Glomerular parietal epithelial cells of adult murine kidney undergo EMT to generate cells with traits of renal progenitors

Swetha G., Vikash Chandra, Smruti Phadnis, Ramesh Bhonde *

Tissue Engineering and Banking Laboratory, National Centre for Cell Science, Pune, India

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Abstract

Glomerular parietal epithelial cells (GPECs) are known to revert to embryonic phenotype in response to renal injury. However, the mechanism of de-differentiation in GPECs and the underlying cellular processes are not fully understood. In the present study, we show that cultured GPECs of adult murine kidney undergo epithelial–mesenchymal transition (EMT) to generate cells, which express CD24, CD44 and CD29 surface antigens. Characterization by qRT-PCR and immunostaining of these clonogenic cells demonstrate that they exhibit metastable phenotype with co-expression of both epithelial (cytokeratin-18) and mesenchymal (vimentin) markers. Transcript analysis by qRT-PCR revealed high expression of metanephric mesenchymal (Pax-2, WT-1, Six-1, Eya-1, GDNF) and uterine bud (Hoxb-7, C-Ret) genes in these cells, indicating their bipotent progenitor status. Incubation of GPECs with EMT blocker Prostaglandin E2, resulted in low expression of renal progenitor markers reflecting the correlation between EMT and acquired stemness in these cells. Additional in vitro renal commitment assays confirmed their functional staminality. When injected into E13.5 kidney rudiments, the cells incorporated into the developing kidney primordia and co-culture with E13.5 spinal cord resulted in branching and tubulogenesis in these cells. When implanted under renal capsule of unilaterally nephrectomized mice, these cells differentiated into immature glomeruli and vascular ducts. Our study demonstrates that EMT plays a major role in imparting plasticity to terminally differentiated GPECs by producing metastable cells with traits of kidney progenitors. The present study would improve our understanding on epithelial cell plasticity, furthering our knowledge of its role in renal repair and regeneration.

Keywords: glomerular parietal epithelial cells • EMT • stem cell phenotype • renal progenitors • metastable cells

Introduction

Kidney is an organ with limited turnover of cells but exhibits remarkable ability to survive injury and restore function [1]. The precise mechanism by which adult kidney replenishes damaged cells remains to be understood. It is still not clear whether cellular repair in adult kidney is carried out by specialized renal progenitors residing in specific niches or by self-duplication/de-differentiation of mature cells. Previous reports suggest the role of renal resident stem cells in kidney repair and regeneration [2–5] and others have suggested the contribution of circulating bone marrow stem cells in renal repair [6]. However, limited contribution of these cells to injured kidneys has led to questions regarding their significance in kidney repair [7]. In vital organs like kidney, with low cell turnover it would be imperative to consider that organ repair would follow a more pragmatic way, wherein somatic cells would acquire phenotypic flexibility rather than activating the quiescent resident stem cell population [8]. It is well established that kidney tubular epithelial cells, respond to injury by de-differentiating into mesenchymal phenotype thus recapitulating the processes active during early nephrogenesis [9]. Furthermore, a recent report suggests that regeneration by surviving tubular epithelial cells is the predominant mechanism of repair in ischemic kidneys [10]. Interestingly, the response of glomerular cells to injury is reported to be more complex, involving considerable phenotypic adaptations [11]. Glomerular parietal epithelial cells (GPECs), lining the inner aspect of Bowman’s capsule is known to respond to injury by de-differentiating into embryonic phenotype, similar to that of myofibroblasts with de novo expression of α-SMA [12]. Under normal physiological conditions, GPECs are known to migrate and differentiate into glomerular podocytes [13]. Moreover, reports suggest that CD133+ CD24+ cell subset of GPEC of adult human kidney have stem cell properties and participates in renal repair [14]. Reparative responses in differentiated glomerular epithelial cells thus repre-
sent an injury-dependent regression from adult phenotype to embryonic mesenchymal phenotype [15]. It is suggested that such phenotypic alterations are primarily conceived by glomerular epithelial–mesenchymal trans-differentiation (GEMT) [16]. However, in certain conditions, these changes are also associated with excessive production of extracellular matrix (ECM) resulting in crescent formation and irreversible renal fibrosis [17]. It is intriguing that renal pathology is caught in this vicious cycle where normal patho-physiological responses to tissue injury such as EMT and fibrosis, can also result in chronic injury culminating in organ failure. Understanding the mechanism of cellular de-differentiation in key glomerular subsets like GPECs would further our knowledge of its role in tissue repair, disease progression and enable more effective targeted therapies for acute and chronic kidney diseases. With this perspective, we investigated the de-differentiation potential of murine glomerular epithelial cells, in vitro. We report here that GPECs of adult murine kidney undergo spontaneous EMT to generate cells with metastable phenotype and acquire gene profile and functional properties similar to that of early renal progenitors.

