Characterization of Adult Canine Kidney Epithelial Stem Cells That Give Rise to Dome-Forming Tubular Cells

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Dome formation can occur in cultured tubular epithelial cells originating from various tissues, including the mammary gland and the kidney. The isolation and characterization of normal kidney epithelial stem cells that give rise to dome-forming tubular cells have never been reported. We attempted to isolate and characterize canine kidney epithelial stem cells using a simple cell culture method that we have previously used to isolate other adult human stem cells. Dome-forming kidney epithelial cells were derived from dissociated adult canine kidney tissues that were cultured in a modified keratinocyte serum-free medium supplemented with *N*-acetyl-L-cysteine, L-ascorbic acid 2-phosphate, nicotinamide, and fetal bovine serum. These cells exhibited high self-renewal capacity in long-term culture (growth for >13 months and 30 cumulative population doublings) and exhibited characteristics of stem cells, including (1) deficiency in gap junctional intercellular communication, (2) anchorage-independent growth, (3) expression of stem cell markers octamer-binding transcription factor 4 and SRY (sex determining region Y)-box 2, (4) expression of cell surface markers CD24 and CD133, and (5) multipotent differentiation into osteoblasts, adipocytes, chondrocytes, and dome-forming tubular cells. Most of these characteristics are shared by the well-known canine renal tubule-derived immortalized Madin–Darby Canine Kidney cell line. Furthermore, the putative canine kidney stem cells developed in this study formed budding tubule-like organoids on Matrigel and required high cell density (>4,000 cells/cm²) for sustained growth and confluency for dome formation. The signal transducer and activator of transcription-3 (STAT3) phosphorylation inhibitor, AG490, inhibited colony-forming efficiency and dome formation, whereas lipopolysaccharide, an activator of STAT3, increased colony-forming efficiency in a dose-dependent manner. These results are consistent with the hypothesis that high cell density induces STAT3 expression, which promotes both stem cell self-renewal and differentiation into tubular cells. Our novel cell culture method should be useful for the future development of normal human kidney stem cells for clinical applications and for studying mechanisms of nephrotoxicity.

Keywords: kidney multipotent stem cells, dome-forming tubular cells, STAT3

Introduction

THE INCIDENCE AND PREVALENCE of end-stage renal disease (ESRD) are increasing globally [1,2]. Patients with ESRD have been treated by renal replacement therapies including peritoneal dialysis, hemodialysis, and kidney transplantation. These treatments, however, have inherent shortcomings, including comorbidities, poor quality of life, and a shortage of organ donors.

Stem cells are undifferentiated cells with the capacity for unlimited or prolonged self-renewal, and the remarkable

potential to give rise to many different cell types in the body. Therefore, stem cells show great potential in regenerative and reparative medicine for the treatment of diseases that are not conventionally curable. There are three major types of stem cells. Embryonic stem (ES) cells might provide an unlimited supply of stem cells due to their infinite lifespan and high differentiation potential. However, drawbacks of ES cells include the potential for teratoma development besides ethical concern. Induced pluripotent stem cells (iPSCs) can be derived after ectopic expression of transcription factors in many different animal and human

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cell types. Although there are no ethical concerns associated with the development of iPSCs, potential drawbacks of using these cells include mutations, dysplasia, or hyperplasia due to multiple retroviral chromosomal integrations, or aberrant expression of transcription factors [3]. Adult stem cells can be derived from various adult tissues using suitable cell culture media and conditions, without the need for ectopic expression of transcription factors. These adult stem cells may have limited proliferative capacity and differentiation potential compared with ES cells and iPSCs. However, adult stem cells are safer for clinical use and are by far the most common type of stem cell used in clinical trials (34, 52, and 573 clinical trials have used ES cells, iPSCs, and adult stem cells, respectively, as of July 30, 2019; <http://clinicaltrials.gov>).

