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(54) Title: ISOLATED POPULATIONS OF ADULT RENAL CELLS AND METHODS OF ISOLATING AND USING SAME

(57) Abstract: A method of generating a nephrospheroid is disclosed. The method comprises culturing human adult kidney cells in a culture medium under non-adherent conditions. Uses thereof and other renal cell populations are also disclosed.

ISOLATED POPULATIONS OF ADULT RENAL CELLS AND METHODS OF ISOLATING AND USING SAME

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to isolated populations of adult renal cells and methods of isolating and using same.

The kidney is a vital organ in mammals, responsible for fluid homeostasis, waste excretion, and hormone production. There are a variety of possible injuries and disorders including cancer, trauma, infection, inflammation and iatrogenic injuries or conditions that can lead to chronic disease or cause reduction or loss of function of a kidney. The incidence of chronic kidney disease in the United States has reached epidemic proportions, and a significant number of these patients will develop end-stage renal disease (ESRD), with glomerular filtration rates too low to sustain life. Dialysis is the major treatment modality for ESRD, but it has significant limitations in terms of morbidity, mortality, and cost. Allogenic kidney transplantation provides significant benefits in terms of mortality and is ultimately less costly, but is hampered by a severe shortage of available donor organs. Acute renal failure (ARF) is also quite common, having a mortality rate that ranges from 20 to 70 %. For a number of reasons, including aggressive care of an older patient population, the mortality rate due to ARF has not changed over the past 20 years despite advances in technology and therapies.

Although kidney disease has a variety of individual types, they appear to converge into a few pathways of disease progression. The functional unit of the kidney is the nephron. There is a decrease in functioning nephrons with the progression of the disease; the remaining nephrons come under more stress to compensate for the functional loss, thereby increasing the probability of more nephron loss and thus creating a vicious cycle. Furthermore, unlike tissues such as bone or glandular epithelia which retain significant capacity for regeneration, it has generally been believed that new nephron units are not produced after birth, that the ability of the highly differentiated tissues and structures of the kidneys have limited reparative powers and, therefore, that mammals possess a number of nephron units that can only decline during post-natal life. There is an increasing interest in developing novel therapies for kidney disease, including artificial organs, genetic engineering, and cell therapy.

Many adult tissues are considered to harbor cells that self-renew and differentiate to form clones of stem, progenitor, and mature cells of the organ, fitting within the criteria of tissue-specific multipotential stem cells, including the skin, the hematopoietic system and the intestine. In contrast to these rapidly-cycling organs, the kidney has a low rate of cell turnover under steady state conditions and it's regenerative capacity is limited. Extra-renal tissue-specific stem cells, including those of the bone marrow do not harbor nephrogenic potential, motivating the search for an adult kidney stem cell. To date, there is no definite evidence for the existence in the adult kidney of a cell that fits within this definition, and is capable of self-renewing and differentiating into the nephron's cell types on the one hand and on the other hand of localizing to sites of injury, thereby contributing to renal repair.

The self-renewing nephron progenitor population residing in the metanephric mesenchyme (MM) and more specifically in the condensed mesenchyme (CM) is entirely exhausted with the completion of nephrogenesis (human-34th gestational week, mice-2 weeks postnatal) and therefore no progenitor population with similar nephrogenic potential to the MM/CM exists in the adult kidney (6, 7). However, a population may exist with a more restricted potential than the CM (for instance a progenitor cell type for proximal tubular cells). This cell type is likely to arise from within the epithelial tubular compartment as Humphreys et al (8) demonstrated by lineage tracing that the cells responsible for tubular repopulation after kidney ischemia are of tubular origin, thereby excluding an extra-tubular source.

Murine studies have elucidated early markers specifying the epithelial renal progenitor population including a unique combination of transcription factors such as *Hox11* paralogs, *Osr1*, *Pax2*, *Eya1*, *Wt1*, *Sall1*, *Six2*, and *Cited1* (9). These early renal progenitor markers have been mostly shown to down-regulate with cessation of nephrogenesis in both murine (6) and human kidneys (7).

International PCT Application IL2010/000158 teaches isolation and characterization of fetal renal progenitor cells.

Bussolati et al [American Journal of Pathology. 2005;166:545-555] teaches isolation and characterization of CD133+ cells derived from normal adult human kidney and suggest that this cell population represent a multipotent adult resident stem cell population that may contribute to the repair of renal injury.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an isolated cell population of human adult kidney cells, comprising at least 80 % adult renal stem cells having a NCAM+ signature.

According to an aspect of some embodiments of the present invention there is provided a method of isolating human adult renal cells comprising enriching for a subpopulation of renal cells from an adult renal tissue, the subpopulation of renal cells having a NCAM+ signature, wherein the enriching is effected such that at least 80 % of the adult renal cells are of the subpopulation of renal cells.

According to an aspect of some embodiments of the present invention there is provided a method of determining clonogenic potential of an adult renal cell population, the method comprising:

(a) culturing the adult renal cell population in serum-comprising medium and conditioned medium from human fetal kidney cells; and

(b) counting a number of clones formed from the adult renal cells of the population, thereby determining clonogenic potential of an adult renal cell population.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising a culture medium and the isolated cell population comprising at least 80 % adult renal stem cells having a NCAM+ signature.

According to an aspect of some embodiments of the present invention there is provided a method of treating a renal damage in a subject in need thereof comprising administering to the damaged kidney of the subject a therapeutically effective amount of the isolated cell population comprising at least 80 % adult renal stem cells having a NCAM+ signature, thereby treating the renal disease in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of identifying an agent capable of regulating differentiation of a renal stem cell, the method comprising contacting the isolated population of cells comprising at least 80 % adult renal stem cells having a NCAM+ signature with an agent, wherein a change in developmental phenotype is indicative of the agent capable of regulating differentiation of the renal stem cells.

According to an aspect of some embodiments of the present invention there is provided a method of generating a nephrospheroid, the method comprising culturing

human adult kidney cells in a culture medium under non-adherent conditions, thereby generating the nephrospheroid.

According to an aspect of some embodiments of the present invention there is provided an isolated nephrospheroid comprising human adult kidney cells.

According to an aspect of some embodiments of the present invention there is provided a cell culture comprising a culture medium and an isolated population of nephrospheroids, the nephrospheroids comprising human adult kidney cells.

According to an aspect of some embodiments of the present invention there is provided a method of identifying an agent capable of regulating differentiation of a renal stem cell, the method comprising contacting an isolated population of nephrospheroids with an agent, the nephrospheroids comprising human adult kidney cells, wherein a change in developmental phenotype is indicative of the agent capable of regulating differentiation of the renal stem cells.

According to an aspect of some embodiments of the present invention there is provided a method of treating a renal damage in a subject in need thereof comprising administering to the damaged kidney of the subject a therapeutically effective amount of an isolated population of nephrospheroids, the nephrospheroids comprising human adult renal cells, thereby treating the renal disease in the subject.

According to some embodiments of the invention, the enriching is effected by detecting surface marker expression of NCAM.

According to some embodiments of the invention, the cells are seeded on a scaffold.

According to some embodiments of the invention, the method further comprises dispersing the human adult kidney cells prior to culturing.

According to some embodiments of the invention, the medium further comprises epidermal growth factor (EGF) and fibroblast growth factor (FGF).

According to some embodiments of the invention, the medium further comprises insulin and progesterone.

According to some embodiments of the invention, the medium is devoid of serum.

According to some embodiments of the invention, the medium comprises serum.

According to some embodiments of the invention, the method further comprises expanding human adult kidney cells in a culture medium under adherent conditions prior to the culturing.

According to some embodiments of the invention, the culture medium comprises serum.

According to some embodiments of the invention, the isolated nephrospheroid is characterized by enhanced expression of at least one polypeptide selected from the group consisting of sal1, pax2, six2 and WT1 as compared to the adult kidney cells grown under adherent conditions.

According to some embodiments of the invention, the isolated nephrospheroid is characterized by enhanced expression of each of sal1, pax2, six2 and WT1 as compared to the adult kidney cells grown under adherent conditions

According to some embodiments of the invention, the isolated nephrospheroid is generated in a serum-free medium.

According to some embodiments of the invention, the isolated nephrospheroid is generated in serum-containing medium.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings and images. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a diagram of the experimental design for ascertaining culture conditions for isolation of adult renal progenitor cells. Adult kidney tissues were collected from patients nephrectomized due to localized renal tumors. The tissues were digested to a single cell suspension. Cells were grown using either in Serum containing media (SCM) or Serum–free media (SFM). Upon receiving confluent adherent culture (after approximately 7 days), cells were harvested and subjected to limiting dilutions, RNA extraction, sphere formation and continuous adherent culture assays. FKCM-Fetal kidney conditioned media, LD- Limiting Dilution.

FIGs. 2A-F are photographs illustrating the growth pattern of hAK cells in two different growth media: SCM and SFM. After one day in culture only a few cells adhered, a few days later these cells started to expand, demonstrating a different pattern of expansion; in SFM expansion was concentric and defined, whereas in SCM, expansion was in less organized manner.

FIGs. 3A-J are photographs illustrating adult kidney cell culture characteristics. In low-passage cultures (passage 1) there is a predominance of proximal tubular cells, indicated by LTA staining and a minority of collecting duct' cells, indicated by DBA staining. While hKEpCs positively stain for markers, negative staining is seen in human foreskin fibroblasts (HFF). As expected, renal proximal tubular epithelial cells (RPTEC) exclusively stain for LTA. Positively stained cells are green. Nuclei are stained with DAPI (blue).

FIGs. 4A-F are photographs illustrating adult kidney cell culture characteristics at passages P3-P5. The cells positively stain for both markers along the represented passages. Nuclei are stained with DAPI (blue).

FIGs. 5A-E are bar graphs illustrating the results of the qRT-PCR analysis of renal stem/progenitor genes (SAL1, SIX2, WT1 and PAX2) and pluripotency gene Nanog in SCM and SFM expanded hKEpC cultures of passages P0-P2. The values for SCM monolayer culture P1 were sued to normalize (therefore equal 1) and all other values were calculated with respect to them. Results are presented as the mean +/- SDEV of 3 separate experiments using cells from different donors.

FIGs. 6A-D are graphs and photographs illustrating the formation of hKEpC spheroids. (Figures 6A and B) Representative micrographs of p2 and p6 spheroid

morphology (Sph P2 and Sph P6 respectively) obtained from the same hKEpC origin. While P2 spheroids are less organized, P6 spheroids are more condensed, well organixed and demonstrate true sphere morphology. (Figures 6C and D) Quantitative representation of P2 vs. P6 spheroid formation from 2 X 10^4 cells/ 2 ml. Figure 6C represents spheroid number formed, showing signficiantly higher spheroid formation at P6. Figure 6D represents the number of spheroids formed according to spheroid size, showing that the small size spheroids (less than 15μm), rather than the bigger ones (more than 15 μm) predominantly contribute to the number difference b eween the P2 vs. P6 spheroids (represented in Figure 6C). Graphs represent mean values from the tripicates from 3 different tissue donors.

FIG.7 are photographs illustrating the characterization of hKEpC spheroid origin. hKEpC cells grown as a monolayer were infected by lenitvirus-based vecgtors, carrying the gene for either green fluorescent protein (GFP, green) or m-cherry (red). Fluorescent cells were mixed at an exact ratio of 1:1 and subjected to low-attachment conditions to allow formation of spheroids. Upper panel: spheroid formation after 7 days in culture (X20). Lower panel: spheroid formation after 6 weeks in culture (X10). Images were taken by Nikon Eclipse TS100 microscope, show abundant m-cherry7 and some GFP expression, indication that at least some cell aggregation had occurred.

FIGs. 8A-J are photographs and graphs illustrating that low attachment conditions induce higher expression of renal progenitor genes. Figures 8A-B. Spheroid –like structures formed in the low attachment conditions from hAK cells. Figure 8C. Adherent hAK culture; Figure 8D. Relative quantification RT-PCR analysis shows higher nanog and fetal kidney progenitor genes expression in the low-attachment conditions (originated from adherent grown in SCM) in comparison to the adherent culture of the p1 hAK cells in SCM. *, $p < 0.05$; **, $p < 0.05$ after logarithmic transformation; Figure 8E-H. Relative quantification RT-PCR analysis shows spheroids generated from adherent culture grown in SFM has higher expression of progenitor genes relative to adherent culture of hAK cell in both SFM and SCM. Mean of 3 different experiments on 3 different hAK tissues. Figure 8I. Elevated transcript levels of Gpc3, in P1 spheroid cells (generated after expansion as a monolayer in SCM) compared to P1 monolayer culture expanded in SCM. P is less than 0.05 after logarithmic transformation. Figure 8J. Elevated progenitor and pluripotency genes

transcript levels of P6 spheroid cells (generated after expansion as a monolayer in SCM) compared to P6 monolayer culture expanded in SCM. SCM-serum containing medium. SFM – serum free medium.

FIG. 9A is a bar graph illustrating surface marker expression and ALDH activity in spheroids vs adherent culture. Spheroids have enchanced ALDH acivity in comparison to adherent culture of hAK cells. Epithelial (EpCAM, CD24), mesenchymal cell (CD44) markers and CD133 has no difference in both culture conditions.

FIG. 9B are time lapse microscopy photographs of the spheroid formation. hKEpC suspension was seeded on the PolyHEMA precoated plates. Micrographs were taken by the CSN 410 Zeiss microscope (x10) with 3 minute intervals. Upper and lower planes show two representative events of cell collisions and aggregation in the process of spheroid formation.

FIG. 9C are representative dot plots showing enhanced ALDH1 activity in spheoids compared to monolayer culture. ALDH1 enzymatic activity was detected using ALDEFLUOR assay. DEAB was used to inhibit the reaction of ALDH with the ALDEFLUOR reagent, providing a negative control.

FIGs. 10A-C illustrate the results of microarray analysis of hKEpC spheroids vs. monolayer cells originating from 3 adult kidney (AK) donors. (A) Unsupervised hierarchical clustering separated samples into two different groups: spheroids and monolayer counterparts; (B) Hierarchical clustering of differentially expressed genes. Genes that were either up- (477 genes) or down-regulated (348 genes) in spheroids (Sph AK1-3) at least twofold compared with their monolayer culture counterparts (Mono AK1-3); (C) Forest plot of the cellular processes gene groups representing percent of up- (red) and down (green) – regulated genes.

FIGs. 11A-B illustrate hKEpC spheroid characterization and proliferation. Figure 11A is a photograph illustrating hematoxylin&eosin staining of paraffin embedded spheroids. Figure 11B is a photograph illustrating immunofluorescence analysis of NCAM and Ki67 of the spheroids. Paraffin embedded spheroids were stained with NCAM (green), Ki67 (red) and Hecht (blue). Low proliferation was observed as evidenced from the low Ki67 staining. Also, low NCAM staining was observed in agreement with FACS analysis.

FIGs. 12A-C are graphs illustrating limiting dilution of primary hAK cultured cells in different media conditions. The graphs represent 3 different experiments on 3 different hAK tissues. hAK seeded in density of 5 and 1 cell per well has higher clonogenic capacity in SCM as opposed to SFM. Highest clonogenic potential was observed with FKCM. (SCM= serum containing media, FKCM= fetal kidney conditioned media (containing serum), SFM= serum free media, SFM from SCM= clones originated in SFM after culture was expanded in SCM).

FIGs. 13A-D are photomicrographs illustrating clone morphology. SCM and FKCM – clones originated from 1 cell/well, SFM - clones originated from 5 cells/well. FKCM clones were more viable and confluent in comparison to SCM.

FIGs. 14A-C are graphs illustrating FACs sorting of NCAM1 expressing hAK cells.

FIGs. 15A-E are bar graphs comparing gene expression between NCAM1+ and NCAM1– cell fractions by quantitative RT-PCR analysis. Cultured hAK cells sorted according to NCAM1 overexpress the renal 'stemness' genes: (a) renal epithelial progenitor genes, wt1, pax2, six2 (Osr1 was also found to be significantly up-regulated, data not shown); (b) Wnt pathway and renal progenitor surface markers, CTNNB1, FZD7, NCAM1 and ACTR2b; (c) polycomb group, EZH2. In addition, analysis of pluripotency genes (d) showed higher Oct4 levels, while analysis for renal maturation genes (e) showed high vimentin and aminopeptidase A (ENPEP) and low E-cadherin and Na–Cl co-transporter (NCCT), disclosing a proximal tubular origin. The values represent average $\pm$ SD of at least 3 different experiments on 3 different hAKs. *, $p < 0.05$;**, $p<0.005$. Aqp1 and 3 were close to statistical significance.

FIGs. 16A-C are bar graphs illustrating the clonogenic potential of hAK NCAM+ cells. Both positive and negative fraction's cells were plated at 1 and 5 cells per well dilution. NCAM+ cells show high clonogenic potential in all concentrations. Graphs represent three experiments originating from three different hAK tissues.