**Immunostaining and confocal microscopy**

Cells on cover slips, cryostat sections and whole mount tissues were fixed in 4% fresh paraformaldehyde, permeabilized with 0.1% Triton-X100 and blocked with 1% bovine serum albumin (BSA). Primary antibodies were incubated overnight at 4°C, washed with PBS and then incubated with secondary antibody at 37°C for 1 hr. DAPI (4’, 6-diamidino-2-phenylindole)/ Hoechst 3342 (Invitrogen, Carlsbad, CA, USA) were used to visualize nuclei. Cells were washed with PBS and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Confocal images were captured using Zeiss LSM 510 laser scanning microscope using 63×/1.3/100× oil objective. Magnification, laser and detector gains were set before saturation. Images were analysed with LSM5 IMAGE EXAMINER software (Carl Zeiss, Microlmaging, Inc., Thornwood, NY, USA). The fluorescence is quantified and represented in 2.5D intensity graphs [18]. Results presented are representative fields confirmed from at least three different experiments. The sources of antibodies and dilutions used are summarized in Table 1.

**Flow cytometry**

Cells were trypsinized, centrifuged and blocked in 1% BSA. For the detection of surface antigens, cells were incubated with freshly diluted fluorescein isothiocyanate (FITC)/phycoerythrin (PE) conjugated antibodies for 1 hr at 37°C. The sources of antibodies and dilutions used are summarized in Table 1. Cells were also stained with FITC- or PE-labelled isotype-matched immunoglobulins, which served as negative controls. Cells were washed with PBS, passed through 40 μm cell stainer to avoid clumps and resuspended in Fluorescence Activated Cell Sorting (FACS) buffer. The cells were analysed using BD FACScanto™ Flow Cytometer and data quantified using BD FACS Diva softwarev5.0. For clonogenic studies, cells labelled with CD24 antibody were sorted as positive and negative fractions into individual wells of a 96-well plate at a density of one cell per well per plate for each fraction using BD FACSAria™ Flow Cytometer in sterile conditions. Wells with single cells were marked and confirmed under phase contrast microscope and maintained in standard culture conditions for colony formation.

**RNA isolation and quantitative real time PCR**

Tissue/Cells samples were frozen in Tri Reagent (Sigma-Aldrich). Total RNA was isolated from samples as per the manufacturers’ instructions, measured on ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Two micrograms of RNA was used for cDNA synthesis per 20 μl reaction. cDNA was amplified using Reverse Transcription System Kit (ImProm II Reverse Transcription system #A3800, Promega Corporation, Madison, WI, USA). The primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) are summarized in Table 1. qRT-PCR was performed in duplicate of total 25 μl reaction mixture containing 1× Power SYBR-Green Master-mix (Applied-Biosystems, Foster City, CA, USA), 600–750 nM each forward and reverse primers using 1/20th of the cDNA preparation. PCR amplification was carried out using Applied-Biosystem/7000 Real-Time PCR Sequence detection System ( SDS-v1 3.1; Applied-Biosystems). Cycling conditions were set as (program: 2 min. at 50°C, 10 min. at 95°C, and 35 cycles of 15 sec. at 95°C and 1 min. at 60°C). All qRT-PCR results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) carried out in duplex reaction to correct differences in RNA input. Gene expression is reported as fold difference in Ct values relative to the expression of gene in cells at P5 over P1. Gene expres-
Table 1 The list of Primers and Antibodies with dilutions used in the present study

| Antibody                  | Company                      | Dilution |
|---------------------------|------------------------------|----------|
| α smooth muscle actin     | Sigma, www.sigma-aldrich.com | 1–100    |
| Aquaporin-6               | (Chemicon) Millipore, www.millipore.com | 1–100    |
| β-Catenin                 | Sigma, www.sigma-aldrich.com | 1–100    |
| Cadherin-11               | Millipore, www.millipore.com | 1–100    |
| Collagen II               | Millipore, www.millipore.com | 1–100    |
| Collagen IV               | Sigma, www.sigma-aldrich.com | 1–100    |
| Cytokeratin-18            | Sigma, www.sigma-aldrich.com | 1–100    |
| E-Cadherin                | Sigma, www.sigma-aldrich.com | 1–100    |
| Fibronectin               | Millipore, www.millipore.com | 1–100    |
| Ki-67                     | Millipore, www.millipore.com | 1–150    |
| Laminin                   | Sigma, www.sigma-aldrich.com | 1–100    |
| Nephrin                   | Santa Cruz www.scbt.com      | 1–100    |
| Nestin                    | Millipore, www.millipore.com | 1–100    |
| PECAM                     | Sigma, www.sigma-aldrich.com | 1–100    |
| Vimentin                  | Sigma, www.sigma-aldrich.com | 1–100    |
| Claudin1                  | Santa Cruz www.scbt.com      | 1:75     |
| Claudin1/2                | Santa Cruz www.scbt.com      | 1:75     |
| CD29-FITC                 | BD, wwwbdbiosciences.com     | 1–100    |
| SCA-1-FITC                | BD, wwwbdbiosciences.com     | 1–100    |
| CD24-FITC                 | BD, wwwbdbiosciences.com     | 1–100    |
| CD31-FITC                 | BD, wwwbdbiosciences.com     | 1–100    |
| CD11b                     | BD, wwwbdbiosciences.com     | 1–100    |
| CD105-PE                  | BD, wwwbdbiosciences.com     | 1–100    |
| CD45-PE                   | BD, wwwbdbiosciences.com     | 1–100    |
| CD38-PE                   | BD, wwwbdbiosciences.com     | 1–100    |
| CD90.1-PE                 | BD, wwwbdbiosciences.com     | 1–100    |
| CD44-PE                   | BD, wwwbdbiosciences.com     | 1–100    |
| C-KIT-PE                  | BD, wwwbdbiosciences.com     | 1–100    |
| CD88-PE                   | BD, wwwbdbiosciences.com     | 1–100    |
| CD133                     | Santa Cruz www.scbt.com      | 1–100    |
| Claudin1/2                | Santa Cruz www.scbt.com      | 1:75     |