We previously reported the development of various animal and human adult stem cells from a range of sources (ie, mammary [4,5], adipose tissue [6,7], gastric [8], liver [9], and amniotic fluid [10]). Successful development of these cells was mainly ascribed to the use of a medium containing *N*-acetyl-L-cysteine (NAC) and L-ascorbic acid 2-phosphate (Asc-2P), which alters the cellular redox state and facilitates the expression of major stem cell transcription factors. In this study, we attempted to isolate stem cells from the adult canine kidney using a similar technique, and found that it is possible to isolate dome-forming canine kidney epithelial cells with stem cell characteristics similar to those exhibited by the canine renal tube-derived Madin–Darby Canine Kidney (MDCK) cell line [11,12]. These canine kidney epithelial cells expressed kidney stem/progenitor cell surface markers, CD24 and CD133, pluripotency-associated stem cell markers, octamer-binding transcription factor 4 (OCT4), and SRY (sex determining region Y)-box 2 (SOX-2), and were capable of extended self-renewal as well as multilineage differentiation. Furthermore, we found that high cell density was required for both sustained growth and dome formation by these cells. Since it was previously shown that MDCK cells at confluency activate signal transducer and activator of transcription-3 (STAT3) and trigger dome formation [13], we hypothesize that high cell density induces STAT3 expression that promotes both self-renewal and differentiation into tubular cells.

Materials and Methods

Cell culture medium

The medium used to develop putative canine kidney epithelial stem/progenitor cell cultures is a modified MCDB 153 medium (Keratinocyte-SFM; Gibco) supplemented with 2 mM NAC (catalog no. A8199; Sigma) and 0.2 mM Asc-2P (catalog no. A8960; Sigma) (referred to as K-NAC medium) [6]. The calcium concentration of this medium is 0.09 mM. The growth factors/hormones added to this medium include recombinant epithelial growth factor (5 ng/mL), bovine pituitary extract (50 µg/mL), insulin (5 µg/mL), hydrocortisone (74 ng/mL), and 3,3',5-triiodo-DL-thyronine (T₃) (6.7 ng/mL). NAC, a potent antioxidant, has been shown to promote the self-renewal of precursor cells [14] and is readily deacetylated intracellularly to yield L-cysteine, thereby enhancing the production of glutathione [15]. Asc-2P is a stable precursor that provides ascorbic acid in cell culture [16,17]. Ascorbate can be antioxidative or pro-

oxidative (due to reversible conversion between ascorbate and dehydroascorbate) depending on its concentration and the concentration of metal ions [18]. The K-NAC medium is also supplemented with nicotinamide (5 mM), which is a poly (adenosine diphosphate-ribose) polymerase inhibitor that regulates sirtuins and the expression of genes related to cell survival and longevity [19,20]. In kidney cell differentiation experiments, a modified Eagle's minimal essential medium (D-medium) [21] was used.

Isolation of dome-forming canine kidney epithelial cells

A whole canine kidney was excised shortly after euthanasia, which was carried out for reasons unrelated to this study. The kidney was washed with phosphate-buffered saline (PBS), and then the outer fascia and fibrotic tissue were removed and minced into small pieces in a 60 mm plate using scalpels. After washing the minced tissue with PBS to remove the majority of erythrocytes, small pieces were seeded into 60 mm plates and incubated with serum-free K-NAC medium supplemented with 0.1% penicillin–streptomycin (catalog no. 15140-122; Gibco). To facilitate the attachment of suspended cells and tissues to the plate, minimal initial volumes of medium were used. Medium was renewed daily, and cell colonies were developed from pieces of tissue after 7–10 days (Fig. 1a). These colonies were isolated using the glass ring/trypsin method, and were transferred into wells of 12-well plates and incubated with K-NAC medium supplemented with 10% fetal bovine serum (FBS) and 5 mM nicotinamide. These cells continued to grow until confluency and exhibited contact-insensitive growth and dome formation (Fig. 1b). Some cells would detach and float in the medium; these floating cells could be repeatedly transferred to 100 mm plates to give rise to a large number of cells. This procedure was found to be more efficient than trypsinization, which tended to induce cell differentiation. Dome-forming colonies in 100 mm plates could be enriched and purified by removing undesired nondome-forming colonies by scraping with a rubber policeman. Every other day, half of the cell culture medium in plate (conditioned medium) was replaced with fresh medium. Cells in 100 mm plates could then be trypsinized and transferred to T-75 flasks to increase number of cells. All cell cultures were incubated at 37°C in incubators supplied with humidified air and 5% CO₂.

Cumulative population doubling levels

The cumulative population doubling level (cpdl) was calculated to determine the proliferation capacity of cells using the equation: $\ln(N_f/N_i)/\ln 2$, where N_i and N_f are initial and final number of cells, respectively, and $\ln 2$ is the natural log. The initial number of cells was 2×10^5 cells in a 100 mm dish for each propagation. To obtain the final cpdl, cpdl in continuous subculture periods was added.