FIG. 16D is a graph illustrating the results of a MTS proliferation assay performed on hAK cells sorted according to NCAM1. Both positive and negative cell fractions were analyzed 4, 5 and 7 days following sorting. NCAM+ cells showed lower proliferation capacity. Representative graph of three experinents, data represents mean of triplicates. *, $p<0.05$.

FIGs. 16E-J are photomicrographs illustrating the results of a spheroid formation assay performed on NCAM $^{+}$ and NCAM^{-} fractions sorted from low-passage cultures and expanded in vitro. NCAM^{+} cells show ability to form well-defined spheroids, as opposed to NCAM^{-} cells, which lack that ability, after 7 days in low-attachment conditions.

FIGs. 17A-K are photographs illustrating the results of an in vivo analysis of shperoid and monolayer hKEpC in the chick embryo. $0.43x10^{6}$ cells, derived from dissociated spheroid and monolayer were grafted on the CAM. Figures 9A-B. CAM grafts (arrowheads) generated from (A) spheroids and (B) monolayer cells, 7 days following grafting. Representative photomicrogrpahs of H&E staining of grafts: grafts originated from P2 cultures of spheroids (C), monolayer (D) and from P6 cultures of spheroids (E) and monolayer (F) cells, (G) whole spheroids (P2) (x20) demonstrating extensive tubule formation exculsively by hKEpC spheroids. Grafts originating from (H) mesenchymal stem cells (MSC) and (I) human embryonic kidney cell line (HEK293) used as controls, failed to generate tubules (x10). Control grafts of human fetal kidney (FK) cells originating from $1.25x10^{6}$ cells (J) did not form any tubules (x10) while $2.5x10^{6}$ cells (K) generated tubules (marked in arrows) (x20).

FIGs. 18A-D are photomicrographs illustrating that P2 hKEpC spheroids generate segment-specific tubules. Immunoperoxidase (brown) staining of (x20 arrowheads) for segment-specific markers (18A) LTA, (18B) anti-Tamm horsfall glycoprotein (THG) and (18C) DBA. Figure 18D: Immunofluorescent DBA staining (red) nuclei counterstained with Hoechst (blue). Original magnification x20.

FIGs. 19A-C are photomicrographs of immunoperoxidase (brown) staining of (x20 arrowheads) for segment specific markers (19A) LTA, (19B) THG and (C) DBA. Original magnification x20.

FIGs. 20A-F are photographs illustrating the results of an in vivo tubulogenesis assay. A. Picture of an explant of hAK cells 7 days after engraftment. Grafting was performed in matrigel B. Flouresence of the explant (cells were labeled with CFSE prior to grating enabling the detection of a flourescent signal). Histological analysis of 7-day grafts (H&E) revealed tubular regeneration by low cell numbers ($0.43x10^{6}$ cells/egg) only when grafting dissosiated hAK spheroids (C) or hAK NCAM+ cells (D). Tubular structures are highlighted in boxes. Similar cell number of adherent cultured hAK cells

did not generate tubular structures (E). Control HEK 293 (F) or mesenchymal stem cells (not shown) did not generate tubular structures and remained as undifferentiated masses.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to isolated populations of adult renal cells and methods of isolating and using same.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Renal failure, whether arising from an acute or chronic decline in renal function, is a severe condition that can result in substantial or complete failure of the filtration, reabsorption, endocrine and homeostatic functions of the kidney. It is therefore desirable to obtain progenitor or stem cells capable of developing into renal cells that could substitute for some or all of the functions of the kidney.

Human adult kidney (hAK) stem/progenitor cells are ideal candidates for cell transplantation and tissue engineering. However, their identity remains elusive.

This does not eliminate the possibility of using expanded populations of adult kidney cells as cell-based therapies for tissue engineering and regenerative medicine aimed at improving and restoring renal function. The autologous approach requires isolation of renal cells from a small human tissue sample, expansion *in vitro* and reintroduction of cells back into the host for renal tissue regeneration. Nevertheless, primary renal epithelia lose their apical-basal polarity, which is characterized by a flattened and elongated morphology and lack of tight junctions, begin to proliferate and dedifferentiate via mechanisms such as epithelial-mesenchymal transition (EMT) after a limited number of passages in monolayer. As the cell number increases, the cells assume the appearance of fibroblasts. Therefore, formation of renal structures may not be adequately achieved by using single kidney cells and appropriate manipulation of renal cells in culture might enhance their functional capacities.

Sphere structures are multicellular globes that develop from cells that survive anchorage-independent conditions *in vitro*, such as growth in ultra-low attachment

plates. Unlike monolayer-based cultures, these structures carry the advantage of mirroring the 3D cellular context. Furthermore, sphere-forming assays have been shown to be a useful means for maintenance and expansion of putative stem/progenitor cell populations.

The present inventor therefore sought to investigate for the first time primary human kidney cells grown in suspension culture over non-adherent plastic surfaces as opposed to monolayer expanded cells. In order to achieve this goal the present inventor isolated primary human renal cells from kidney surgical samples, established heterogeneous cultures of human kidney epithelial cells (hKEpC) and demonstrated their ability to efficiently generate 3D aggregates or spheroids.

It was discovered that the use of serum free medium (SFM) and subsequent low-attachment conditions lead to formation of "nephrospheroids". Epithelial cells present in the nephrospheroids expressed enhanced levels of progenitor and 'stemness' genes including *Pax2*, *Sall1*, *Six2*, *Wt1* and as well as the pluripotency gene, *Nanog*, when compared to adherent culture (Figures 8E-H).

In addition, the present inventors have identified a cell surface progenitor marker (NCAM) in cultured human adult kidney which provides for a signature for the isolation of renal stem/progenitor cells. Identification of this marker was very surprising considering it is not expressed in vivo in the adult kidney.

The present inventor showed that $NCAM^+$ enriched adult renal cells overexpressed early renal epithelial progenitor markers (*Six2, Osr1, Sall1, Pax2* and *Wt1*) and early surface antigens (*FZD7, AVR2b*) (11), polycomb group (*Bmi-1, Ezh2*), Wnt pathway (*Beta-catenin, FZD7*) as well the pluripotency marker, *Oct4* (Figures 9A-E), indicating the presence of stem/progenitor cells. The $NCAM^+$ subpopulation was highly clonogenic (Figures 16A-C) and further comprised sphere generating capabilities (Figures 16E-J) further indicating the presence of stem/progenitor cells.

Whilst further reducing the present invention to practice, the present inventor showed that both spheroid-forming and NCAM+ cells efficiently regenerated tubular structures when grafted in the chick embryo (Figures 17A-C).

These results provide a feasible approach for experimental cell sorting of adult human renal progenitors as well as a framework for developing cell selection strategies for renal cell-based therapies.

Thus, according to one aspect of the present invention there is provided an isolated cell population of human adult kidney cells, comprising at least 50 %, 60 %, 70 % 80 % or 90 % adult renal stem having a NCAM+ signature.

As used herein, the term "isolated" means that a cell population is removed from its natural environment. As used herein, the term "purified," means that a cell population is essentially free from any other cell type (e.g., feeder fibroblasts).

As used herein the phrase "renal stem cell" refers to a cell which is not terminally differentiated as a renal cell but which has the ability to differentiate into specialized cell having one or more structural and/or functional aspects of a physiologic kidney. According to specific embodiments the renal stem cells are not embryonic stem cells.

According to an exemplary embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ CD133+ signature.

According to another embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ CD133- signature.

According to another embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ CD24+ signature.

According to another embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ CD24- signature.

According to another embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ nestin+ signature.

According to another embodiment, at least 50 %, 60 %, 70 % 80 % or 90 % of the renal stem cells have a NCAM+ nestin- signature.

NCAM+ populations of the present invention further comprise a gene expression profile as provided in Figures 15A-E. Assaying expression of any of the genes of the provided expression profile may be used to qualify cells of the NCAM +, signature as further described herein below.

The present invention further provides for a method of isolating the aforementioned cells. This is effected by enriching for a subpopulation of renal cells from a human adult renal tissue, the subpopulation of renal cells having an NCAM+ signature.

Thus a human adult kidney is provided. The kidney may comprise a whole kidney or fragments thereof (e.g., renal capsule). Typically the cells of the adult kidney are of a heterogeneous population.

The cells of the adult kidney may be dispersed prior to selection. Exemplary agents that may be used to disperse the kidney cells include collagenase, dispase and trypsin.

According to one embodiment, the cells of the adult kidney are expanded prior to sorting. Typically the cells are cultured for less than three passages, more preferably for less than two passages.

Below is a list of some of the exemplary markers of the present invention with their accession numbers.

NCAM1 (3 variants): NM_181351, NM_000615, NM_001076682; FZD7: NM_003507; CD24: NM_013230; CD133 (PROM1): NM_006017; NTRK2: AF410902; PSA-NCAM, Polysialylated NCAM1 same ID as NCAM1; ACVRIIB: NM_001106; ROR2 (2 variants): M97639 NM,_004560; oct4 (POU5F1): NM_203289 NM_002701; six2: NM_016932 (accession number:AF136939); sall1: NM_002968; ctnnb1 NM_001098210 (NM_001098209 XM_001133660 XM_001133664 XM_001133673 XM_001133675 NP_001091679 XP_001133660 XP_001133664 XP_001133673 XP_001133675); vimentin: NM_003380 (accession number: M14144); Bmi1: NM_005180 (accession number BC011652); ezh2 (2 variants): NM_152998 NM_004456; nanog: NM_024865 (accession number: AB093576 (complete); aqp1 - NM_000385 (accession number: M77829); aqp3: NM_004925; e-cadherin (CDH1): NM_004360 (accession number: L08599); nestin (NES) NM_006617.1.

Antibodies for the above mentioned cell markers are commercially available. Examples include but are not limited to, NCAM1 (eBioscience), EPCAM (MiltenyiBiotec), FZD7 (R&D Systems), CD24 (eBioscience), CD133 (MiltenyiBiotec), NTRK2 (R&D Systems), PSA-NCAM (MiltenyiBiotec) ACVRIIB (R&D Systems), ROR2 (R&D Systems), nestin (Abcam).

As used herein, the term "enriching" refers to a procedure which allows the specific subpopulation of renal cells to comprise at least about 50 %, preferably at least about 70 %, more preferably at least about 80 %, about 95 %, about 97 %, about 99 % or more renal stem cells having the desired signature (e.g. NCAM+).

The enriching may be effected using known cell sorting procedures such as by using a fluorescence-activated cell sorter (FACS).

As used herein, the term "flow cytometry" refers to an assay in which the proportion of a material (e.g. renal cells comprising a particular maker) in a sample is determined by labeling the material (e.g., by binding a labeled antibody to the material), causing a fluid stream containing the material to pass through a beam of light, separating the light emitted from the sample into constituent wavelengths by a series of filters and mirrors, and detecting the light.

A multitude of flow cytometers are commercially available including for e.g. Becton Dickinson FACScan and FACScalibur (BD Biosciences, Mountain View, CA). Antibodies that may be used for FACS analysis are taught in Schlossman S, Boumell L, et al, [Leucocyte Typing V. New York: Oxford University Press; 1995] and are widely commercially available.

Another method of cell sorting is magnetic cell sorting as further described in the Examples section below.

It will be appreciated that the enriching may also be effected by depleting of non-relevant subpopulations such as renal stromal cells or interstitium (interstitial) cells.

Once isolated, cells of the present invention may be cultured and their "stemness" properties may be further analyzed as described below.

Since clonogenicity is a function of stem cells, the cells may be analyzed for their clonogenic potential. The present inventors have shown that isolated adult renal cells having an NCAM+ signature are highly clonogenic.

An exemplary method for ascertaining clonogenic potential is described in the Example section below.

The present inventor has discovered that culturing cells at low dilution in serum-comprising medium, preferably in the presence of conditioned medium from human fetal kidney cells is an optimal way to ascertain clonogenic potential. By counting the number of clones formed after a predetermined time (e.g. one month), one can determine the clonogenic potential of a renal cell population.

(b) counting a number of clones formed from the adult renal cells of the population, thereby determining clonogenic potential of an adult renal cell population.

An exemplary method for obtaining conditioned medium from human fetal kidney cells is by combining (e.g. in q 1:1 ratio) SCM and SCM from FK cultures of passages 1 to 3.

The ability to form spheres is also a function of stem cells. Accordingly, the cells may be analyzed for their sphere-forming potential. The present inventors have shown that isolated adult renal cells having an NCAM+ signature have a high sphere forming potential.

An exemplary method for ascertaining sphere-forming potential is described in the Example section below.

Another way to confirm the presence of renal stem cells is by testing for expression of stem cell-specific genes. An upregulation of such genes infers the presence of renal stem cells. Such genes include, but are not limited to Six2 (NM_016932-accession number: AF136939), osr1 (NM_145260.2), Pax2 (NM_003987.3 NM_000278.3, NM_003988.3, NM_003989.3, NM_003990.3), Sall1 (NM_002968) and Cited 1 (NM_001144885.1, NM_001144886.1, NM_001144887.1 NM_004143.3). Methods for analyzing for the expression of stem cell-specific genes include RT-PCR, Northern blot, Western blot, flow cytometry and the like.

As mentioned, the present inventor has found optimal conditions for culturing adult kidney cells such that they form spheroids. The present inventor found that these spheroids expressed stem cell-specific genes to a greater extent than adult kidney cells that were cultured under adherent conditions.

Thus, according to another aspect of the present invention there is provided a method of generating a nephrospheroid, the method comprising culturing adult kidney cells under non-adherent conditions, thereby generating the nephrospheroid.

As used herein, the term "nephrospheroid" refers to a 3 dimensional (spherical or partially) aggregate of kidney cells. It may also be referred to as a tubular organoid. The nephrospheroid comprises at least two cell types and is not derived from a single cell-type (i.e. is not of a clonal origin).

According to one embodiment the nephrospheroid is capable of generating proximal distal tubules and collecting ducts when allowed to differentiate in vivo following grafting to the chorioallantoic membrane (CAM) of the chick embryo.

According to another embodiment, the nephrospheroid is not capable of generating proximal distal tubules and collecting ducts when allowed to differentiate in vivo following grafting to the chorioallantoic membrane (CAM) of the chick embryo.

The phrase "non-adherent conditions" refers to conditions in which the cells do not attach to the surface of a container in which they are cultured such that a substantial portion of the cells can be removed from the surface of the container by mechanical manipulations that do not cause significant damage to the cells. It is understood that the cells can still be retained in or on a non-adherent matrix (e.g., on Hydrogel spheres) and be removed from the surface of the container. Such manipulations include, for example, gentle agitation, massage, or manual manipulation of the container, or rinsing the container with growth media As used herein, a substantial portion of the cells to be removed is at least 70%, preferably at least 75%, 80% or 85%, more preferably at least 90% or 95%. Manipulations that cause damage to the cells can be identified by determining the viability of the cells before and after manipulation, for example by trypan blue staining. Mechanical manipulations should cause damage to less than 20%, preferably less than 15%, or 10%, more preferably less than 5%, 2%, or 1% of the cells. Numerous methods are known for culturing cells under non-adherent conditions. These include growth of cells encapsulated in matrices such as Hydrogel and Matrigel™, on in between layers of agarose, or in Teflon™ bags. An exemplary hydrogel which may be used is PolyHEMA. It will be appreciated that the cells can grow in contact with the non-adherent matrices, but do not adhere to plastic culture containers.

Contemplated culture mediums include, but are not limited to IMDM (Invitrogen) or DMEM (Invitrogen).

According to one embodiment, the culture medium comprises serum.

According to another embodiment, the culture medium is devoid of serum.

The medium may comprise additional components which further encourage the cells to form spheroids. Thus, for example, the medium may further comprise growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF). Other contemplated components include insulin and progesterone.

Typically, prior to culturing the adult kidney cells, the cells are dispersed as described herein above.

Optionally, the adult kidney cells are cultured prior to forming the spheroids in order to expand the number of cells.

According to one embodiment, the adult kidney cells are expanded in serum containing medium for about 4, 5, 6, 7 or more passages under adherent conditions prior to generation of the spheroids.

The phrase "adherent conditions" refers to conditions in which the cells attach to the surface of a container in which they are cultured such that a substantial portion of the cells cannot be removed from the surface of the container by mechanical manipulations that do not cause significant damage to the cells.

Using the above described method, the present inventor generated nephrospheroids and proceeded to characterize these structures.

According to one embodiment, an isolated nephrospheroid may be characterized by enhanced expression of at least one polypeptide selected from the group consisting of sal1, pax2, six2 and WT1 or combinations thereof, as compared to identical adult kidney cells grown under adherent conditions.

According to one embodiment, an isolated nephrospheroid may be characterized by enhanced expression of each of sal1, pax2, six2 and WT1 as compared to identical adult kidney cells grown under adherent conditions.