Continued

| Genes | Primer sequence |
|-------|-----------------|
| WNT4  | F GCA GAT GTG CAA ACG GAA CCT TGA |
|       | R ATG TGG CTT AAG CTG TGG ACC TTA |
| WNT7b | F AAC TCGCAC CAC GAC GTT ACC TTA |
|       | R TGG CAT TTG AAG CAA CAC CAG |
| WNT9b | F TGC CAC CTT GTC TCC TGT TGG |
|       | R ATC TCC AGG CAT TTG GAC CTA |
| WNT11 | F TGC CTC CCT GGA AAC GAA GTG TAA |
|       | R TGT CAC TCG CTT TGG AAG TCT TGT |
| WNT2b | F CAA ATT CCA GTG CTG TCT GTC TGT |
|       | R AGG CAG AGT AGC ATC AAA CAC GGA |
| WNT6  | F TGT CAG TCC CTG TCG TTT GAA |
|       | R GCT TGT GCT GCG CAT CCA TAA AGA |
| WNT5b | F TGG AAA TCC ACA ACC AGT GGG AGA |
|       | R AGC AGG TGA CAG AAC CTG GTT TCT |
| TGF-β1| F TAA AGA GTT CAC CCG GTG GCT AAT |
|       | R AAA GAC AGC CAC TCA GGC GTA TCA |
| TGF-β2| F AAG GCG TTA GTG ATC TCA CCT |
|       | R AAT CAT GCT GGC TTC TAG ACC GGT |
| TGF-β3| F CAG GGC AAG GCA AAG AGC TGG ATT |
|       | R TAT CTG ATA TCG CCC AAC GCT GCT |
| BMP-7 | F GAA ACC AGC AGT GAC CA |
|       | R GGT GGC GGT CAT GTA GGG GT |
| Pax-2 | F TCC CAG TGT GTC ATC CAT CA |
|       | R GTT AGA GGC GGT GGA AAC AG |
| WT-1  | F TAC AGA TGC ATG GGC GGA AGC ACA |
|       | R TCA CAC CTG TGT GTC TTC TGG TGT |
| Eya-1 | F AGC AGC TTT ACC AGC TCA GCA GGA |
|       | R ATG GGT CTA GTG GGA GCT TGG ATT |
| Six-1 | F ACC ACT GTT TCT TCT CCA CAG CCT |
|       | R TAC AAA GCA TGA GCA AGC CAA CCC |
| Odd 1 | F AGA GTG TGG GAA AGG ATT CTG CCA |
|       | R TGC TGT GGA AGG ATC CCG AAA |
| K-Cadherin | F AGG AGG AAT GAG CCT GGA TT |
|       | R TTT CTC TTT GCT GGG AAG CCA CTA |
| GDNF  | F AAA GCA TTC GCG TAA AGC GTG TGG |
|       | R TTT CTG TAG CTG GGC CTT CCA |
### Table 1 (Continued)

| Genes     | Primer sequence |
|-----------|-----------------|
| Hox b7    | F AGA AAG CCA AAG GGA GGA AAG CGA |
| R GGC ACA TTC CAG AAG GCC ACA GAA |
| C-Ret     | F AGC ATG ACA CAG GAG AAG CGG ATT |
| R TAG CAC TGG CTT CGT GAG TGA CAA |
| Vimentin  | F ATG CTT CTC TGG CAC GTC TT |
| R AGC CAC GCT TTC ATA CTG TT |
| Nestin    | F ATA CAG GAC TCT GCT GGA GG |
| R AGG ACA CCA GTA GAA CTG GG |
| Cytokeratin19 | F AGT TTG AGA CAG AAC AGC CCT TGC |
| R TCA GGC TCT CAA TCT GCA TCT CCA |
| E-Cadherin| F TGA CTC GAA ATG ATG TGG CTC CCA |
| R ACT GCC CTC GTA ATC GAA CAC CAA |
| Claudin 3 | F TGA CAG AGC ACA CAC AGT CTG CTT |
| R TCC ATT CGG CCT GGA CAG TCT CTA |
| Claudin 4 | F ATG GTC ATC AGC ATC ATC GTG GGT |
| R TGT AGA AGT CGC GGA TGA CTG TGT |
| Occludin  | F AGC AGG CCT CAG GGTT ACT ATT TT |
| R ACG ACAG TTA ACT CCT GAA CCA GCA |
| Snail 1   | F ACA GGT GCT TCG AGC CAT AGA ACT |
| R TGT ACC TCA AAG AAG GTG GCC TGA |
| Snail 2   | F CAC ATT CGA ACC CAC ACA TTG CCT |
| R TGT GCC CTC AGG TTT GAT CTG TCT |