Anchorage-independent growth assay

To determine anchorage-independent growth (AIG), agarose (0.5%, Type I; Sigma) was prepared in K-NAC medium containing 5% FBS at 39°C, and was added to 60 mm dishes and allowed to solidify in a 37°C incubator. A total of 50,000 cells suspended in medium containing 0.33% agarose were

FIG. 1. Primary canine kidney epithelial cell cultures were successfully established from canine kidney tissue. Initial cell growth from minced canine kidney tissue is shown (a). Colonies with epithelial morphology were developed in a week. Confluent cell culture with dome-forming colonies exhibited contact-insensitive growth. Repeated transfer of the detached floating cells from the cell culture was able to give rise to cell colonies and a large number of cells (b). Domes were distinct from adherent primitive cells and showed three-dimensional structures (c, d). Images (c, d) were acquired at different focal planes. Scale bars: (a, b) 100 μ m; (c, d) 50 μ m.

overlaid on top of the solidified 0.5% agarose layer. The plates were incubated at 37°C, and K-NAC medium containing 5 mM nicotinamide and 10% FBS was added 3 days after cell inoculation and renewed every 3 days. After 4 weeks, colonies were stained and scored using a microscope.

Gap junctional intercellular communication

Gap junctional intercellular communication (GJIC) was studied by the scrape loading/dye transfer technique reported previously [22]. In brief, this technique involves (1) the growth of a confluent cell culture in 35 mm plates over a period of 1–2 days; (2) the addition of 2 mL Lucifer Yellow (catalog no. L0259, 0.5 mg/mL in PBS; Sigma); (3) scraping of the cell monolayer with a surgical blade to allow entry of the dye into damaged cells, which transfer the dye to neighboring cells within 3 min if the cells are GJIC competent; and (4) removal of the dye solution and washing of the cell monolayer with PBS. Dye transfer between cells was visualized with a Nikon Eclipse TE300 UV fluorescence microscope and recorded using a digital camera connected to a computer.

Flow cytometry

Subconfluent canine kidney epithelial cells (clone DK-701) were grown as described previously in 60 mm dishes and dissociated from the dishes using a nonenzymatic dissociation solution (Cellstripper; Corning). Fluorescence-activated cell sorting (FACS) buffer (Hank's balanced salt solution, 1% bovine serum albumin, 0.1% NaN₃) was used to wash cells between staining and fixing steps. Cells were incubated with FACS buffer containing 20% fetal calf serum and antimouse CD16/CD32 (BD Biosciences) to help block Fc receptors, if present. Cells were then incubated with antibodies CD24 (clone M1/69; Biolegend) and CD133 (clone 13A4; eBiosciences) and washed three times with FACS buffer. BD Cytotfix™ (BD Biosciences) was used to fix cells per the manufacturer's protocol. Cells were analyzed on an FACS BD Canto II™ (BD Biosciences). After acquisition, data analysis was performed using FLOWJO v10 software.

Reverse transcription polymerase chain reaction

Total RNA was extracted from cells using a Versagene RNA purification kit (Gentra) and treated with DNase I to remove contaminating DNA. cDNA was synthesized from 1 μ g total RNA using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). For amplification of cDNA, we used primers specific for OCT4, SOX-2, and NANOG (stem cell transcription factors) that were derived from coding regions of the corresponding genes [7]. A total of 25 μ L polymerase chain reaction (PCRs) were prepared with 2 μ L cDNA, 5 pmol of each primer, 0.5 U of Taq polymerase (Invitrogen), and final concentrations of 40 μ M deoxyribonucleotide triphosphate, 2 mM MgCl₂, 20 mM Tris-HCl, and 50 mM KCl. Cycling conditions were as follows: 94°C for 4 min; 30–35 cycles at 94°C for 1 min, annealing for 1 min, and 72°C for 1 min; followed by 72°C for 5 min. PCR products were separated on 2% agarose gels by electrophoresis, stained with ethidium bromide, and visualized under UV light. The digital images were captured using AlphaImager software. PCR products were gel purified using a Qiaex II Gel Extraction Kit (Qiagen), sequenced with an automated sequencer, and verified by alignment to the canine genome.