As used herein, the term enhanced expression refers to an increase in expression by at least 1.5 fold, more preferably at least 2 fold and even more preferably at least 3 fold.

Once generated the cell populations of the present invention (including the NCAM+ populations and the nephrsopheres) are typically allowed to proliferate under conditions that preserve their stem/progenitor cell phenotype.

Cell populations of the present invention can be genetically modified to express a transgene. This may be used to increase survival of the cells, render them immortalized or differentiated to a desired lineage. Examples of such transgenes and methods of introducing the same are provided below.

Candidate genes for gene therapy include, for example, genes encoding the alpha 5 chain of type IV collagen (COL4A5), polycystin, alpha- galactosidase A, thiazide-sensitive sodium chloride cotransporter (NCCT), nephrin, actinin, or aquaporin 2.

Further, genes encoding erythropoeitin or insulin can be introduced into a kidney stem cell. For treatment of anemia associated with renal failure or diabetes it can be useful to introduce into a patient a stem cells modified to express erythropoeitin or insulin. The renal stem cells can be stably or transiently transfected with DNA encoding any therapeutically useful polypeptide.

The cell populations of the invention can also be provided with a transgene encoding VEGF or some other factor that can promote growth and or differentiation of cells.

These genes can be driven by an inducible promoter so that levels of the transgen can be regulated. These inducible promoter systems may include a mutated ligand binding domain of the human estrogen receptor (ER) attached to the protein to be produced. This would require that the individual ingest tamoxifen to allow expression of the protein. Alternatives are tetracyclin on or off systems, RU486, and a rapamycin inducible system. An additional method to obtain relatively selective expression is to use tissue specific promoters. For instance, one could introduce a transgene driven by the KSP-cadherin, nephrin or uromodulin-specific promoter.

Cells isolated or generated by the method described herein can be genetically modified by introducing DNA or RNA into the cell by a variety of methods known to those of skill in the art. These methods are generally grouped into four major categories: (1) viral transfer, including the use of DNA or RNA viral vectors, such as retroviruses (including lentiviruses), Simian virus 40 (SV40), adenovirus, Sindbis virus, and bovine papillomavirus for example; (2) chemical transfer, including calcium phosphate transfection and DEAE dextran transfection methods; (3) membrane fusion transfer, using DNA-loaded membrane vesicles such as liposomes, red blood cell ghosts, and protoplasts, for example; and (4) physical transfer techniques, such as microinjection, electroporation, or direct "naked" DNA transfer. Cells can be genetically altered by insertion of pre-selected isolated DNA, by substitution of a segment of the cellular genome with pre-selected isolated DNA, or by deletion of or inactivation of at least a portion of the cellular genome of the cell. Deletion or inactivation of at least a portion of the cellular genome can be accomplished by a variety of means, including but not limited to genetic recombination, by antisense technology (which can include the use of peptide nucleic acids, or PNAs), or by

ribozyme technology, for example. Insertion of one or more pre- selected DNA sequences can be accomplished by homologous recombination or by viral integration into the host cell genome. The desired gene sequence can also be incorporated into the cell, particularly into its nucleus, using a plasmid expression vector and a nuclear localization sequence. Methods for directing polynucleotides to the nucleus have been described in the art. The genetic material can be introduced using promoters that will allow for the gene of interest to be positively or negatively induced using certain chemicals/drugs, to be eliminated following administration of a given drug/chemical, or can be tagged to allow induction by chemicals (including but not limited to the tamoxifen responsive mutated estrogen receptor) for expression in specific cell compartments (including but not limited to the cell membrane).

Calcium phosphate transfection, which relies on precipitates of plasmid DNA/calcium ions, can be used to introduce plasmid DNA containing a target gene or polynucleotide into isolated or cultured cells. Briefly, plasmid DNA is mixed into a solution of calcium chloride, then added to a solution which has been phosphate-buffered. Once a precipitate has formed, the solution is added directly to cultured cells. Treatment with DMSO or glycerol can be used to improve transfection efficiency, and levels of stable transfectants can be improved using bis-hydroxyethylamino ethanesulfonate (BES). Calcium phosphate transfection systems are commercially available (e. g., ProFection from Promega Corp. , Madison, WI).

DEAE-dextran transfection, which is also known to those of skill in the art, may be preferred over calcium phosphate transfection where transient transfection is desired, as it is often more efficient.

For isolated cell populations, microinjection can be particularly effective for transferring genetic material into the cells.

The developmental potential of the cell populations thus obtained can be investigated using methods which are well known in the art. For example by injection into other organs (liver, muscle, heart and bone marrow) to test their multipotency Clarke et al. describes protocols for investigating the development potential of stem cells (Clarke et al. 2000 Science 288:1660). The cell popualtions may also be grated into chick embryos so as to ascertain their developmental potential as described in the Examples section herein below.

The cell populations of the invention (or cells which have been differentiated therefrom) can be used to supplement or substitute for kidney cells that have been destroyed or have reduced function. Thus, they can be used to treat patients having poor or no kidney function. The cell populations of the invention or cells derived there from may be capable of performing the filtration and reabsorptive/secretive functions of the kidney.

Thus according to an aspect of the present invention there is provided a method of treating a renal damage in a subject in need thereof comprising administering to the damaged kidney of the subject a therapeutically effective amount of the isolated population of cells described herein, thereby treating the renal disease in the subject.

Cells of the present invention can be used to treat any form of acute or chronic kidney disease, diabetic nephropathy, renal disease associated with hypertension, hypertensive acute tubular injury (ischemic, toxic), interstitial nephritis, congenital anomalies (Aplasia/ dysplasia / obstructive uropathy/reflux nephropathy); hereditary conditions (Juvenile nephronophtisis, ARPCKD, Alport, Cystinosis, Primary Hyperoxaluria); Glomerulonephritides (Focal Segmental Glomerulosclerosis); Multisystem Diseases (SLE, HSP, HUS).

The present inventor contemplates administration of single cell suspensions of dissociated spheroid-cells, partly dissociated spheroid-cells or non-dissociated spheroid cells.

The cells may be administered per se or as part of a pharmaceutical composition where they are mixed with a suitable carrier or excipient.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the renal progenitor cells (or cells differentiated therefrom) accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not

abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

The cell populations or cells derived (e.g. differentiated therefrom) can be administered into a subject such as surgically or by infusion. For example, renal cells are injected in vivo into a kidney that is in the postischemic recovery phase. This can be tested easily in an animal model predictive of ischemic kidney damage, the renal pedicle of an anesthetized mouse is clamped for 30 minutes to induce kidney ischemia. Renal stem cells are then injected into the juxtamedullary region (approximately 2000 cells at a depth of 2-4 mm). After 2 weeks of recovery, immunohistochemical analysis is used as described above to look for differentiated cells surface markers GP330, Tamm-Horfall, Dolichos Biflorous, and the like. Post-incorporation differentiation status can then be compared to pre-injection marker status.

The cells of the invention, or cells derived there from (e.g., epithelial cells endothelial cells, mesangial cells, vascular smooth muscle cells, and pericytes) can be used to construct artificial kidney systems. Such a system can be based on a hollow fiber filtration system.

In one example of a filtration device, the stem cells of the invention or differentiated progeny thereof are grown on the interior of hollow fibers having relatively high hydraulic conductivity (i.e., ultrafiltration coefficient). The hollow fiber passes through a chamber that is provided with a filtrate outlet port. Arterial blood containing metabolic waste and other unwanted material is introduced into one end of the hollow fiber through an inlet port. Blood passed through the fiber and exits the other end of the fiber through an outlet port where it passed into the patient's vascular venous flow. As blood passes through the fiber, filtrate pass through the cells lining the interior of the fiber and through the hollow fiber itself. This filtrate then passes out of the chamber containing the fiber through the filtrate outlet port. The device preferably includes many such hollow fibers each of which can be in its own chamber.

Alternatively many, many hollow fibers (100-100,000 or even more) can be bundled together in a single chamber.

The cells of the invention can be used to create a tubule-processing device. In such a device the stem cells of the invention or differentiated cells derived from the stem cells of the invention can be grown in a layer on the exterior of the semipermeable hollow fiber (i.e. a scaffold). The fiber is placed in a chamber that is provided with an inlet port and an outlet port. As ultrafiltrate from filtered blood flows through the chamber, reabsorbant passes through the cell layer and through the wall of the fiber into the lumen of the fiber from which it can be directed back into the patient's systemic circulation. Ultrafiltrate that is not reabsorbed passes through the outlet port of the chamber.

In the devices described above, it can be desirable to coat the fiber surface that will bear the cell layer with extracellular matrix components. For example, the fiber can be coated with materials such as collagen (e.g., Type I collagen or Type IV collagen), proteoglycan, fibronectin, and laminin or combinations thereof. It can be desirable to combine various cell types on the inner or outer surface of the fibers. For example, it can be desirable to include endothelial cells and pericyte, vascular smooth muscle cells or mesangial cells or fibroblasts or combinations thereof. It can also be useful to provide a feeder layer of cells, e.g., irradiated fibroblasts or other cells that can provide soluble factors and structural support to cells they are indirectly or directly in contact with.

The above-described filtration system and the above-described tubule processing system can be combined to create an artificial kidney. Such systems are described in U.S. Pat. No. 6,150,164, hereby incorporated by reference. A number of suitable materials for forming the hollow fiber are described in U.S. Pat. No. 6,150,164, hereby incorporated by reference.

The present invention provides a method of using the cell populations of the present invention to characterize cellular responses to biologic or pharmacologic agents involving isolating the cells as described s, culture expanding the cells to establish a plurality of MRPC cultures, contacting the MRPC cultures with one or more biologic or pharmacologic agents, identifying one or more cellular responses to the one or more biologic or pharmacologic agents, and comparing the one or more cellular responses of the cultures. Tissue culture techniques known to those of skill in the art allow mass

culture of hundreds of thousands of cell samples from different individuals, providing an opportunity to perform rapid screening of compounds suspected to be, for example, teratogenic or mutagenic.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to". This term encompasses the terms "consisting of" and "consisting essentially of".

The phrase "consisting essentially of" means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the claimed composition or method.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory

Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND METHODS

Cell cultures of human adult kidney (hAK): Normal hAK samples were retrieved from borders of RCC tumors from partial and total nephrectomy patients. This procedure was done following informed consent and has been approved by the local ethical committee. The samples were minced in HBSS, soaked in collagenase for 2 hours and then cultured in serum containing medium, (SCM) or in serum free medium (SFM). SCM comprised IMDM medium supplemented with FBS 10 %, L-Glutamin 1

%, Pen-Strep 1 % and growth factors: 50 ng/ml of bFGF, 50 ng/ml of EGF and 5 ng/ml of SCF (R&D systems). SFM comprised 500ml DMEM:F12 (ratio 1:1, Invitrogen), 1 % Pen-strep, 2 ml B27 supplement (Gibco), 4 μg/ml heparin, 1 % Non essential Amino acids (invitrogen), 1 % of sodium pyruvate (invitrogen), 1 % L-glutamine, 1 ml Lipid mix (Sigma), 5 ml N2 supplement 100X (Gibco), 5 ml growth factor mix (for 200 ml of growth factor mix: 100 ml DMEM:F12, 4 ml 30 % glucose, 200 mg transferin, 50 mg insulin in 20 ml of water, 19.33 mg putrescine in 20 ml ddw, 200 μl sodium selanite (0.3 mM stock), 20 μl progesterone (2 mM stock)), FGF 10 ng/ml, EGF 20 ng/ml. Upon 90 % confluence, cells were split. Medium was changed every 3 days. Spheroid formation was tested by seeding the cells in PolyHEMA pre-coated plates, in the SFM. Fetal kidney conditioned medium (FKCM) was obtained by combining in the 1:1 ratio SCM and SCM from FK cultures of passages 1 to 3.

Antibodies for FACS analysis: Primary fluorochrome conjugated anti-human antibodies against: CD133/1:APC, CD133/1:PE, CD34:FITC, PSA-NCAM:PE (Miltenyi Biotech), NCAM:APC (Biolegend), NCAM:PE (eBioscience), CD90:FITC (Biosciences Pharmingen, BD), CD105:FITC (Serotec), CD24:PE (eBioscience), C-Kit :APC(eBioscience), CD45:FITC (R&D systems). Primary unconjugated anti human antibodies against: ACVR2B, FZD7, NTRK2 (R&D systems). In order to visualize the primary unconjugated antibodies, appropriate secondary antibodies were used conjugated to either Alexafluor-488 or Alexafluor-647 (Molecular Probes, INC.). 7-amino-actinomycin-D (7-AAD, BD Biosciences) was used for dead cells exclusion from the analysis.

FACS Analysis: Monolayer cells were detached from culture plates with 0.25% trypsin (Gibco), spheroids were collected and dissociated by 5-10 min digestion with Accutase (Sigma-Aldrich). Viable cell number was determined using Trypan blue staining (Invitrogen). Cells (1×10^5 in each reaction) were suspended in 50 μl of FACS buffer [0.5% BSA and 0.02% sodium azid in PBS (Sigma-Aldrich and Invitrogen, respectively)] and blocked with FcR Blocking Reagent (MiltenyiBiotec, Auburn, USA) and human serum (1:1) for 15 min at 4°C. Cells were then incubated for 45 min with a respective antibody or a matching isotype control. Cell viability was tested using 7AAD viability staining solution (eBioscience). Cell labeling was detected using FACSCalibur (BD Pharmingen). Flow cytometry results were analyzed using FlowJo analysis

software. Viable cells were defined by their FSC/SSC profiles and, in addition, their lack of 7AAD. Detection of cells with high ALDH1 enzymatic activity was performed using the ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) according to the kit's protocol.

Magnetic Cell Sorting: CD56 (NCAM) microbeads (Miltenyi Biotec) were used for single marker cell separation. Positive and negative fractions were separated using Mini or MidiMACS cell columns (Miltenyi Biotech) according to the manufacturer's protocols. Briefly, cell suspension was obtained and, for the removal of clumps, was passed through a 30 μm nylon mesh. Cells were labeled by adding 20 μl CD56 microbeads per 10^7 total cells for 15 minutes in refrigerator. Then the cells were washed, resuspended and magnetically separated. For increased purity, the fractions were passed a second time through fresh columns. Separated cells were plated for limiting dilution, differentiation assays and FACS analysis. A part of cells was used for RNA extraction.

On the next day, purity of sorted fractions was checked by FACS analysis, after fluorescent labeling.

FACS sorting: Cells were harvested as described above, filtered through a 30 μm nylon mesh before final centrifugation, then re-suspended in flow cytometry buffer consisting of 2 mM bovine serum albumin (BSA; Sigma-Aldrich) and 10 % sodium azide in PBS. Cells were labeled with anti NCAM:PE (eBioscience) or other needed antibody. Fluorescence-activated cell sorter FACSAria and the FACSDiva software (BD Biosciences) were used in order to enrich for cells expressing these markers. Single viable cells were gated on the basis of 7-amino-actinomycin-D (7-AAD, BD Biosciences) stained cell exclusion, and then physically sorted into collection tubes for limiting dilution plating and RNA extraction. Data were additionally analyzed and presented using FlowJo software (Tree Star). Purity of sorted fractions was checked visually and by FACS reanalysis.

Gene expression analysis of the separated cell fractions: Quantitative real time reverse transcription PCR (qPCR) reactions were carried out to determine fold changes in expression of the selected renal 'stemness' genes (57) as well of differentiation markers in the sorted hAK cells.

The following nephron segment-specific genes were analyzed: Aminopeptidase-A (ENPEP), Aquaporin-1(AQP1), Aquaporin-3 (AQP3), Na/CL co-transporter (NCCT), Podocin; renal stem/progenitor genes: PAX2, SALL1, SIX2, WT1 and pluripotency gene: NANOG.

Primers were obtained from Applied Biosystems. RNA was extracted using the micro or miniRNeasy kits (Qiagen) according to the manufacturer's protocols. cDNA synthesis was carried out using the High Capacity cDNA RT kit (Applied Biosystems). Each analysis reaction was performed in triplicate. GapDH or HPRT1 were used as endogenous controls throughout all experimental analyses. Gene expression analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems). Analysis was performed using the -ΔΔCt method, which determines fold changes in gene expression relative to a comparator sample (the positive fraction of each hAK).

Clonogenicity of hAK stem/progenitor cells: Limiting dilution assay was performed on separated cell fractions NCAM1 positive vs. NCAM1 negative. Briefly, sorted cells were plated in 96-well micro well plates (Greiner Bio-One) in 150μl of culture media, at 0.3 or 1 cells per well dilution. The low cell concentration was achieved by serial dilutions reaching 1000 cells per ml. The number of colonized wells was recorded after one month.