### Microinjection of CD24⁺ cells to E13.5 kidney

The cells were trypsinized and stained with fluorescent cell marker chloromethylfluorescein diacetate (CMFDA) (Invitrogen) as per manufacturer’s instruction. Briefly, cells were incubated with 5 μM of CMFDA dye at 37°C for 30 min. in plain medium. The cells were then washed and incubated in normal culture medium. Fluorescent labelled cells were then resuspended in 20 μl of PBS to make a final concentration of 10⁶ cells/ml and microinjected with a very fine needle into E13.5 embryonic kidneys on a transwell membrane. E13.5 kidneys were cultured in air media interface and microinjected with a very fine needle into E13.5 embryonic kidneys on a transwell membrane. For in vitro induction experiments, cells were co-cultured with E13.5 spinal cord using cell culture inserts in serum-free media (RPMI1640 with 1% BSA and 0.25 μl beta-mercaptoethanol (BME)]. Cells were seeded in 6-well plates and insert containing spinal cord were slowly lowered. Media were changed every 24 hrs and conditioned media collected. After 5 days in culture, to promote tubulogenic differentiation, the embryoid bodies (EBs; nephrospheres) formed were transferred to matrigel coated plates and cultured for additional 5 days in E13.5 spinal cord conditioned media.

### In vitro tube formation assay

For endothelial differentiation, 2 × 10⁵ cells were seeded onto Matrigel (250 μl) (BD Biosciences, San Diego, CA, USA) coated 24-well plates in normal culture media. The cells were incubated in 37°C, 5% CO₂ and observed for 24 hrs. Cells were then separated from the matrigel by 1 hr/37°C incubation with Dispase (1 mg/ml). Isolated cells were then analysed by flow cytometry for the expression of endothelial specific markers.

### Unilateral nephrectomy in mice

Unilateral nephrectomy was performed on 6–8-week-old Swiss albino mice as described earlier [19]. Under anaesthesia (ketamine [150 mg/kg] and xylazine [10 mg/kg]), right kidney was exposed through a small lumbar incision. The renal artery was ligated and the kidney excised. The right kidney was removed in all experimental groups (n = 10). The animals were kept warm during and after the procedure, using a heating lamp. Incisions were closed using 4–0 absorbable sutures. Animals returned to cages and were kept in postoperative care with adequate food and water.

### Cell implantation under kidney capsule

Before implantation under kidney capsule, the cells were labelled with fluorescent dye PKH26 (Red Fluorescent Cell linker Kit, Sigma-Aldrich) as per manufacturer’s instructions. Briefly, 4 μM of PKH26 cell linker dye was incubated with 2 × 10⁵ cells. Cells were washed separately with PBS and staining was stopped by adding complete media with FCS. The cell implantation was carried out following the protocol described earlier [20]. Briefly, under anaesthesia, the labelled cell suspensions were mixed with equal volume of mouse autologous tail vein blood and the resulting clot was then implanted under the kidney capsule. Use of blood clot helps in the localization of the grafts and prevents loss of cells. Implants were removed 2 weeks after nephrectomy of the contra-lateral kidney and processed for further analysis. For histological evaluation of the grafted cells, sections were stained with haematoxylin and eosin.

### Statistical analysis

Values are expressed as mean ± S.E.M. from three different experiments unless otherwise indicated. Statistical analysis was done using paired two-tailed, t-test to determine the significance between different conditions. P-values < 0.05 were considered significant Prism4, Graphpad Software; (http://www.graphpad.com) was used for all analysis.
Results

Glomerular parietal epithelial cells of adult murine kidney generate CD24+ mesenchymal cells in vitro

Capsulated glomerular explants derived from collagenase digested murine kidney gave rise to a number of epithelioid colonies in vitro (Fig. 1A). The epithelial nature of these cells was established by their polygonal shape and cobblestone appearance at confluence. The parietal epithelial nature of these cells was established by positive immunostaining for Claudin-1 and 2 [21], also co-expressing CD24 (Fig. 1B). GPECs were positive for E-Cadherin and CD24 (Fig. 1C) but negative for podocyte marker, nephrin (Fig. 1D) and endothelial marker, Von-Willibrand factor (VWF) (Fig.1E). Immunostaining of normal murine kidney for the expression of CD24 showed that the antigen is localized in cells lining the Bowman’s capsule and certain cells of proximal tubules (Fig. 1F). By 5–6 days in culture, epithelial outgrowths from individual glomeruli were isolated by ring cloning and cultured in 24-well plates. Under in vitro conditions, the typical cobblestone pattern of the cultured epithelial cells was gradually (7–8 days) replaced by spindle-shaped fibroblast-like cells. After the cells were expanded to sufficient numbers for cloning they were sorted to CD44+ and CD24+ dual positive cells and designated as passage1(P1). At P1, 99.7 ± 0.3% cells were dual positive for CD44 and CD24 antigens and further characterization showed that these cells exhibited uniform expression for CD44 (94 ± 3.1%) and CD29 (99 ± 0.25%) A detailed CD marker analysis showed that the cells were negative for CD133 and other lineage specific markers (CD45, CD11b, CD38, etc.) (Fig. 2A). All clones from GPEC (n = 6) consistently exhibited this surface antigen profile throughout passages. However, in certain clones (n = 2), we observed intermittent fluctuation with respect to CD24 antigen (Fig. 2B). Single cell cloning of CD24+ and CD24− fractions and subsequent analysis of the colonies revealed that the antigenic flux is not due to co-existence of independent cell populations since culturing of isolated CD24+ and CD24− fractions restored cells with original expression profile (Fig. 2C).