Multipotent differentiation

Adipogenesis. A total of 4×10^5 cells were plated in 35 mm dishes and treated with rosiglitazone (5 μ M), 3-isobutyl-1-methylxanthine (IBMX; 500 μ M), dexamethasone (1 μ M), indomethacin (100 μ M), insulin (10 μ g/mL), glucose (4.5 g/L), and 5% rabbit serum for 21 days. The induction medium with supplementations was renewed every 3 days. Oil red O staining was performed to reveal lipid droplet formation.

Osteogenesis. A total of 4×10^5 cells were plated in 35 mm dishes and treated with dexamethasone (0.1 μ M), Ascorbic acid (50 μ M), and β -glycerophosphate disodium (10 mM; DAG cocktail) in D-medium containing 10% FBS for 6–8 weeks, with medium change every 3 days. Alizarin red staining was carried out to detect calcified extracellular matrix deposit.

Chondrogenesis. Micromass cultures of cells (1×10^5 cells/10 μ L) were allowed to form in 24-well plates for 2.5 h before treatment with transforming growth factor- β 1 (10 ng/mL), Asc-2P (50 μ M), and insulin (6.25 μ g/mL) (TAI cocktail) for 14 days, with medium change every 3 days. The micromass was stained with Alcian blue for the presence of sulfated proteoglycan-rich matrix.

Three-dimensional growth on Matrigel

Liquid Matrigel was added to 35 mm dishes (0.5 mL/plate) and allowed to solidify in an incubator at 37°C. A total of 1×10^7 cells in 0.5 mL K-NAC medium containing 10% FBS and 5 mM nicotinamide were then added onto the Matrigel for three-dimensional cell growth.

Regulation of self-renewal and dome-forming tubular differentiation of canine stem/progenitor cells by STAT3

For this study, 4×10^4 cells were plated in 35 mm dishes for 1 day and then treated with AG490 (2.5, 5, or 10 μ M and dimethyl sulfoxide [DMSO] as vehicle control) or lipopolysaccharide (LPS) (0.1, 0.2, or 0.4 μ g/mL). The medium was renewed once every 3 days. Both dome-forming and nondome-forming colonies were counted after 4 weeks.

Statistical analysis

Mean $\pm$ SD data were analyzed using one-way analysis of variance followed by post hoc Dunnett's multiple comparison test (GraphPad Prism).

Results

Canine kidney epithelial cell cultures were successfully established from canine kidney tissues

Canine kidney cell colonies were initially derived from minced kidney tissues. We used the serum-free K-NAC medium [6] to develop cell populations with characteristics of stem cells. The use of this cell culture medium and the methods described previously yielded colonies displaying epithelial cell morphology after 7–19 days, as shown in Fig. 1a. These colonies were isolated by glass ring/trypsin method and propagated for further characterization.

Canine kidney epithelial cells that were capable of forming dome-like structures can be continuously derived from single cells of the initial dome-forming cell culture

Although canine kidney cell colonies were initially derived in serum-free K-NAC medium [6], we observed that these cells grew more efficiently in K-NAC medium supplemented with 5 mM nicotinamide and 10% FBS. We, therefore, used this serum-containing medium to determine the cells' proliferation potential and colony-forming capacity on plastic or soft agar.

The isolated kidney epithelial cell colonies proliferated in wells and displayed contact-insensitive growth (Fig. 1b). Dome-like structures appeared in confluent cell cultures (Fig. 1c, d), similar to those reported for the canine MDCK cell line [11]. These cells showed density-dependent growth after

subculture, that is, optimum cell proliferation density $>4,000/\text{cm}^2$. Furthermore, confluent dome-forming canine kidney epithelial cell cultures in 12-well plates could be maintained >13 months with medium change. Floating cells that detached from these adherent cultures could also be repeatedly transferred to new wells or plates to initiate new dome-forming cultures.

Canine kidney epithelial cells express stem cell markers on the cell surface

To examine whether the population of canine kidney epithelial cells express the well-known cell surface markers for renal stem/progenitor cells (see references in "Discussion" section), we performed flow cytometry to analyze the expression of CD24 and CD133 on the surface of normal canine cells (clone DK-701). We found that 22.2% of the cells express CD24 on the surface (Fig. 2a, c) and 5.13% of the cells expressed CD133 on their surface (Fig. 2b, c). In addition, double positive cells expressing both CD24 and CD133 were present at 2.66% (Fig. 2c).