Immunohistochemical staining of hAK: Sections, 4-μm thick, from whole blocks of normal hAK were cut for immunohistochemistry. The sections were processed within 1 week to avoid oxidation of antigens. Before immunostaining, sections were treated with 10 mM citrate buffer, PH 6.0 for 10 min at 97 ^{0}C in a microwave oven for antigen retrieval, followed by treatment of 3 % H_2O_2 for 10 minutes. The slides were subsequently stained by the labeled – (strept) avidin-biotin (LAB-SA) method using a histostain plus kit (Zymed). Anti-human CD56 antibody (LifeSpan Biosciences, Inc.) and anti-FZD7 antibody, at a dilution of 1:50, were used. Controls were prepared by omitting the primary antibodies or by substituting the primary antibodies with goat IgG isotype. The immunoreaction was visualized by an HRP-based chromogen/substrate system, (Zymed).

***Immunofluorescent staining of nephrospheroids*:** Spheroids were collected, fixed in PFA 4%, embedded in agarose gel and then in paraffin. Immunocytochemistry for Ki67 (mammalian-specific monoclonal rabbit antibody, Lab Vision clone SP6) was

performed on the sections containing spheroids using microwave antigen retrieval. Detection was performed with Alexa-594 anti-rabbit antibodies (Molecular Probes), and slides were counterstained with Hoechst.

Nephron segment specific staining of AK cells in culture: Tubular segments were identified by use of the following markers: Proximal tubule (PT) with *Fluorescein labeled Lotus tetragonolobus* lectin (LTL), collecting duct (CD) with *Fluorescein labeled Dolichos biflorus agglutinin* (DBA) 1:200 for 30 minutes (Vector Laboratories); distal tubules and thick ascending limb of Henle with anti-Tamm-Horsfall Glycoprotein antibody (anti-THG) (Millipore, Chemicon), secondary antibody used for this staining NorthernLights anti-sheep IgG-NL637 (R&D systems).

Grafting of AK cells on the chick embryo chorioallantoic membrane (CAM): Fertile chicken eggs were obtained from a commercial supplier, and incubated at 37 ^{0}C at 60-70 % humidity in a forced-draft incubator. At 3 days of incubation, an artificial air sac was established dropping the CAM. A window was opened in the shell, and the CAM exposed on 9 or 10th day of incubation. AK cells derived from AK adherent vs spheroid cultures or NCAM+/- sup-populations. AK cells were suspended in 50 µl medium and Matrigel (1:1 by volume) and pipetted into a plastic ring placed on the membrane. The egg was then sealed with adhesive tape and returned to the incubator. After one week, the graft was removed, paraffin embedded, and serially sectioned at 6 µm for histological and immunocytochemical analyses. Sample sections were stained with hematoxylin and eosin at intervals of 100-150 µm in order to find the grafted cells in the large mass of Matrigel (not shown). Biotin-labeled LTA (1:500), DBA (1:2000) (Vector Laboratories) and mouse anti-THG (1:800) (Millipore, Chemicon) were used for distinguishing parts of the renal tubules. Before immunostaining, sections were boiled for 10 minutes in 10mM citrate buffer, PH 6.0 in a microwave oven for antigen retrieval (only for anti-THG). Endogenous peroxidates was blocked using 3% H_2O_2 in methanol for 10 min. The lectins and primary antibodies were subsequently stained by the avidin-biotin method, using peroxidase conjugated avidin (lectins), proceeded by anti-mouse biotin (antibody) (Vector laboratories). Controls were prepared by omitting the lectin or the primary antibody. DAB substrate kit (Zymogen) was used for detection of the peroxidase.

Double-immunocytochemistry for Ki67 (mammalian-specific monoclonal rabbit antibody, Lab Vision clone SP6) and NCAM (mouse monoclonal, Santa Cruz) was performed on the sections containing AK cells using microwave antigen retrieval. Detection was performed with Alexa-488 anti-mouse and Alexa-594 anti-rabbit antibodies (Molecular Probes), and slides were counterstained with Hoechst, and all serial sections were examined. Photomicrographs were made with digital cameras (CFW-1312M and CFW-1612C, Scion Corporation) on Olympus SZX12 and BX51 microscopes. All changes in the images (contrast, brightness, gamma, sharpening) were made evenly across the entire field, and no features were removed or added digitally.

Sphere-forming assay: To establish genetically marked hKEpC, HEK293 cells were initially transformed. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin (Biological Industries, Israel), at 37°C and 5% CO2. Cells were transfected using calcium phosphate with three lentiviral vectors, pHR-CMV-GFP/m-Cherry (7.5 μg), ΔR8.2 (5 μg) and pMD2.G (2.5 μg). After 6 h, the supernatants were replaced with 5 ml of fresh medium. Supernatants of transfected cells were supplemented with HEPES (pH 7.0; 50 mM final concentration), filtered through a 0.45-μm-pore-size filter, and 2ml was placed on the targeted cells for 2h with the addition of 8μg/ml Polybrene (hexadimethrine bromide; Sigma) then 3ml of fresh medium was added. These viral like particles were used to infect hKEpC cells ($2x10^5$ cells in 60-mm-diameter dishes) Expression of reporter genes was analyzed two days post infection. GFP and m-Cherry labeled hKEpC cells were mixed in 1:1 ratio and seeded on PolyHEMA pre-coated 6 well plates, 1-2 $x10^4$ cells/well.

Microarray analysis: Adult renal spheroids and monolayer cells were obtained from 3 different adult donors. All experiments were performed using Affymetrix HU GENE1.0st oligonucleotide arrays (url1). Total RNA from each sample was used to prepare biotinylated target DNA, according to manufacturer's recommendations. The target cDNA generated from each sample was processed as per manufacturer's recommendation using an Affymetrix GeneChip Instrument System (url2). The quality and amount of starting RNA was confirmed using an agarose gel or by Bioanalyser (Agilent). After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. The

signals derived from the array will be assessed using various quality assessment metrics. Details of quality control measures can be found at (url1). Gene level RMA sketch algoritm (Affymetrix Expression Console and Partek Genomics Suite 6.2) was used for crude data generation. Significantly changed genes were filtered as changed by at least 2 fold (P value 0.05). Genes were filtered and analysed using unsupervised hierarchical cluster analysis, and supervised hierarchical cluster analysis (Partek Genomics Suite and Spotfire DecisionSite for Functional Genomics; Somerville,MA) to get a first assessment of the data. Further processing included functional analysis and over-representation calculations based on Gene Ontology and publication data: DAVID (www.apps1.niaid.nih.gov/David/upload.asp), Ingenuity, Database for Annotation (GO), Visualization, and Integrated Discovery. Over- representation calculations are done using Ease (DAVID).

url1:www.worldwidewebdotaffymetrixdotcom/support/technical/datasheets/gene_1_0_st_datasheet.pdf

url2:www.worldwidewebdotaffymetrixdotcom/support/downloads/manuals/wt_sensetarget_label_manualdotpdf

Time lapse microscopy: hKEpC were seeded on the poly-HEMA pre-coated plate. Photomicrographs were taken every 3 minutes by CLSN 410 Zeiss microscope (x10) in DIC mode. Images were stacked to the movie file by ImageJ 1.42q software.

Statistical analysis: Results are expressed as the mean values ± STDEV. Statistical differences of two group data were compared by Student's *t* test. Where indicated, *t* test was performed after logarithmic transformation in order to achieve normality. For all statistical analysis, the level of significance was set as $P<0.05$.

EXAMPLE 1

Adherent hFK cell cultures

Following the retrieval of a small specimen of hAK from nephrectomized patients, tissue was dissociated into a single cell suspension and cultured in low densities in T75 flasks so as to allow clonal growth (see scheme in Figure 1). To achieve expansion to a confluent adherent monlayer culture (P0), low cell numbers were initially grown using either serum containing media (SCM) or defined serum free media (SFM). Cell growth was initiated from small cell foci. However, while both media enabled cell expansion, SFM promoted more concentric, well defined expansion and

SCM displayed rapid expansion in a less-organized manner (Figures 2A-F). Staining of cultures for segment-specific markers [lotus tetragonolobus (LTA)-proximal tubules; Tamm-Horsfall glycoprotein (THG)-distal tubules; DBA-collecting tubules] revealed the presence of heterogeneous tubule cell types with predominance of proximal (70 %) and distal (20 %) tubules and to a lesser extent collecting ducts (<10 %; Figures 3A-D). Staining of renal proximal tubular epithelial cells (RPTEC) and human foreskin fibroblasts (HFF) were used as positive and negative controls respectively (Figures 3E-J). This heterogeneity was preserved along hKEpC culture passages (P3-P5) – Figures 4A-F.

Analysis of renal epithelial segment specific gene (aminopeptidase A(ENPEP), aquaporin-1 (AQP1), aquaporin-3 (AQP3), Na/Cl co-transporter (NCCT) and podocin) expression in the primary kidney cultures compared to human foreskin fibroblasts (HFF) indicated the tubular epithelial nature of the cells as illustrated in Table 1. Shown are relative quantification (RQ) values, normalized to expression values of HFF (which therefore equal 1). Two different P) hKEpC cultures are shown to express all the nephron segment-specific genes: proximal tubule – aminopeptidase-A (ENPEP) and Aquaporin-(AQP1)-collecting duct – Aquaporin-3 (AQP3), distal convoluted tubule – NA/Cl co-transporter (NCCT), podocyte – Podocin.

Table 1 – Gene expression in RQ values

	HFF	***hKEpC#1***	***hKEpC#2***
ENPEP	1	28.36	9.40
AQP1	1	281.14	70.19
AQP3	1	6.34	1.97
NCCT	1	3845.58	3387.39
Podocin	Undetectable	Detectable	Detectable

Analysis of renal progenitor gene expression in the heterogeneous P0 adherent SCM and SFM cultures showed similar gene levels (Figures 5A-E). Cells were then harvested and propagated and expanded as adherent cultures in SFM/SCM or subjected to sphere formation and limiting dilutions to assess for clonogenicity in various culture conditions (see Figure 1).

EXAMPLE 2

Low-attachment conditions in heterogeneous hAK cultures promote formation of 'nephrospheroids'.

The present inventors considered that culture conditions that support proliferation of human kidney cells that form spheroids may represent a strategy for isolation of cells with progenitor potential. Accordingly, heterogeneous P0 adherent SCM and SFM cultures originating from five hAK samples were subjected to low attachment conditions – specifically they were seeded on polyHEMA plates at a density of 20-40,000 viable cells/ml.

After 7–10 days, floating cellular aggregates, termed nephrospheroids or hKEpC spheroids, 100-130 micrometer in diameter, were obtained from 10 of the 10 cases (Figure 6A). Primary kidney-spheres, once enzymatically disaggregated into single cells and replated at a density of 20,000 cells/ml in ultra-low attachment plates could give rise to secondary spheres within 5–7 days of culture. Having determined that P2 spheroids could be reproducibly generated following the seeding of $2x10^4$ cells/2ml of P1 monolayer hKEpCs, the present inventors determined hKEpC spheroid formation after long-term monolayer culture and observed reproducible generation of P6 spheroids following seeding of similar cell numbers of P5 monolayer hKEpCs (Figure 6B). Interestingly, a comparison between low and high passage hKEpCs spheroids showed the latter to display a well-organized, more condensed and "true" sphere-like morphology. In addition, it was found that a significantly higher number of spheroids were generated at P6 compared to P2 cultures (Figure 6C). Close examination of this difference revealed it to be attributed mainly to formation of small size spheroids (<15μm), rather then medium size (15-130μm) or larger ones (>130μm) (Figure 6D).

***Origin of hKEpC spheroids*:** In order to analyze whether hKEpC spheroids are of clonal origin, kidney-derived cells from two donors were grown as a monolayer and stably labeled with either red or green fluorescent proteins using lentivirus-based vectors, directing constitutive expression of mCherry and GFP, respectively. For efficient infection and antibiotic selection of monolayer hKEpC, cells were propagated to P2-P3. Fluorescent hKEpC were detached, mixed at a ratio of 1:1 and subjected to low-attachment conditions at low densities 10^4 cells/well to generate spheroids. Continuous microscopic examination from 7-10 days to six weeks after seeding

revealed that spheroids contained both red and green cells. More than 75 % of cells in each kidney-spheroid were comprised of one color (Figure 7), suggesting that aggregation into hKEpC spheroids had occurred and that cells were not entirely clonally derived. In addition to genetic labeling, time-lapse microscopy was utilized to follow initial events after hKEpC seeding ($2x10^4$ cells/well) in non-adherent conditions, as cells were filmed every 3 minutes for 48 hours. Cell collision and aggregation were noticed to occur within five hours after seeding, indicating this as the initiating process for spheroid formation.

Phenotypic characterization of hKEpC spheroids: The present inventors initially determined whether the generation of hKEpC spheroids promoted the expression of 'stemness' genes.

Elevated expression of *Pax2*, *Sall1*, *Six2*, *Wt1* as well as the pluripotency gene, *Nanog*, when compared to adherent culture (Figure 8D) was found. Moreover, this expression profile was especially prominent when spheroids were generated from hAK cells grown in SFM and not SCM (including *Six2*), while expansion in SFM adherent cultures did not significantly promote renal progenitor markers by comparison to SCM adherent cultures (Figures 8E-H).

Interestingly, among genes characteristic of the early ureteric lineage a strong elevation in kidney-spheroids of the heparan sulfate proteoglycan *GPC3*, an extra-cellular matrix (ECM) molecule, which functions in the uerteric bud niche was found (Figure 8I), but expression of the other ureteric lineage genes (*Wnt11* and *c-Ret*) was undetectable (data not shown). Finally, generation of kidney-spheroids after long-term monolayer expansion (P6) also resulted in significant re-expression of the renal progenitor genes (Figure 8J). Thus, in contrast to monolayer cells hKEpC spheroids promoted, at least in part, expression of renal developmental markers and 'stemness' profile.

Flow cytometry was used (Figure 9A) to analyze the percentage of cells expressing the epithelial, renal and mesenchymal stem cell antigens EpCAM, CD24, CD133, CD44 (15) in spheroid and monolayer hKEpC. High expression levels of CD24 and CD44 (80-100% of cells) in both spheroids and monolayer cells was found (Figure 9A and Figure 9B), while EpCAM and CD133 levels found to be further elevated in spheroids indicating mostly an $EpCAM^+CD24^+CD133^+CD44^+$ phenotype of spheroid

cells. In addition, analysis of the activity of aldehyde dehydrogenase1 (ALDH1), an enzyme which increased activity has been detected in stem/progenitor cell populations, showed significantly higher levels in hKEpC spheroid cells compared to monolayer counterparts (Figure 9C). It was found that 29.93 ± 11.78% of spheroid cells displayed high levels of ALDH1 activity, compared to 8.06 ± 4.53% of monolayer cells.

Thus, hKEpC spheroids have a distinct antigenic profile with enhanced ALDH1 activity. ***Global transcriptional changes associated with kidney spheroid formation:*** Having illuminated specific characteristics of hKEpC spheroids the present inventors wanted to asses on a global level the transcriptional alternations taking place in relation with spheroid formation. For this spheroid and monolayer hKEpC were generated from three different human adult kidney sources and their global gene expression profile using oligonucleotide microarrays were compared. Unsupervised clustering (Partek 6.5) of the entire human microarray data set clearly distinguished among samples separating them into two major groups: hKEpC spheroids and hKEpC grown as monolayer and indicating a different biological entity and fundamental difference in gene expression patterns (Figure 10A). Kidney spheroids were closer to each other rather than to their monolayer counterpart of the same adult kidney origin. 825 genes differentially expressed by spheroid and monolayer hKEpC (>2 fold change, ANOVA, $P < 0.05$,) were identified. These included 477 genes upregulated and 348 downregulated in spheroids compared to monolayer cells (Figure 10B). The 20 genes most highly expressed in hKEpC spheroids and monolayer cells are respectively shown in Table 2.