Metastable mesenchymal cells are generated in vitro through EMT

The phenotypic shift in parietal epithelial cells to CD24+ mesenchymal cells indicated that EMT may be operational in these cells under in vitro culture conditions. Transcript analysis of CD24+ cells at P5 as compared to that of P1, for epithelial and mesenchymal genes confirmed that these cells have undergone EMT. Quantitative real time PCR performed on the sorted cell fractions showed significant down-regulation of the epithelial genes, E-Cadherin, Occludin, Cytokeratin-19), Claudin-3, claudin-4 and a simultaneous up-regulation of mesenchymal genes, vimentin and nestin in CD24+ cells from P-1 to P-5. The down-regulation of cell adhesion molecules was concomitant with the high expression of major regulators of EMT, Snail-1 and Snail-2. Further, we examined the level of common initiators of EMT, which include members of transforming growth factor (TGF)-β family of genes in these cells. Transcript level of TGF-β1 gene, the key EMT modulator, was high in CD24− cells at P1 and maintained its expression throughout passages. Significant up-regulation in the expression of TGF-β2 transcript and slight up-regulation of TGF-β3 transcripts was observed in these cells. The up-regulation of TGF-β1 transcript from P1 to P5 was followed by concurrent down-regulation of BMP-7(Fig. 3A), which is known to counteract TGF-β1 induced EMT [22].

Sequestration of E-Cadherin from the cell surface in P-1 to the perinuclear vesicles in P-5 of these cells (Fig. 3B) reinforced the occurrence of EMT like changes in these cells. The enriched CD24+ cells showed positive staining for mesenchymal proteins nestin, fibronectin, α smooth muscle actin and collagen-I (Fig. 3C). Co-expression of epithelial and mesenchymal transcripts in the CD24+ clonal cells indicated metastable phenotype of these cells. To clarify that individual cells in CD24+ colony express both epithelial and mesenchymal markers and not that a sub-population of these cells are becoming mesenchymal regardless of the CD24 status, we carried out clonogenic assay (n = 5) for cells at P1 and P5. The colonies were probed for the co-expression of the mesenchymal protein, vimentin and epithelial marker cytokeratin-18. Our data indicate that at P1, the cells express high abundance of CK-18 but low levels of vimentin which was localized in the nuclei. By P5, vimentin expression was up-regulated along with CK-18 (Fig. 3D) suggesting that EMT rendered metastable phenotype to these cells.

CD24+ metastable cells generated through EMT intrinsically express markers of early kidney progenitors

Studies have shown that the process of EMT renders stem cell like characteristic to cells during embryonic development [23]. To examine whether CD24+ cells demonstrate any such progenitor profiles, we checked for the co-expression of Cadherin 11 with CD24, surface markers of renal progenitors [24]. We found that 94.7 ± 2.1% of these cells showed co-expression of CD24 and Cadherin-11 (Fig. 4A) indicating that these cells have acquired stem cell like properties. These cells also exhibited high expression of CXCR4 (78.7 ± 2.3%), the chemokine required for homing and regenerative potential of renal progenitors [25] (Fig. 4B). Data obtained from qRT-PCR analysis (n = 3) of CD24+ cells at P1 for kidney progenitor markers, revealed transcript abundance of Pax-2, WT1, GDNF, Eya-1, Six-1 and Odd1, which are a set of pivotal genes involved in mammalian kidney development [26]. As EMT progressed from P1 to P5, significant up-regulation in the expression of major progenitor makers (Eya-1, Six-1 Odd1, WT-1 and GDNF) was observed. No change in transcript abundance was
Fig. 1 Generation and characterization of GPECs. Capsulated glomeruli isolated from collagenase digested adult murine kidney were plated on culture dishes (n = 6). By day 7, compact cell bodies were abundant, surrounding the parent glomeruli, in vitro culture of GPECs led to loss in epithelial phenotype and resulted in clonogenic cells with mesenchymal phenotype (A). Primary GPECs showed CD24 (green) immunostaining which co-expressed with Claudin-1/2 (red) and claudin-1 (cyan) (B). The cells exhibited tight intercellular junctions; confocal images showing E-Cadherin (red) and CD24 (green) co-expression in these cells (C). Nuclei stained with DAPI. Lack of nephrin (D) and VWF (E) expression in the cellular outgrowth ruled out the possibility of podocytes or endothelial cells in culture. Cryosections of mouse kidney showing CD24 (green) localization in cells lining the Bowman’s capsule, nuclei are stained by DAPI, represented in red (F). Results are representatives of at least three independent experiments. Abbreviations: VWF – Von-Willibrand factor, DAPI-4’, 6-diamidino-2-phenylindole.
Fig. 2 Immunophenotypic profile of the flow sorted CD44⁺ CD24⁻ cells. Flow cytometry histograms show the expression of selected CD markers (CD44, CD29, CD24 and SCA-1 (low intensity) and no expression of other mesenchymal/lineage specific markers (CD31, CD11b, CD133, CD105, CD90.1, etc.) (A). CD24⁻ cells undergo antigenic flux in vitro. Cells show homogenous expression of CD44 but show distinct variation in the expression of CD24 (B), isotype control is shown. Analysis of single cell clones of CD24⁻ and CD24⁺ cells for CD24 expression. CD24⁻ and CD24⁺ Cells were singly sorted into 96-well plates, 10–13 wells showed presence of single cells which were further expanded and analysed. Follow-up studies show that the CD24⁻ population after two population doublings reverts to the CD24⁺ profile (C).
Fig. 3 GPECs undergo epithelial EMT in vitro. qRT-PCR analysis for the expression of epithelial and mesenchymal markers as well as transcript levels of major EMT regulators in CD24⁺ cells at passage 5 over P1 (n = 3). Graphs are represented as fold difference in Ct value of passage 5 over passage 1. RNA was obtained from single cell colony of CD24⁺ cells at passages 1 and 5. All mRNA expression levels were normalized to the house keeping gene GAPDH expression. Data are represented as mean ± S.E.M., (*P < 0.05, **P < 0.01, ***P < 0.001). Transcript abundance of the EMT specific genes is estimated by duplex quantitative real-time PCR for 35 cycles (A). Confocal image showing the sequestration of membrane bound E-Cadherin in cells at P1 to perinuclear space in cells at P5, nuclei stained with DAPI (B). Confocal fluorescent images showing the expression of mesenchymal cytoskeletal proteins in CD24⁺ cells (P5) nestin, fibronectin, α-smooth muscle actin, collagen 2 (C). Clonogenic assay of the cells show co-expression of epithelial and mesenchymal marker, indicating metastable phenotype in these cells confocal image of a representative CD24⁺ cell colony at P1 show low expression of Vimentin (red) and high level of CK-18 (green). By passage 5, the cells exhibit uniform expression levels of vimentin (red) and CK-18 (green) (D). Fluorescence is quantified and represented in 2.5D intensity graphs. Nuclei stained with DAPI (scale bar = 10 μm). Abbreviations: qRT PCR, quantitative reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.
noted in case of Pax-2 and Hoxb-7. However, the expression of K-Cadherin was significantly down-regulated as EMT progressed from P1 to P5. Interestingly, we found that along with the markers of metanephric mesenchyme, CD24+ cells consistently (n = 3) co-expressed markers of uterinc bud (Hoxb-7 and C-Ret) suggesting a possible bi-potent progenitor status of these cells.