Canine kidney epithelial cells express pluripotency-associated transcription factors

Experiments were carried out to detect the expression of important stem cell markers by reverse transcription PCR. Both the canine kidney epithelial cells developed in this study, and MDCK cells, expressed OCT4 and SOX-2 as shown by the expression of mRNA encoding OCT4 in Fig. 2d (274 bp product) and SOX-2 in Fig. 2e (142 bp product) in normal canine kidney (NCK) cells and MDCK cells. However, NANOG expression was not detected in these cells (data not shown).

Canine kidney epithelial cells proliferate in culture for finite time and display AIG in soft agar

The proliferation potential of dome-forming canine kidney epithelial cells was determined by estimating their cpdl in continuous culture. We found that these cells had a finite lifespan, with ~ 30 population doublings in a period of 42 days. These cells were also capable of colony formation in soft agar (ie, AIG) (Fig. 3a). The frequency of AIG was found to be $\sim 4\%$ (Fig. 3a) compared with an AIG frequency of $\sim 8\%$ for MDCK cells (Fig. 3b).

Canine kidney epithelial cells are deficient in GJIC

The lack of GJIC is a common property of stem cells and cancer cells [23]. Therefore, we carried out experiments to determine whether the canine kidney epithelial cells developed in this study, and immortalized canine MDCK cells, were capable of GJIC. Experiments using the scrape-loading dye transfer technique [22] revealed that both of these cell types are GJIC deficient as shown in Fig. 3c and d for normal canine epithelial cells and in Fig. 3e and f for MDCK cells. As shown, the dye retained in the damaged cells without transferring to neighboring cells as shown by GJIC-deficient cells [22].

Canine kidney epithelial cells are capable of multilineage differentiation

We have previously reported reliable protocols for osteogenic, adipogenic, and chondrogenic differentiation for

FIG. 2. Canine kidney epithelial cells express kidney stem cell markers. Flow cytometry analysis shows that canine kidney epithelial cells (clone DK-701) contain cell populations that express CD24 and CD133 on the surface. Histogram plots for CD24 (a), CD133 (b), and two-dimensional plots (c) are shown. Reverse transcription polymerase chain reaction revealed the expression of mRNA encoding OCT4 (d) (274 bp product shown by arrow head) and SOX-2 (e) (142 bp product shown by arrowhead) in both NCK cells and MDCK cells. MDCK, Madin–Darby canine kidney; NCK, normal canine kidney; OCT4, octamer-binding transcription factor 4; SOX2, SRY (sex determining region Y)-box 2.

human and canine adipose-derived mesenchymal stem cells [6,7]. Applying these protocols, we found that canine kidney epithelial cells were capable of osteogenic, adipogenic, and chondrogenic differentiations as revealed by positive Alizarin red (Fig. 4b), Oil red O (Fig. 4d), and Alcian blue staining (Fig. 4f), respectively, as compared with the control treatment shown in Figs. 4a, c, and e.

Canine dome-forming kidney cells form ductal tubule-like organoids on Matrigel

After canine kidney epithelial cells were plated on Matrigel as described in the Materials and Methods section, tubule-like structures formed in 10–14 days and ductal tubule-like organoids emerged 4–6 weeks later. Tubule-like

FIG. 3. Canine kidney epithelial cells exhibit several features of stem cells. The AIG frequency of dome-forming canine kidney epithelial cell cultures on Matrigel (**a**) was comparable with MDCK cell culture (**b**) ($\sim 4\%$ in canine kidney epithelial cells vs. $\sim 8\%$ in MDCK cells). In addition, normal canine epithelial cells (**c**, **d**) and MDCK cells (**e**, **f**) were deficient in GJIC. Scrape-loading dye transfer technique revealed that, in both cell types, the dyes were retained in damaged cells without any transfer to neighboring cells. Scale bars: (**a**) 100 μm ; (**b**) 200 μm . AIG, anchorage-independent growth; GJIC, gap junctional intercellular communication.

structure and tubular budding from ductal tubule organoids formed by NCK epithelial cells are shown in Fig. 5a and b, respectively. Figure 5c and d shows the ductal tubule-like organoids formed by NCK epithelial cells on Matrigel.