Table 2

Up-regulated

Gene category	*Gene name*	*Symbol*	*Fold-Change*	*RefSeq*	*Probeset ID*
	chromosome 9 open reading frame 71	C9orf71	43.20	NM_ 153237	8161610
	low density lipoprotein receptor-related protein 2	LRP2	18.30	NM_ 004525	8056611
ECM protein	spondin 2	SPON2	15.33	NM_ 012445	8098870
sodium ion transport	solute carrier family 10	SLC10A2	12.97	NM_ 000452	7972692

	polymeric immunoglobulin receptor	PIGR	11.75	NM_ 002644	7923929
	adenylate cyclase 5	ADCY5	11.55	NM_ 183357	8090070
	transmembrane protein 176A	TMEM176A	11.50	NM_ 018487	8137264
	aldehyde dehydrogenase 1 family, member A1	ALDH1A1	11.30	NM_ 000689	8161755
	transmembrane protease, serine 4	TMPRSS4	9.44	NM_ 019894	7944164
	leucine-rich repeat kinase 2	LRRK2	8.57	NM_ 198578	7954810
	aquaporin 1	AQP1	8.21	NM_ 198098	8132118
	solute carrier family 17 , member 1	SLC17A1	7.91	NM_ 005074	8124337
	V-set domain containing T cell activation inhibitor 1	VTCN1	7.24	NM_ 024626	7918936
	solute carrier family 27	SLC27A2	6.92	NM_ 003645	7983650
	solute carrier family 34, member 2	SLC34A2	6.90	NM_ 006424	8094441
ECM	mucin 20	MUC20	6.60	NM_ 152673	8084895
	acyl-CoA synthetase medium-chain family member 3	ACSM3	6.41	NM_ 005622	7993756
	aldo-keto reductase family 1, member B10	AKR1B10	6.19	NM_ 020299	8136336
	phospholipase A1 member A	PLA1A	6.13	NM_ 015900	8081890
	complement component 4A	C4A	6.09	NM_ 007293	8118409

Down-regulated

Gene category	*Gene name*	*Gene Symbol*	*Fold-Change*	*RefSeq*	*Probeset ID*
	solute carrier family 14	SLC14A1	-16.06	NM_00 1128588	8021081
	serpin peptidase inhibitor	SERPINE1	-11.15	NM_ 000602	8135069
	Thy-1 cell surface	THY1	-6.99	NM_	7952268

	antigen			006288	
	filaggrin	FLG	-6.82	NM_ 002016	7920165
	lysyl oxidase	LOX	-6.17	NM_ 002317	8113709
	cadherin 13, H-cadherin	CDH13	-5.41	NM_ 001257	7997504
	metallothionein 1L	MT1L	-5.25	NR_ 001447	7995793
	metallothionein 1A	MT1A	-5.20	NM_ 005946	7995806
	plasminogen activator, tissue	PLAT	-4.90	NM_ 000930	8150509
	semaphorin 7A	SEMA7A	-4.89	NM_ 003612	7990345
	serpin peptidase inhibitor, clade B	SERPINB7	-4.88	NM_ 003784	8021623
	DNA-damage-inducible transcript 4	DDIT4	-4.85	NM_ 019058	7928308
	small nucleolar RNA, C/D box 30	SNORD30	-4.67	NR_ 002561	7948900
	cyclin A2	CCNA2	-4.66	NM_ 001237	8102643
	histone cluster 1, H2bm	HIST1H2BM	-4.63	NM_ 003521	8117594
	small nucleolar RNA, C/D box 25	SNORD25	-4.57	NR_ 002565	7948910
	sema domain, immunoglobulin domain (Ig)	SEMA3A	-4.53	NM_ 006080	8140668
	small nucleolar RNA, C/D box 74	SNORD74	-4.50	NR_ 002579	7922418
	kynureninase (L-kynurenine hydrolase)	KYNU	-4.47	NM_ 003937	8045539
	cyclin B2	CCNB2	-4.45	NM_ 004701	7983969

To infer the function of the 825 differentially expressed genes, the Gene Ontology (GO) enrichment analysis tool and DAVID were used. Up and down regulated genes in hKEpC spheroids were categorized into cellular processes, according to Partek (Figure 10C) and DAVID (Table 3), showing the most significantly elevated genes to group into cell-cell adhesion/ECM/cell recognition, ion transport, regulation of

cellular component biogenesis, while down-regulated genes were related to cell growth/mitosis/cell cycle and cell locomotion.

Table 3

Up-regulated

	Count
cell adhesion	23
sodium ion transport	8
cell-cell adhesion	10
anion transport	7
cell motion	13
response to hormone stimulus	11
phosphate transport	3
regulation of cell motion	7
cell migration	8
extracellular structure organization	6
cellular aldehyde metabolic process	3
cell motility	8
cell morphogenesis involved in differentiation	7
regulation of cellular localization	7

Down-regulated

	Count
M phase	47
cell cycle	67
cell cycle phase	51
cell cycle process	57
mitosis	37
mitotic cell cycle	44
cell division	38
chromosome organization	26
cell proliferation	23
meiosis	9
blood vessel morphogenesis	13
blood vessel development	14
vasculature development	14
chromatin organization	14
regulation of locomotion	9
regulation of cell motion	9
cell migration	11
regulation of cell migration	8
cell motion	15

growth	8
localization of cell	11
cell motility	11

Table 4 further elaborates 23 genes categorized in biological adhesion, which were up-regulated in spheroid cells (DAVID, $p<0.00001$).

Table 4

Cell and biological adhesion

Symbol	*Gene name*	*Gene assignment*	*Fold change*
CLDN7	claudin 7	Involved in the formation of tight junctions between epithelial cells	2.74
PCDHB5	Protocadherin beta-5	Member of the protocadherin beta gene cluster	3.32
CLDN3	Claudin 3	Member of the claudin family, is an integral membrane protein and a component of tight junction strands.	3.93
CNTN6	contactin 6	Contactins mediate cell surface interactions during nervous system development. Participates in oligodendrocytes generation by acting as a ligand of NOTCH1.	3.09
PKHD1	polycystic kidney and hepatic disease 1	Localized predominantly at the apical domain of polarized epithelial cells, suggesting it may be involved in the tubulogenesis and/or maintenance of duct-lumen architecture.	3.38
PCDHB2	protocadherin beta 2	The extracellular domains interact in a homophilic manner to	2.94

		specify differential cell-cell connections.	
CDH1	E-cadherin (epithelial)	cell adhesion molecule	3.27
CX3CL1	hemokine (C-X3-C motif) ligand 1	CX3CL1 elicits its adhesive and migratory functions by interacting with the chemokine receptor CX3CR1	2.86
CXADR	coxsackie virus and adenovirus receptor		2.95
BCL2L11	BCL2-like 11 (apoptosis facilitator)		2.42
SEMA5A	sema domain seven thrombospondin repeats		4.01
PVRL4	poliovirus receptor -related 4	Involved in cell adhesion through trans-homophilic and -heterophilic interactions. It is a single-pass type I membrane protein.	3.18
ARVCF	Armadillo repeat protein deleted in velo-cardio-facial syndrome	Member of the catenin family which play an important role in the formation of adherens junction complexes, which are thought to facilitate communication between the inside and outside environments of a cell.	2.22
CDH16	cadherin 16, KSP-cadherin	cell adhesion molecule	2.99
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Essential component of the renin-angiotensin system (RAS)	5.67
ITGB6	integrin beta 6	Integrin alpha-V/beta-6 is a receptor for fibronectin and cytotactin.	3.41

VNN1	vanin 1	May play a role in oxidative-stress response	4.79
RHOB	ras homolog gene family member B	Mediates apoptosis in neoplastically transformed cells after DNA damage. Affects cell adhesion and growth factor signaling in transformed cells.	2.58
CNTN4	contactin 4	Member of the immunoglobulin superfamily. It is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule.	2.61
GPNMB	glycoprotein (transmembrane) nmb	transmembrane glycoprotein	6.03
SPON2	Spondin 2	Extracellular matrix protein	15.33
CHL1	cell adhesion molecule with homology to L1CAM	cell adhesion molecule	4.58
CEA CAM1	carcinoembryonic antigen-related cell adhesion molecule	cell adhesion molecule	2.07

Thus, hKEpC spheroids generated a quiescent niche enriched in cell-cell and cell matrix interactions. The quiescent nature of spheroids was confirmed by analysis of proliferating cells in whole spheroids fixed and embedded in paraffin and stained for hematoxylin and eosin (Figure 11A) and for the cell proliferation marker, Ki-67 (Figure 11B). All hKEpC spheroids exhibited a low proliferation index of <10% of Ki-67-positive cells per spheroid/section, indicative of the quiescent nature of the spheroids.

EXAMPLE 4

Heterogeneous hAK cells cultured in fetal kidney conditioned media show enhanced clonogenicity

Following the observation that heterogeneous cultures of kidney epithelial cells maintain the ability to form spheres, the present inventors analyzed culture conditions that enhance cells' clonogenic capacities and would allow for clonal analysis of hAK cell subpopulations. Cells were plated in limiting dilution (LD) concentrations under 4 different growth conditions: a) SCM, b) SFM, c) culture expanded in SCM and LD analysis performed in SFM, d) SCM in 1:1 ratio with fetal kidney conditioned media (FKCM). Analysis of the number of colonized wells, recorded after 4 weeks, showed that SCM promotes higher clonogenic capacities compared to SFM. This was especially evident when combined with FKCM (condition d) (Figures 12A-C). FKCM clones showed improved viability and confluence as opposed to SCM (Figures 13A-D). Clonogenic expansion indicative of self-renewal could be performed for more than 11 passages.

EXAMPLE 5

NCAM1 expressing cells isolated from heterogeneous hAK cultures are highly clonogenic and preferentially form spheres

Following elucidation of various culture conditions that allow for enhanced expression of the renal progenitor genes, clonogenic capabilities and nephrospheroid formation the present inventors determined surface markers that could identify cells within the heterogeneous hAK cultures preferentially exhibiting these characteristics. Accordingly, cell subpopulations positive for surface markers which have been shown to mark the renal progenitor population of the developing human kidney such as NCAM1 and FZD7 (10, 11) were sorted. NCAM1 which during nephrogenesis is localized to cells of the MM and its early derivatives, including condensed mesenchyme and early nephron, is not expressed in the adult kidney in vivo (11, 17, 18). Efficient fractionation of NCAM+ cells was achieved with FACS sorting (Figures 14A-C) and to a lesser extent via microbeads. Analysis of renal 'stemness' genes in NCAM+ cells compared to NCAM- fraction obtained from heterogeneous cultures of five different

hAK revealed overexpression of the early renal epithelial progenitor markers (*Six2, Osr1, Sall1, Pax2* and *Wt1*) and early surface antigens (*FZD7, AVR2b*) (11), polycomb group (*Bmi-1, Ezh2*), Wnt pathway (*Beta-catenin, FZD7*) as well the pluripotency marker, *Oct4* (Figures 15A-E). Analysis for renal differentiation markers revealed elevated aminopeptidase (ENPAP) and aquaporin1 (AQP1) and low Na/Cl co-transporter (NCCT) and aquaporin3 (AQP3) as well as high vimentin and low E-cadherin, all indicating NCAM+ cells to originate from the proximal tubule (Figures 15A-E)

Having identified the unique clonogenic ability of hAK cells cultured with FKCM, the present inventors next examined both the ability of NCAM+ and NCAM- cells to form single cell clones under these conditions as well as their proliferative capacity in culture. Experiments performed on cultures originating from 3 different kidneys revealed the NCAM+ fraction to be highly clonogenic in all instances (Figures 16A-C). Although highly clonogenic, NCAM+ cells were found to be less proliferative on MTS proliferation assay compared to the negative counterpart (Figure 16D). Sphere-forming activity in the NCAM+ cell population sorted from heterogeneous hAK cultures was then assessed. Strikingly, immediately after sorting of low-passage cultures, only NCAM+ cells generated spheres while the NCAM- fraction was devoid of this capacity. Short-term expansion of NCAM+ cells after sorting resulted in the appearance of spheres in the NCAM- population; nevertheless, well-defined spheres were exclusively observed in the NCAM+ cells (Figures 16E-J). Analysis of sorted populations from high-passage cultures showed both the NCAM+ and NCAM- fractions to possess sphere-forming abilities (data not shown)). Accordingly, while well-defined spheres generated from NCAM+ cells in low-passage cultures demonstrated enhanced expression of the renal progenitor genes compared to spheres formed by NCAM- cells and also to an adherent NCAM+ fraction, in high-passage cultures both NCAM+ and NCAM- spheres showed similar elevated gene levels when compared to adherent NCAM+ cells (data not shown). Thus, NCAM strongly enriched for sphere-forming capability in low-passage heterogeneous cultures. In high-passage cultures, sphere-formation irrespective of NCAM expression enriches for the renal progenitor genes.

EXAMPLE 6

In vivo generation of human tubular structures in the chick embryo by nephrospheroids and NCAM+ cells

Having determined that hKEpC spheroids have enhanced renal "stemness" profile and recapitulate a microenvironment rich in ECM and cell contact molecules the present invnentors tested whether this leads to improved functional potency to generate renal structures. Accordingly, human cell grafting was performed onto the chorioallantoic membrane (CAM) of the chick embryo and their fate 7 days post-implantation was analyzed (Figure 17A-k). Chick embryos were grafted with either whole hKEpC spheroids, or single cell suspensions of dissociated spheroid-cells (immediately after disassociation) and monolayer hKEpC. The suspended cells are especially important as they represent an injectable form of cells. Implantation of whole human kidney-spheroids onto the CAM resulted in tubule formation (Figure 17G). Comparison of single cell implantation of spheroid and monolayer hKEpC demonstrated that grafts generated from spheroid cells were much bigger then their counterparts (Figures 17A-B). H&E staining revealed robust tubule formation capacity by spheroid-cells, e.g. 0.43 X 10^6 spheroid cells induced formation of multiple tubular structures, while few tubuli were observed in grafts generated by similar numbers of monolayer hKEpC (Figures 17C-D). Additional experiments were performed to determine whether hKEpC spheroids generated after long-term expansion of monolayer cells (P6) can recapitulate tubule formation. 0.43 X 10^6 dissociated single spheroid cells grafted onto the CAM reconstituted tubular structures, while long-term expanded monolayer hKEpC (P6) completely failed to generate similar structures (Figures 17E-F). Importantly, grafting of control cell types, mesenchymal stem cells (MSCs) and human embryonic kidney cell line (HEK293) generated disorganized cell masses (Figures 17H-I), indicating that only kidney-derived cells bear nephrogenic potential in this model. Interestingly, when using human fetal kidney (FK) cells as additional controls no tubular formation was observed after grafting of 1.25x10^6 FK cells (Figure 17J), while grafting of 2.5x10^6 FK cells (Figure 17K) showed tubular formation. Therefore much fewer (0.43 x10^6) adult kidney spheroid cells generate tubular structures further emphasizing there high tubulogenic capacity.

To better define the tubular structures that were formed by dissociated spheroid hKEpC, graft sections were stained for segment-specific tubular markers (LTA, proximal; THG, distal; DBA, distal/collecting). It was found that reconstituted renal structures showed LTA, THG and DBA positive tubules and were reminiscent of a wide adult human tubular spectrum (Figures 18A-D). To clarify specificity of DBA expression immunofluorescent staining was performed and DBA(+) tubules were found to comprise a portion of the reconstituted tubules (Figure 18D). Spheroid-cells obtained from high-passage cultures also showed more than one type of differentiated tubules with positive staining of the THG and DBA markers and to a much lesser extent LTA staining (Figures 19A-C). Thus, hKEpC spheroids enhance functional potency for tubule formation.

The regenerative ability of NCAM+ sorted, adherent cells was also analyzed. In this experiment, strong tubular reconstitution by 0.43 X 10^6 $NCAM^+$ cells was observed with the NCAM- fraction failing to form similar structures (Figures 20A-F). Thus, low numbers of both spheroid- and sorted NCAM+ cells can recapitulate kidney structures *in vivo* indicative of high renal potential.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

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WHAT IS CLAIMED IS:

1. A method of generating a nephrospheroid, the method comprising culturing human adult kidney cells in a culture medium under non-adherent conditions, thereby generating the nephrospheroid.

2. The method of claim 1, further comprising dispersing said human adult kidney cells prior to culturing.

3. The method of claim 1, wherein said medium further comprises epidermal growth factor (EGF) and fibroblast growth factor (FGF).

4. The method of claim 3, wherein said medium further comprises insulin and progesterone.

5. The method of claim 1, wherein said medium is devoid of serum.

6. The method of claim 1, wherein said medium comprises serum.

7. The method of claim 1, further comprising expanding human adult kidney cells in a culture medium under adherent conditions prior to said culturing.

8. The method of claim 7, wherein said culture medium comprises serum.

9. An isolated nephrospheroid comprising human adult kidney cells.

10. The isolated nephrospheroid of claim 9, characterized by enhanced expression of at least one polypeptide selected from the group consisting of sal1, pax2, six2 and WT1 as compared to said adult kidney cells grown under adherent conditions.

11. The isolated nephrospheroid of claim 9, characterized by enhanced expression of each of sal1, pax2, six2 and WT1 as compared to said adult kidney cells grown under adherent conditions.

12. The isolated nephrospheroid of claim 9, having being generated in serum-free medium.

13. The isolated nephrospheroid of claim 9, having being generated in serum-containing medium.

14. An isolated cell population of human adult kidney cells, comprising at least 80 % adult renal stem cells having a NCAM+ signature.

15. A method of isolating human adult renal cells comprising enriching for a subpopulation of renal cells from an adult renal tissue, said subpopulation of renal cells having a NCAM+ signature, wherein said enriching is effected such that at least 80 % of the adult renal cells are of said subpopulation of renal cells.