**Fig. 4** CD24+ cells express markers of embryonic renal progenitors. FACS analysis exhibits the co-expression of surface markers of embryonic renal progenitors in CD24+ cells, CD24 and Cadherin-11 (95.5 ± 2.1%), isotype control (A). The cells showed expression of CXCR-4 (78.7 ± 1.6%) (B). qRT-PCR was performed on CD24+ cells for the expression of genes specific to metanephric progenitors (n = 3) (C). RNA was obtained from single cell colony of CD24+ cells at passages 1 and 5. All mRNA expression levels were normalized to the house keeping gene GAPDH expression. Graphs are represented as fold difference in Ct value of passage 5 over passage 1. Data are represented as mean ± S.E.M. (*P < 0.05, **P < 0.01, ***P < 0.001). qRT-PCR analysis for Wnt gene expression of the cells at P5 over P1 (n = 3) (D). Confocal immunostaining of β-catenin localization in cells of P1 and P5. β-catenin translocated from the cell membrane at P1 to cytoplasm at P5. Fluorescence is quantified and represented in 2.5D intensity graphs. Scale bar represents 10 μm (E).

**CD24+ cells exhibit dynamic pattern of Wnt gene expression in vitro**

During the reciprocal inductive mechanisms, active Wnt signalling is observed in the epithelial progenitors and metanephric
In order to confirm that EMT rendered traits of renal progenitors to GPECs, we carried out EMT blocking experiments with Prostaglandin-E2, a potent inhibitor of EMT [33]. The PEC outgrowth from renal glomeruli (P1) were treated with 0.1 μM PGE2 and cultured. We observed that PECs treated with PGE2 had lower proliferative potential and no apparent EMT like changes were observed in these cells. By 8–10 days, untreated cells proliferated and showed EMT like changes, (Ki-67 and nestin expression) (Fig. 5A.a) while no such changes were observed in GPECs treated with PGE2 (Fig. 5A.b). Further characterization by qRT-PCR revealed that incubation with PGE2 lowered cell proliferation (low expression of Ki-67 transcript) (Fig. 5B) and successfully inhibited EMT (low transcript abundance of mesenchymal markers) in these cells. GPECs treated with PGE2 had higher expression of all epithelial markers and low levels of mesenchymal markers when compared with untreated cells at P1 and P5 (Fig. 5C). Interestingly, we found that EMT blockage also resulted in low expression levels of most progenitor markers except epithelial markers K-Cadherin and Hoxb-7 whose levels were higher (Fig. 5D). Additionally, we observed that blocking EMT modulated Wnt gene expression pattern in these cells. Wnt genes associated with epithelial progenitors were up-regulated (Wnt7, Wnt9b and Wnt-11) over the mesenchymal Wnt genes (Wnt2d, Wnt-4, Wnt5b and Wnt6) whose transcript levels were low (Fig. 5E).

CD24+ cells exhibit renal commitment in vitro as well as in vivo

Encouraged by the transcript profile of the CD24+ metastable mesenchymal cells, the functional renal commitment potential of these putative progenitors was assessed by in vitro and in vivo nephron development assays.