Regulation of self-renewal and dome-forming tubular differentiation of canine stem/progenitor cells is STAT3 dependent

The transcription factor STAT3 is required for the maintenance and self-renewal of ES cells [24]. In canine MDCK cells, cell confluence has been reported to activate STAT3 and trigger epithelial dome formation [13]. Therefore, we tested the effect of AG490, a STAT3 phosphorylation inhibitor, or LPS, an activator of STAT3, on self-renewal and dome formation. Experiments to determine the effect of STAT3 phosphorylation inhibitor AG490 on colony-forming efficiency were carried out. As shown in Fig. 6a, AG490 inhibited the formation of colonies in a dose-dependent manner. An average of 99, 81, 71, and 22 colonies per plate were found for DMSO vehicle control and treatments with 2.5, 5, or 10 μM AG490, respectively (the respective P values were 0.0942, 0.0251, and 0.0005). AG490 treatment significantly affected dome formation only at concentrations $>5 \mu\text{M}$. In contrast, treatment of these cells with

LPS, which activates STAT3, increased colony-forming frequencies in a dose-dependent manner (an average of 13, 15, 23, and 26 colonies per plate were found for the control and treatments with 0.1, 0.2, or 0.4 $\mu\text{g/mL}$ LPS, respectively (the respective P values were 0.5542, 0.0201, and 0.0079) (Fig. 6b).

Discussion

Dome formation can occur in cultured tubular epithelial cells originating from various tissues including the mammary gland [25] and the kidney [11]. This study demonstrates that dome-forming cells can be isolated from adult canine kidney tissues. Other than the well-known immortal canine kidney epithelial MDCK cell line, the isolation of dome-forming normal canine epithelial cells has not been reported. Furthermore, we show that these cells possess several characteristics of stem cells, that is, (1) high self-renewal potential; (2) the ability to differentiate into multiple cell lineages; (3) expression of the stem cell markers OCT4 and SOX-2; (4) expression of cell surface markers CD24 and CD133; and (5) deficiency in GJIC.

In continuous subculture, these normal kidney epithelial cells were able to undergo ~ 30 population doublings. In confluent cultures in 12-well plates, floating cells that

FIG. 4. Canine kidney epithelial cells can differentiate into mesenchymal lineages. NCK epithelial cells underwent osteogenic, adipogenic, and chondrogenic differentiation after treatment with respective induction cocktails (**a–f**). Cells treated with osteogenic induction cocktail were stained positive with Alizarin red (**b**), those treated with adipogenic induction cocktail were stained positive with Oil red O (**d**), whereas those treated with chondrogenic induction cocktail were stained positive with Alcian blue stain (**f**). Uninduced control cells (**a**, **c**, **e**) remained unstained after staining with the corresponding dye. Scale bars: 50 μ m.

detached from the cell monolayer could be continuously collected and transferred into new plates to establish new cultures for >13 months. These new cell cultures maintained their dome-forming abilities. This appears to be a novel method for the long-term growth of stem cells that are sensitive to terminal differentiation after trypsinization. These NCK epithelial cells were capable of multilineage

differentiation into adipocytes, osteoclasts, and chondrocytes, as well as tubular structure-like organoids on Matrigel. Whether these cells can also differentiate into other cell types remains to be examined.

The canine kidney epithelial stem cells derived in this study express OCT4 and SOX-2, but not NANOG. This does not cast doubt on their qualification as stem cells, since

FIG. 5. Canine kidney epithelial cells form ductal tubule-like structures. Ductal tubule-like organoids formed by NCK epithelial cells on Matrigel are shown (**a–d**). Tubule-like structures were formed after 10–14 days (**a**). The *circle* highlights tubular budding in ductal tubule organoids (**b**). Ductal tubule-like organoids emerged 4–6 weeks later (**c**, **d**). Scale bars: (**a**) 200 μ m; (**b**) 100 μ m; (**c**) 200 μ m; (**d**) 50 μ m.

FIG. 6. STAT3 modulators can alter the ability of NCK epithelial cells to form colonies. The STAT3 phosphorylation inhibitor AG490 inhibited colony formation by NCK epithelial cells in a dose-dependent manner (**a**). Asterisks indicate statistically significant difference between each dose of AG490 treatment and vehicle control (*** $P < 0.001$; * $P < 0.05$; ns, nonsignificant, $P = 0.0942$). In contrast, LPS, an activator of STAT3, increased colony formation by NCK epithelial cells in a dose-dependent manner (**b**). Asterisks indicate statistically significant difference between each dose of LPS treatment and vehicle control (** $P < 0.01$; * $P < 0.05$; ns, nonsignificant, $P = 0.5542$). LPS, lipopolysaccharide; STAT3, signal transducer and activator of transcription 3.