16. The method of claim 15, wherein said enriching is effected by detecting surface marker expression of NCAM.

17. A method of determining clonogenic potential of an adult renal cell population, the method comprising:

(a) culturing the adult renal cell population in serum-comprising medium and conditioned medium from human fetal kidney cells; and

(b) counting a number of clones formed from the adult renal cells of said population, thereby determining clonogenic potential of an adult renal cell population.

18. A cell culture comprising a culture medium and the isolated cell population of claim 14.

19. The cell culture of claim 18, wherein said cells are seeded on a scaffold.

20. A method of treating a renal damage in a subject in need thereof comprising administering to the damaged kidney of the subject a therapeutically effective amount of the isolated cell population of claim 14, thereby treating the renal disease in the subject.

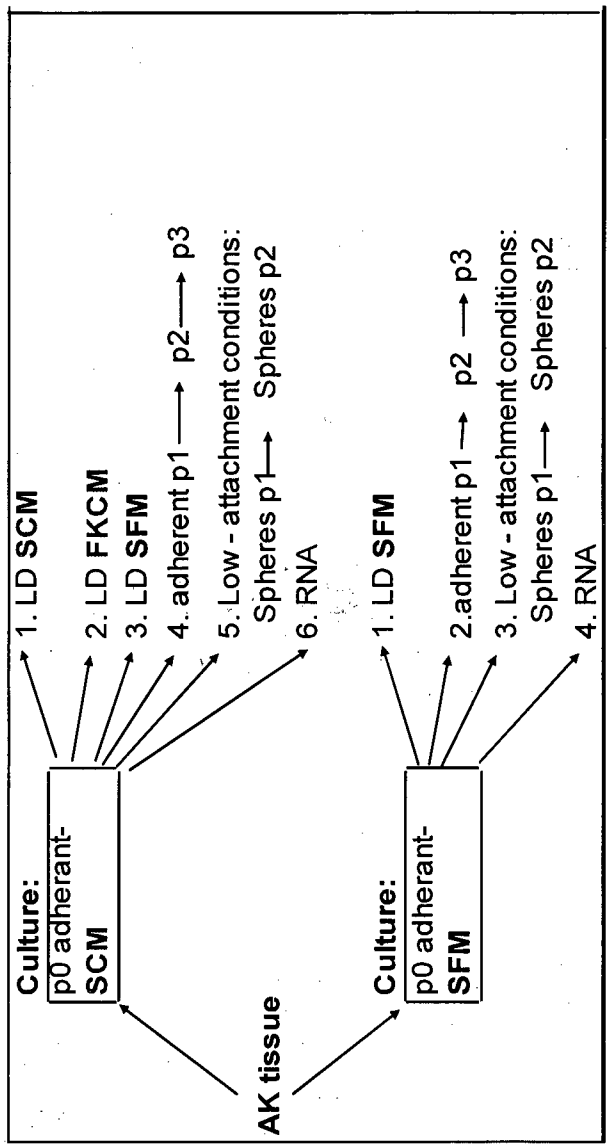
21. A method of identifying an agent capable of regulating differentiation of a renal stem cell, the method comprising contacting the isolated population of cells of claim 14 with an agent, wherein a change in developmental phenotype is indicative of the agent capable of regulating differentiation of said renal stem cells.

22. A method of treating a renal damage in a subject in need thereof comprising administering to the damaged kidney of the subject a therapeutically effective amount of an isolated population of nephrospheroids, said nephrospheroids comprising human adult renal cells, thereby treating the renal disease in the subject.

23. A cell culture comprising a culture medium and an isolated population of nephrospheroids, said nephrospheroids comprising human adult kidney cells.

24. A method of identifying an agent capable of regulating differentiation of a renal stem cell, the method comprising contacting an isolated population of nephrospheroids with an agent, said nephrospheroids comprising human adult kidney cells, wherein a change in developmental phenotype is indicative of the agent capable of regulating differentiation of said renal stem cells.

FIG. 1


AK tissue
Culture:
p0 adherant-
SCM
1. LD SCM
2. LD FKCM
3. LD SFM
4. adherent p1 → p2 → p3
5. Low - attachment conditions:
Spheres p1 → Spheres p2
6. RNA
Culture:
p0 adherant-
SFM
1. LD SFM
2.adherent p1 → p2 → p3
3. Low - attachment conditions:
Spheres p1 → Spheres p2
4. RNA