In vitro co-culture of CD24+ cells with developing embryonic kidney (E13.5)

Here, we demonstrate the ability of CD24+ cells to subsist, proliferate and integrate into the developing E13.5 kidney in an in vitro system. The CD24+ cells (1000–1500 cells/tissue) tagged with green fluorescent dye marker CMFDA were injected into the kidney rudiments dissected from E13.5 kidney and cultured for 5 days on transwell filters without additional growth factors. Whole mount and antibody staining of the rudiments showed that the CD24+ cells have integrated into the developing kidney. Optical sectioning of the whole mount kidney rudiment showed clusters of CD24+ cells integrated with the 3D kidney primordia (Fig. 6A). Anti-laminin staining showed that the cells have integrated into the developing tubules and uterical-bud stalk [34] (Fig. 6B). PGE2 treated cells failed to integrate into the developing kidney. However, few cells remained attached to kidney surface (Fig. 6B).

In vitro co-culture of CD24+ cells with E13.5 spinal cord

CD24+ cells were co-cultured with E13.5 embryonic spinal cord, the heterologous inducer of metanephric mesenchyme and uterical bud [35]. After 2–3 days in culture, these cells aggregated to form EBs. Extended culture (5–6 days) of these EBs on matrigel in the presence of E13.5 spinal cord conditioned medium resulted in sprouting and network branching in more than 60% of cell aggregates (n = 10) (Fig. 6C). PGE2 treated cells on the other hand failed to form intact nephrophereses due to poor migration and no branching pattern were observed in these cells when grown on matrigel with E13.5 spinal cord conditioned medium(Fig. 6D).

CD24+ cells exhibit endothelial differentiation potential in vitro

To analyse whether CD24+ cells can differentiate into endothelial lineage and participate in angiogenesis, matrigel tube formation assay was carried out. A 16 hr culture on matrigel demonstrated the capillary formation ability of CD24+ cells (n = 6) (Fig. 6D). The matrigel culture induced the expression of platelet endothelial cell adhesion molecule (PECAM) (CD31), an endothelial marker in (92.2 ± 6.5%) of these cells over undifferentiated cells (Fig. 6E).
Fig. 5 EMT inhibition in GPECs by Prostaglandin E2. GPECs (P1) were incubated with 0.1 \( \mu \text{M} \) PGE2 and cultured for 2 weeks. Control GPECs without PGE2 treatment showed rapid proliferation, Ki-67 staining, inset and appearance of nestin positive cells within the colony (A.a) while cells incubated with PGE2 exhibited no nestin expression and low Ki-67 staining (A.b). qRT-PCR analysis showed that in cells incubated with PGE2, Ki-67 was highly down-regulated as compared to cells at P1 and P5 (B). qRT-PCR analysis of GPECs treated with PGE2 over cells at P1 and P5 for the expression of epithelial and mesenchymal markers as well as major EMT regulators (C). qRT-PCR analysis was carried out to compare the expression level of renal progenitor genes (D) and Wnt genes (E) in GPECs treated with PGE2 over untreated cells at P1 (grey bar) and P5 (black bar). Graphs are represented as fold difference in Ct value of cells treated with PGE2 over untreated cells at P1 and P5, Ct (PGE2-P1) (PGE2-P5). All mRNA expression levels were normalized to the house keeping gene GAPDH expression. Data are represented as mean ± S.E.M. (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).
These results demonstrate that CD24⁺ cells are capable of producing capillary structures in vitro by differentiating into endothelial cells.

CD24⁺ cells exhibit in vivo differentiation potential when implanted under contra-lateral kidney capsule of unilaterally nephrectomized mice

Compensatory renal growth after unilateral nephrectomy is a remarkable regenerative mechanism exhibited by the kidney. After unilateral nephrectomy, the remaining kidney increases in size, the phenomenon is known as compensatory hypertrophy [19]. We reasoned that the mechanism of compensatory hypertrophy would provide an ideal growth and differentiation milieu for the putative kidney progenitors to exhibit renal commitment in vivo if any. FACS purified and PKH26 labelled cells (2 × 10⁵ cells) were grafted under the left kidney capsule of normal mice (n = 5). One-week after implantation, unilateral nephrectomy of the right kidney was performed. Two weeks after nephrectomy mice were killed to visualize the status of the graft. The recovered grafts exhibited increase in size, were vascularized and no neoplasia were observed (Fig. 7A–D). Grafted CD24⁺ cells generated neoglomerular, vascular and ductal structures at 3 weeks after engraftment, as determined by histological assessment of fixed glomerular, vascular and ductal structures at 3 weeks after implantation. Further, immunostaining for CD31 (92.2 ± 6.3%) in the cells after differentiation over undifferentiated cells (0.3 ± 1%) (F). (Scale bar = 50 μm). Abbreviations: CMFDA, 5-chloromethylfluorescein diacetate; SFM, serum-free media; E-embryonic day; d, day; f, field; EB, embryoid body.

