gise H. Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney: a morphometric contribution. *Am J Nephrol* 1987; **7**: 421–433.
34. Grimwood L, Masterson R. Propagation and culture of renal fibroblasts. *Methods Mol Biol* 2009; **466**: 25–37.
35. Howie AJ, Ferreira MA, Adu D. Prognostic value of simple measurement of chronic damage in renal biopsy specimens. *Nephrol Dial Transplant* 2001; **16**: 1163–1169.
36. Robertson D, Savage K, Reis-Filho JS *et al.* Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biol* 2008; **9**: 13.