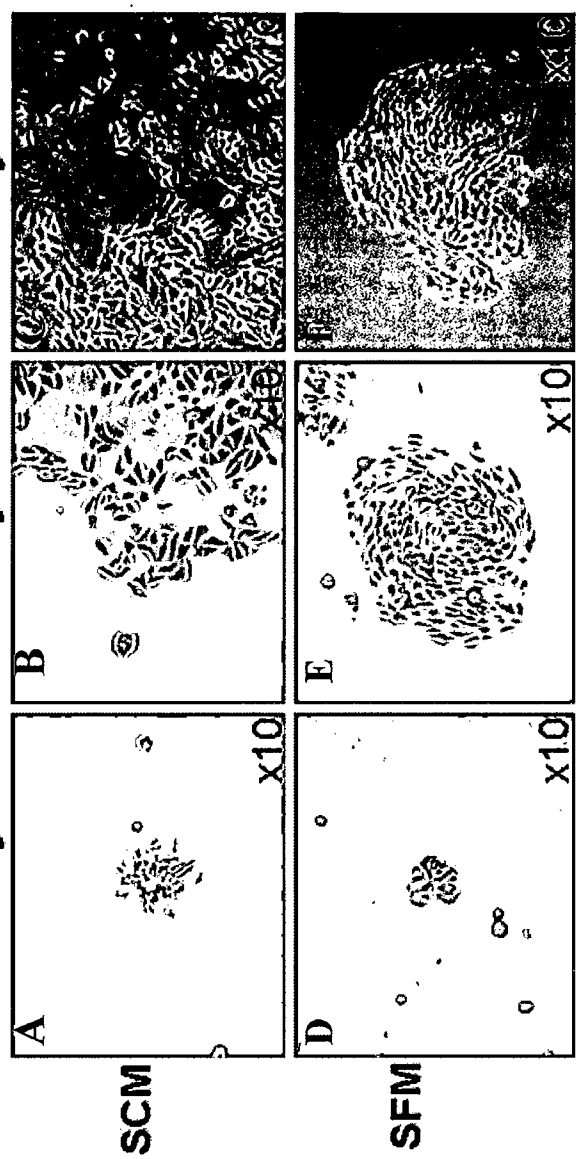


FIG. 2A FIG. 2B FIG. 2C

FIG. 2D FIG. 2E FIG. 2F

FIG. 3A

FIG. 3B

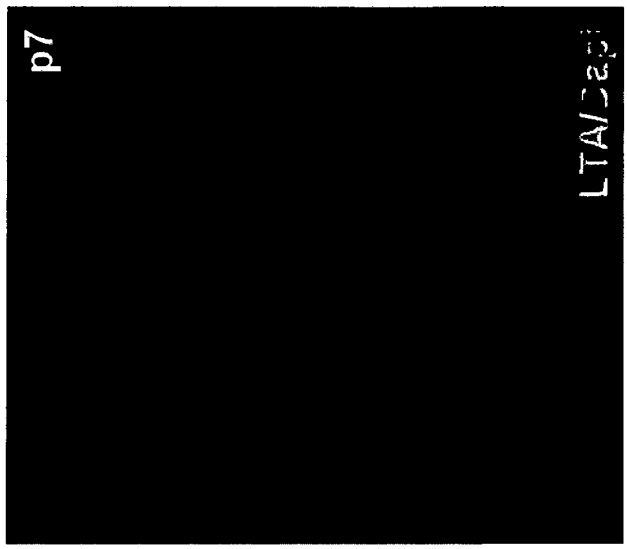


FIG. 3C

FIG. 3D

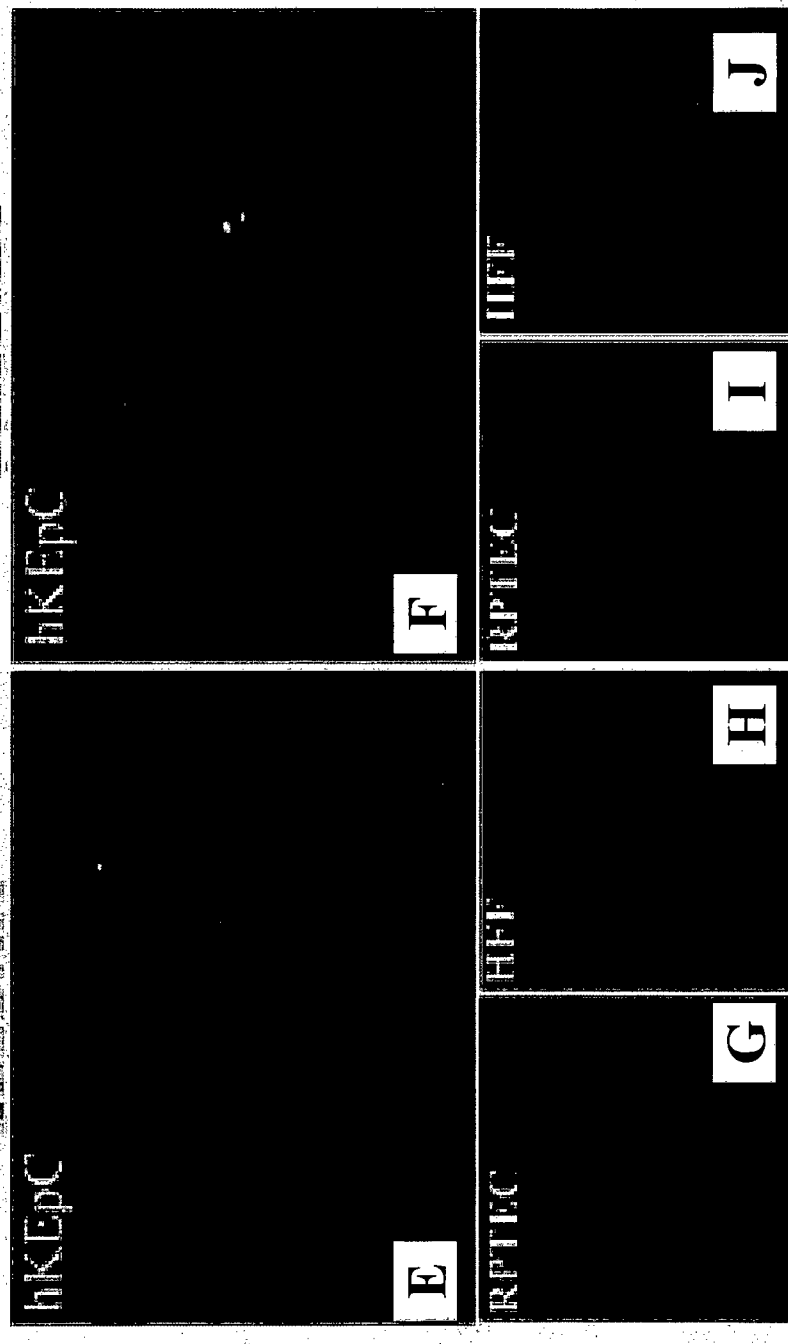


FIG. 3E FIG. 3F FIG. 3G FIG. 3H FIG. 3I FIG. 3J

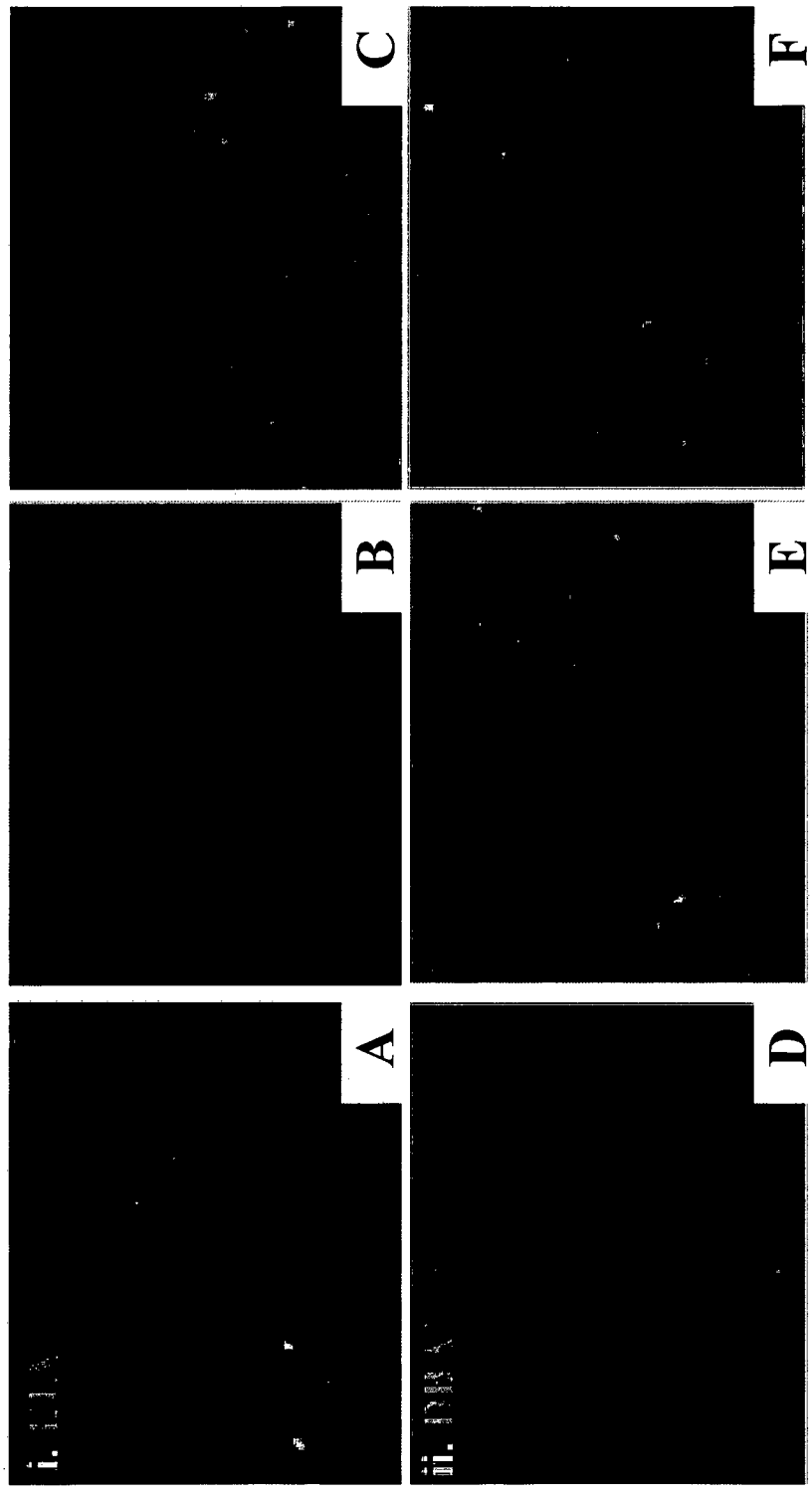


FIG. 4A FIG. 4B FIG. 4C

FIG. 4D FIG. 4E FIG. 4F

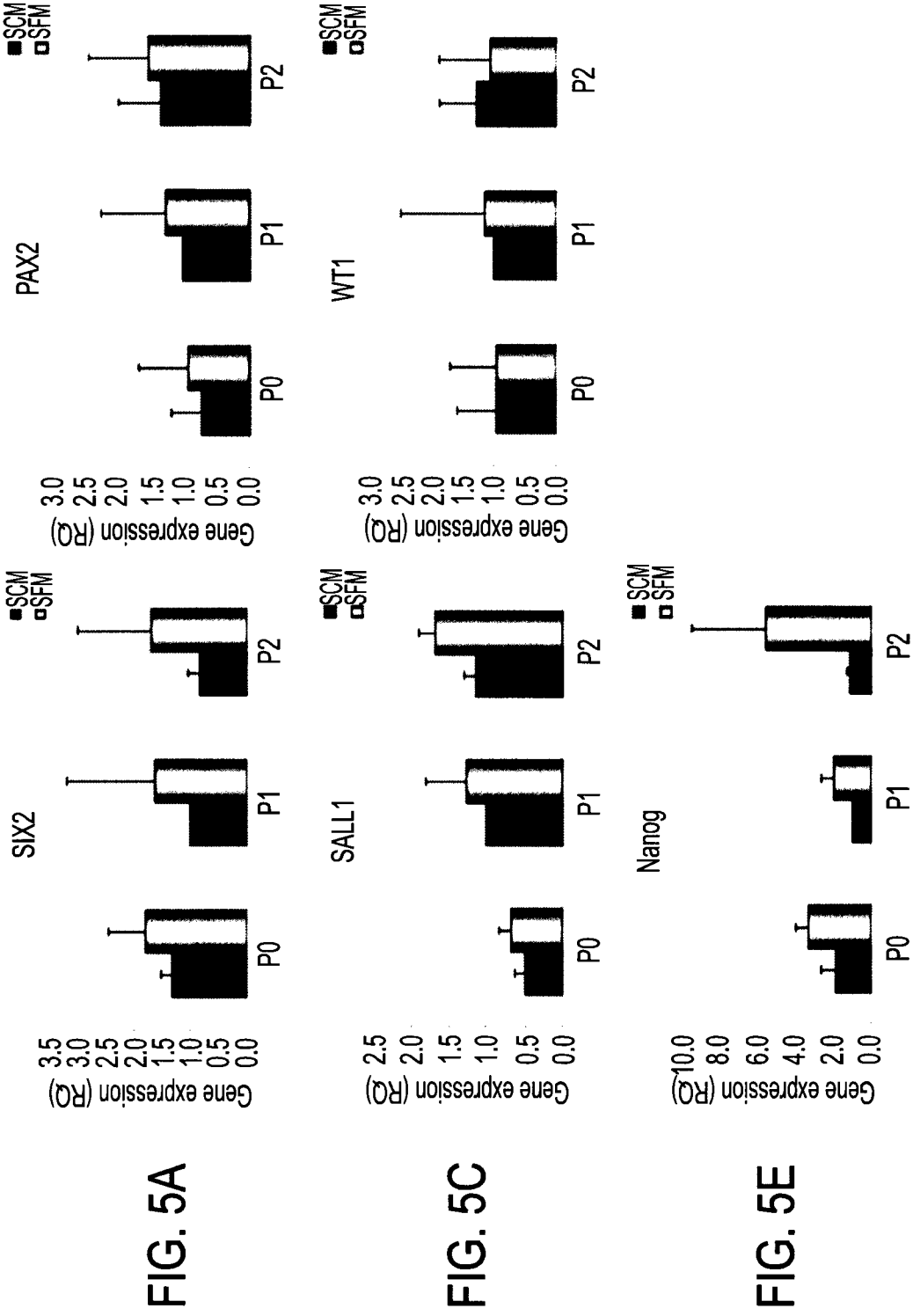


FIG. 5A FIG. 5B FIG. 5C FIG. 5D FIG. 5E

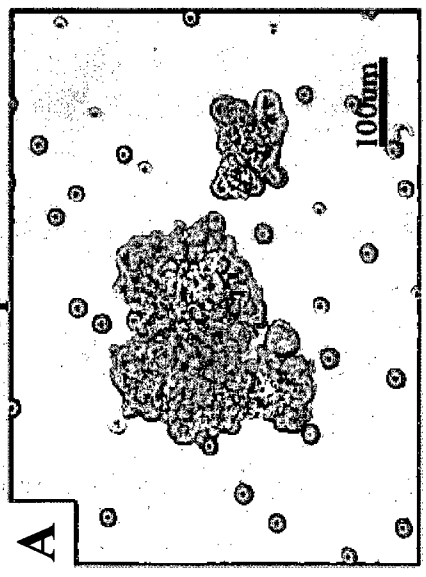


FIG. 6A

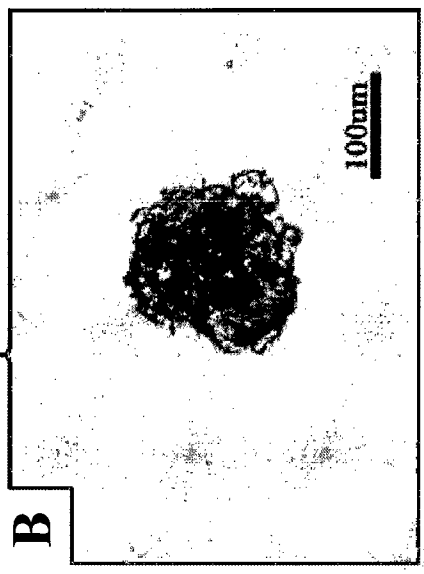


FIG. 6B

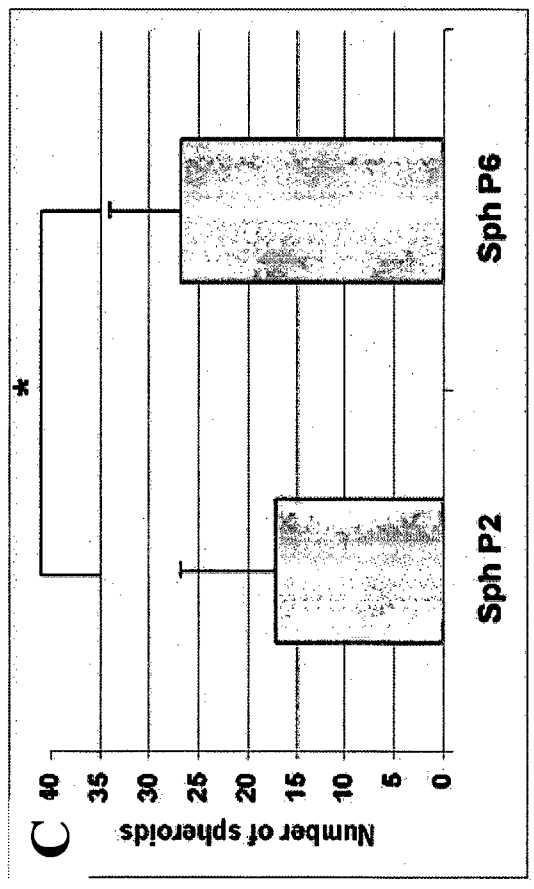


FIG. 6C

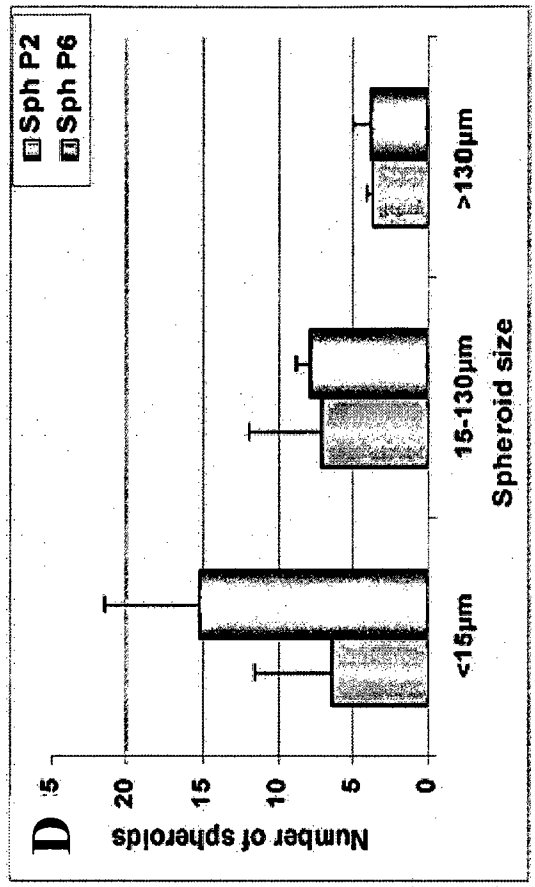


FIG. 6D

FIG. 7

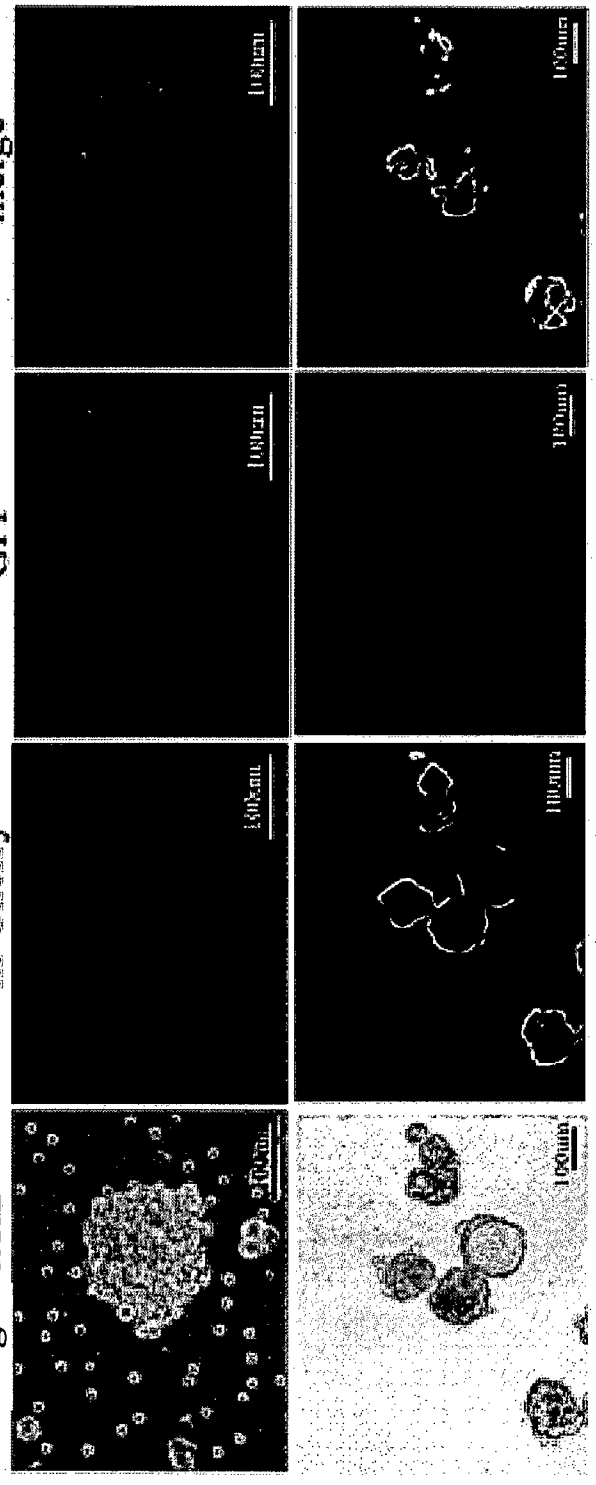


FIG. 8A

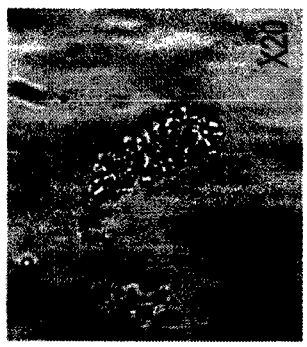


FIG. 8B

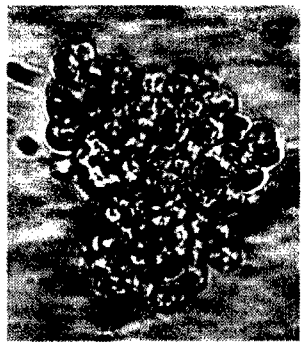


FIG. 8C

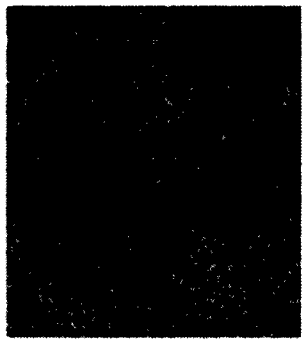
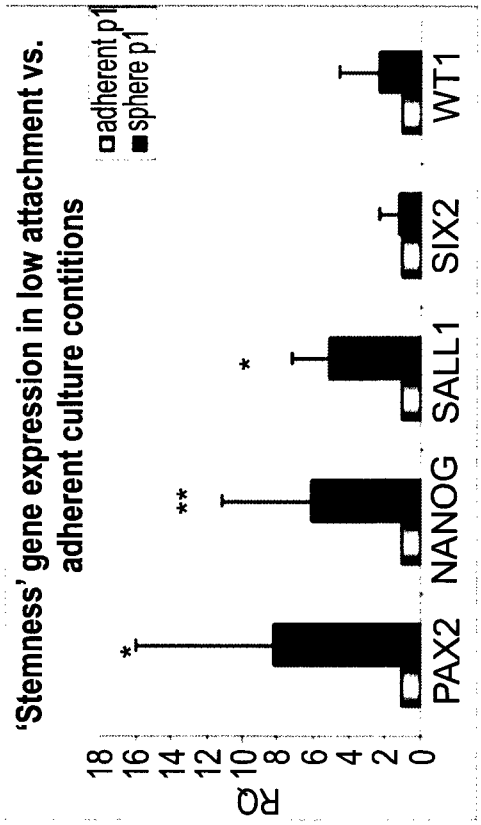