Discussion

In this study, we demonstrate that GPECs of adult murine kidney undergo spontaneous EMT in vitro to generate cells with characteristics and functional properties of embryonic renal progenitors. More importantly, by clonal assays we demonstrate that these cells adopt metastable phenotype, with attributes of both epithelial and mesenchymal markers. This suggests that parietal epithelial cells of the glomeruli act as potential stem cells and EMT could increase this ability. Earlier reports have shown the isolation of CD24⁻ CD133⁻ renal stem cells from PEC of human kidney [14]. In our study, GPECs were strongly positive for murine CD24 antigen but the cells did not show any expression for CD133. We point out that these cells are resultant of EMT. Parietal epithelial cells in the renal capsule of normal (un-nephrectomized) mice produced no visible differentiation within the stipulated time (data not shown).
Fig. 7 CD44<sup>+</sup> CD24<sup>+</sup> cells exhibit in vivo renal commitment when grafted under the renal capsule of contra-lateral kidney of unilateral nephrectomized mice. CD44<sup>+</sup> CD24<sup>+</sup> cells were grafted under the left kidney capsule of normal mice. One week after implantation, right kidney was nephrectomized. Grafts were harvested 2 weeks after nephrectomy. Photographs showing contra-lateral kidney of unilateral nephrectomized mice-control kidney (without graft) (A) and test kidney (with graft) (B). Haematoxylin and eosin stained section of the control kidney (without graft) (C) and test kidney (with graft) (D). Note the thick layer of cellular graft under the renal capsule in the test kidney, which is absent in the control kidney. (Magnification = 100×). High magnification view of the graft sections showing neo-glomeruli like structures, vascularization (erythrocytes could be seen suggesting blood flow to the graft tissue) after 14 days of implantation (E). The dashed line separates the graft from the host. (Magnification = 200×). Grafts contained immature glomerular like structures (E.a, b), ducts (E.c, d) and vasculature with RBCs (E.e). The results are representative of five separate grafts.

Abbreviations: CP, capillary; NG, neo-glomeruli; RBC, red blood cells.
C-Ret, K-Cadherin) genes, indicating that these cells might be bipotent renal progenitors. Taken together, our results suggest that GPECs have undergone partial EMT to generate bipotent progenitor cells. Emerging evidence suggests direct correlation between EMT and stemness [38]. In order to confirm that acquisition of progenitor markers by GPECs was EMT driven, we carried out PGE2 mediated EMT blocking experiments. Low transcript abundance was observed for most progenitor markers as well as Wnt genes, when EMT was blocked. Further, we observed that on EMT arrest; the rapid proliferative potential of GPECs was compromised, suggesting that EMT imparts survival and proliferative potential to the terminally differentiated GPECs. Despite the fact that EMT process in our culture system was spontaneous, possibly contributed by a number of growth factors, and there by blocking EMT using PGE2 might be partial, we observe that our results reflect a direct link between EMT and acquired stemness. This inference was further confirmed when CD24⁺ putative kidney progenitors were tested in nephron development assays. Injection of fluorescently tagged CD24⁺ cells into embryonic kidney organ culture resulted in the integration of the cells to kidney primordia. Additionally, co-culturing of CD24⁺ cells with E13.5 spinal cord resulted in the formation of EBs, which on extended culture in matrigel exhibited distinct events of tubulogenesis such as formation of cellular processes, development of branching multi-cellular cords and establishment of tubules with lumens [39]. These results provide more convincing evidence on the functional progenitor status of these cells. Next, to definitively prove that CD24⁺ cells could exhibit in vivo renal commitment,
FACS sorted PKH-26 labelled CD24⁺ cells were grafted under renal capsule of normal Swiss albino mice. One week after grafting, nephrectomy of the contra-lateral kidney was carried out. We observed that the grafted cells were capable of renal regeneration during compensatory renal growth and expressed markers associated with developing kidney. Co-expression of PKH-26 label with the assessed markers confirmed the donor origin of the graft. In summation, we show here that parietal epithelial cells of renal glomeruli, despite being highly committed could acquire properties of embryonic renal progenitors under the influence of EMT. Animal and human studies have often associated EMT to pathological conditions such as fibrosis and cancer [40]. Understanding the cellular processes initiated during organ repair which ironically, at times, lead to organ failure, is very important for designing efficient therapeutic strategies and rescue renal pathology from this vicious cycle. Extrapolating our results to an in vivo scenario, we suggest that metastable cells with progenitor profile are generated through partial EMT by GPECs in response to glomerular injury. These metastable cells contribute to epithelial cell plasticity and reorganize the damaged tissue without losing all the epithelial characteristics [23]. Pathological situations like glomerular crescent formation and fibrosis may arise when these epithelial cells undergo total EMT, possibly

**Fig. 9** Expression of kidney specific markers in the grafted cells: Expression of collagen-4 (A) and laminin (B) show that the grafted cells are surrounded by glomerular basement membranes indicating renal differentiation in these cells. Mature podocyte marker, Nephrin (C) and tubular water channel protein, Aquaporin-6 (D) were expressed by certain cells within the graft tissue. Note that non-grafted CD24⁺ cells showed very low or no expression of the mature kidney markers. Grafted cells exhibited good proliferation potential in vivo as indicated by the expression of proliferative marker Ki-67 (E). Negative control (F) (Scale bar = 20 μm).
due to conditional aberrations in the regenerating milieu. However, in order to arrive at a conclusion, in vivo studies using animal models of renal injury need to be carried out to track cells undergoing EMT.

We ideate that the present study would improve our understanding on the plasticity exhibited by GPECs under EMT stimulation which would further our knowledge of its role in tissue homeostasis and disease progression and for possible intervention.

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