the reprogramming of human renal proximal tubular cells into iPSCs required only OCT4 and SOX-2 [26]. Compared with the immortal canine MDCK cell line, NCK cells expressed lower levels of OCT4 and SOX-2. This is likely due to the lower frequency of stem cells in the population as indicated by their lower colony-forming efficiency (0.18%). We found that ~22% cells in the canine kidney epithelial cell population expressed CD24, ~5% cells expressed CD133, and ~2.7% cells expressed both CD24 and CD133. It was previously shown that CD24 was found to be highly expressed by renal progenitor cells [27]. Similarly, CD133⁺ kidney cells expressed several stem cell markers and integrated into developing tubules [28]. In fact, kidney cells expressing both CD24 and CD133 exhibited stem cell features such as self-renewal, multilineage differentiation, and expression of stem cell transcription factors including OCT4 [29–33]. The lower percentage of cells expressing both CD24 and CD133 compared with cells expressing only CD24 or CD133 found in this study may indicate that CD24⁺/CD133⁺ cells are indeed the original stem cells that could undergo self-renewal or give rise to cells expressing only one of these two markers. This can be tested in a future study.

The canine kidney epithelial cells developed in this study share many stem/progenitor cell characteristics with the well-known immortal MDCK canine kidney cell line, that is, dome formation, deficiency in GJIC, expression of OCT4 and SOX-2, and the ability of AIG. The frequency of AIG of the cells described here was ~4%, which is higher than the colony-forming efficiency from single cells on plastic surfaces (0.18%), but lower than the frequency of AIG (~8%) for the MDCK cell line.

The mentioned lines of evidence strongly suggest that both the adult canine kidney epithelial cells developed here, and the MDCK cell line, are canine kidney stem/progenitor cells. MDCK cells at confluency were found to activate STAT3 transcription and to trigger dome formation [13]. For our NCK epithelial cells, we observed that sustained cell growth required a high cell density (>4,000/cm²) and that

dome formation was found in high cell density areas. We, therefore, suspect that, in both cell types, high cell density induces STAT3 expression, which promotes both stem cell self-renewal and differentiation into tubular cells. In agreement with the mentioned hypothesis, the STAT3 inhibitor AG490 was found to reduce colony-forming efficiency (Fig. 6a), whereas the STAT3 activator LPS was found to increase colony-forming efficiency (Fig. 6b). Putative adult stem cells were found at multiple locations in the kidney (proximal tubule, renal papilla, interstitium, Bowman's capsule, and glomerular parietal epithelium) of mice, rats, and humans [34,35]. Various methods have been used to isolate these putative renal stem cells, including retention of dyes, side populations, specific markers, and cell culture methods [35]. These renal/progenitor stem cells may have therapeutic potential [36] as exemplified by the three-dimensional reconstruction of kidney structures in vitro [37] and the repair of renal damage in mice with severe combined immunodeficiency [30].

Human kidney stem/progenitor cells can be developed from embryonic kidneys [30], tubular renal cells after iPSC reprogramming with OCT4 and SOX-2 [26], and fetal [38] or adult [29,39–46] kidneys. The isolation of kidney stem/progenitor cells from embryonic and adult tissues relies primarily on the expression of cell surface markers, that is, CD133/CD24 [30,39–46]. After isolation, the potential therapeutic use of these cells depends on identifying proper media for long-term growth of a large number of cells. Human fetal kidney stem cells were found to be contact insensitive (ie, colonies formed on a layer of human fibroblasts that had been lethally irradiated with X-rays), GJIC deficient [38], and positive for OCT4 expression [46]. To our knowledge, ours is the first study to demonstrate that kidney stem/progenitor cells can give rise to dome-forming tubular cells. We believe that our cell culture method will be useful in the development of human stem cells from adult kidneys. This method is straightforward and requires relatively little tissue to develop sufficient quantities of stem cells for potential therapeutic applications.

Conclusion

We have isolated and characterized the adult canine kidney epithelial cells with stem cell characteristics that can give rise to dome-forming tubular cells. Our results are consistent with the hypothesis that high cell density induces STAT3 expression, which promotes both stem cell self-renewal and differentiation into tubular cells. Our cell culture method should be useful for the development of normal human kidney stem cells for clinical applications and for studying mechanisms of nephrotoxicity.

Author Disclosure Statement

No competing financial interests exist.

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