FIG. 8D



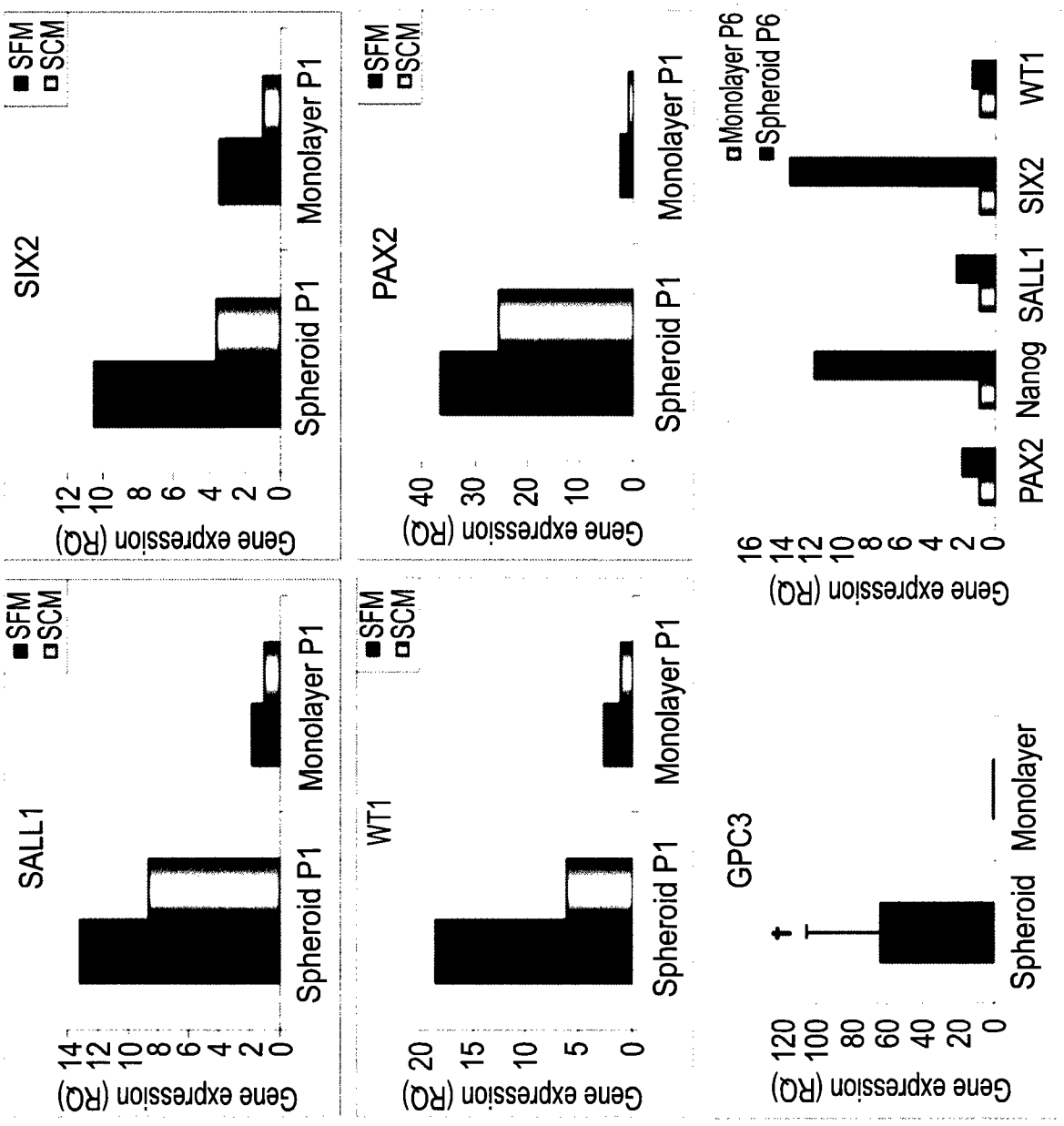


FIG. 8G FIG. 8H FIG. 8J

FIG. 8E FIG. 8F FIG. 8I

FIG. 9A

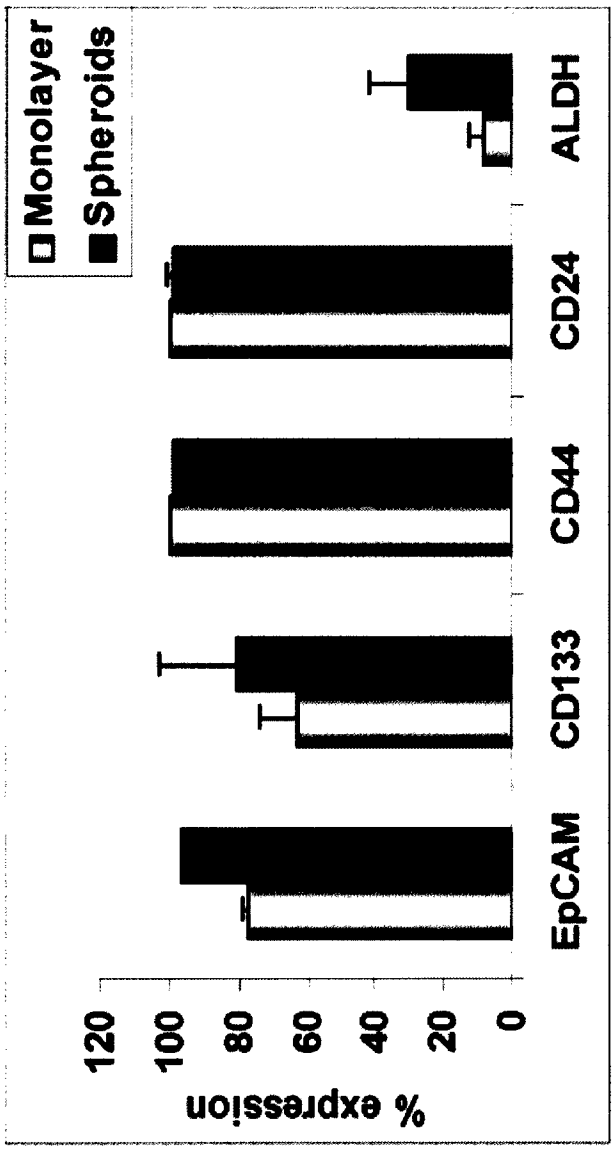
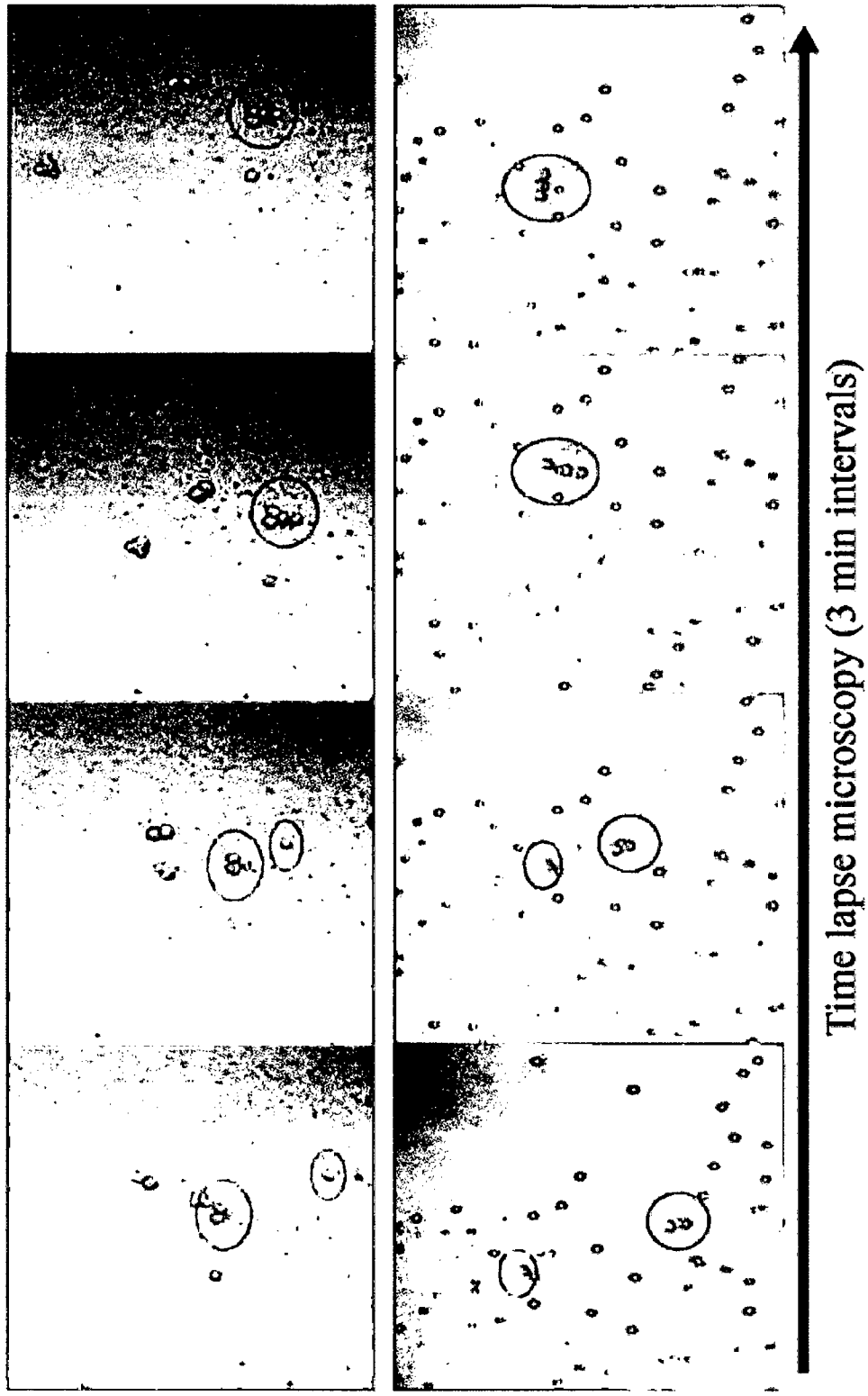

Monolayer
Spheroids
% expression
120
100
80
60
40
20
0
EpCAM
CD133
CD44
CD24
ALDH

FIG. 9B



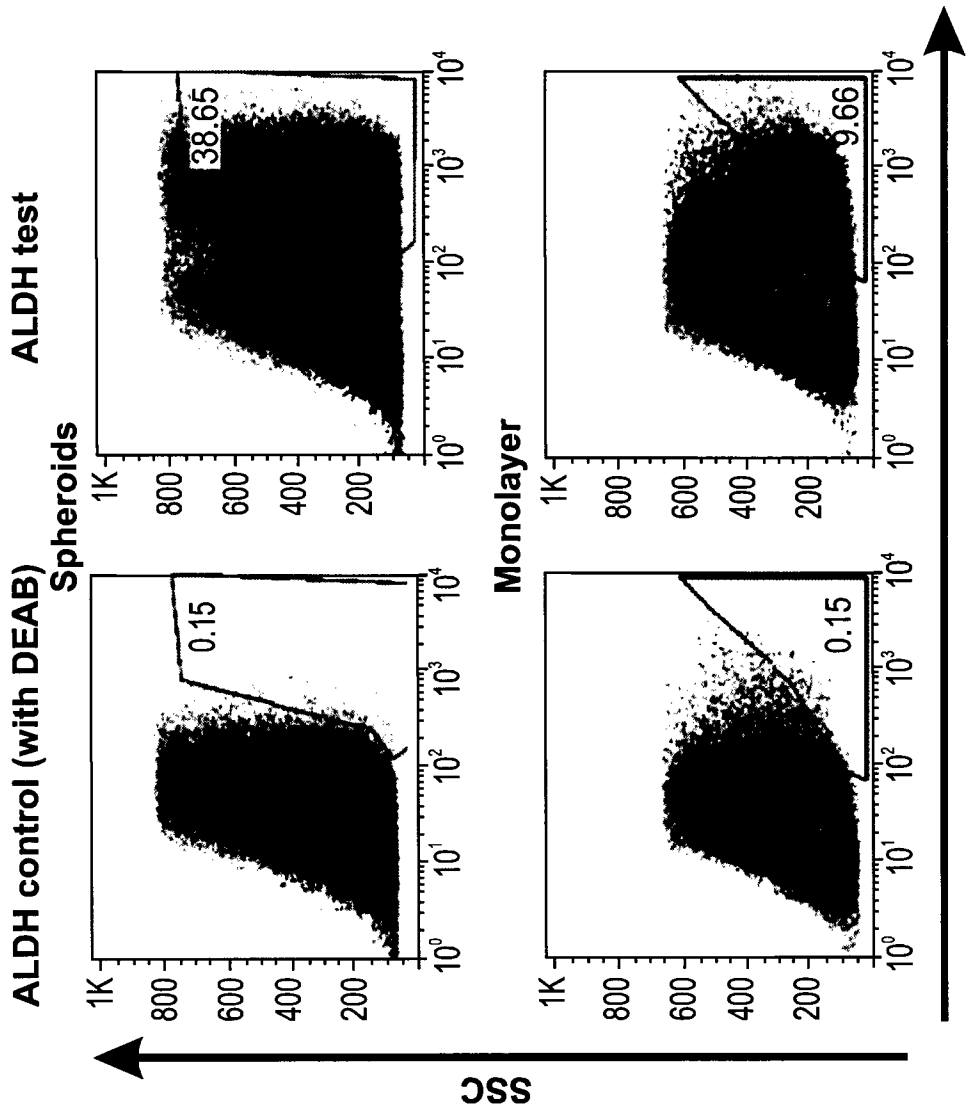
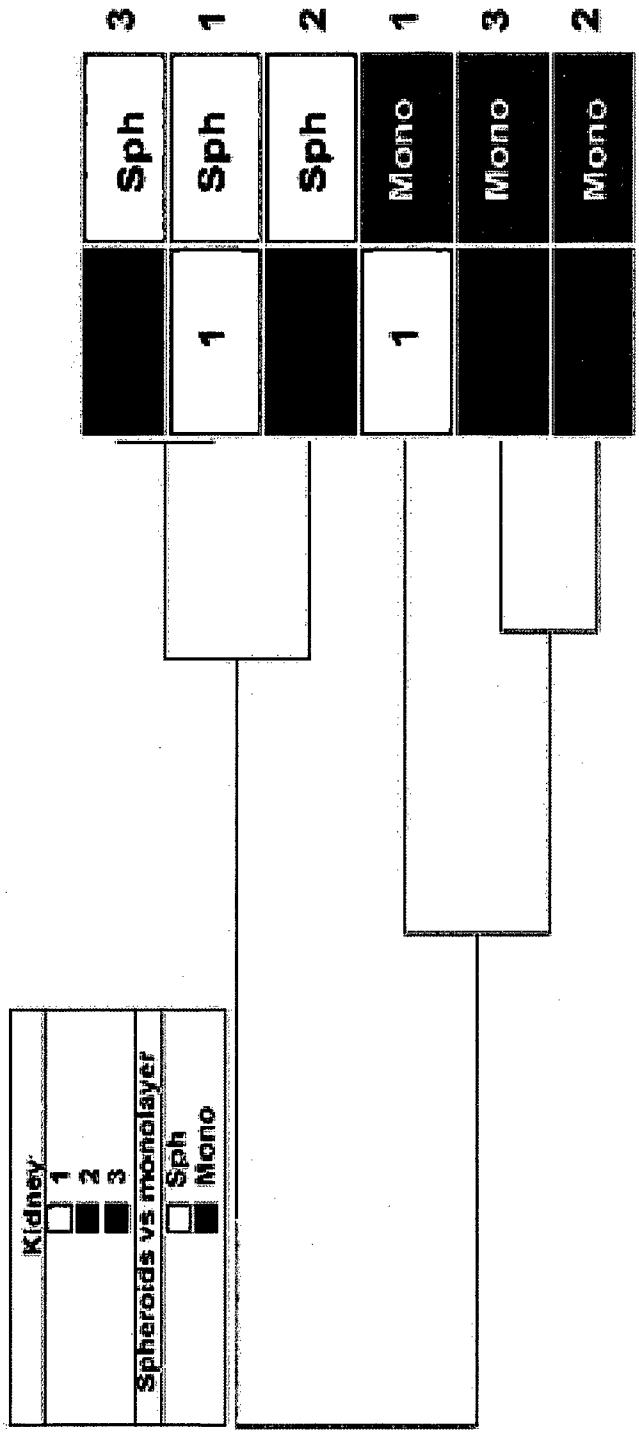

ALDH test
ALDH control (with DEAB)
Spheroids
Monolayer
38.65
9.66
0.15
0.15
SSC
ALDH 1
1K
800
600
400
200
10^0
10^1
10^2
10^3
10^4

FIG. 9C

FIG. 10A


3
1
2
1
3
2
Sph
Sph
Sph
Mono
Mono
Mono
1
1
Kidney
1
2
3
Spheroids vs monolayer
Sph
Mono

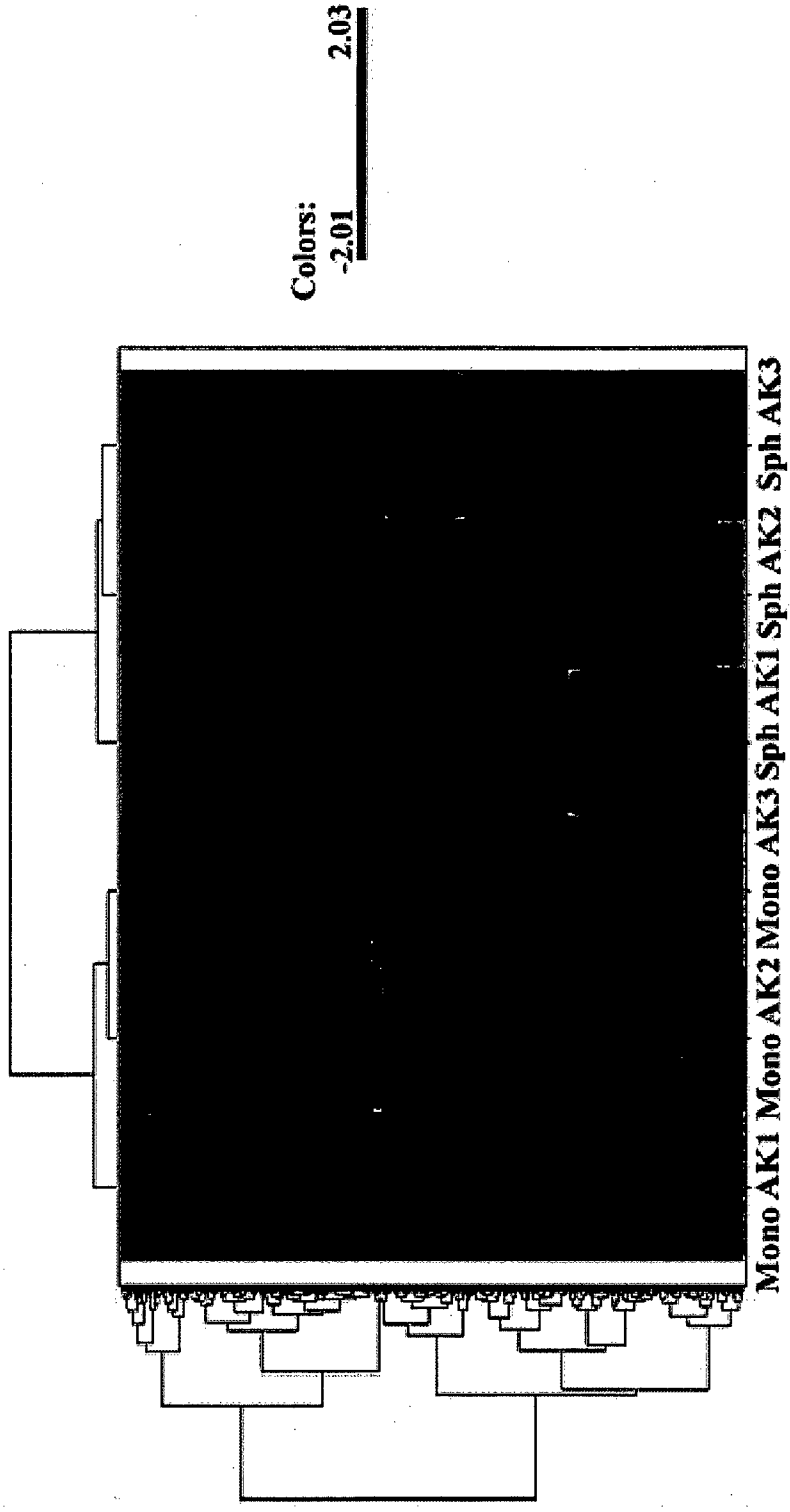

Colors:
2.03
-2.01
Mono AK1
Mono AK2
Mono AK3
Sph AK1
Sph AK2
Sph AK3

FIG. 10B

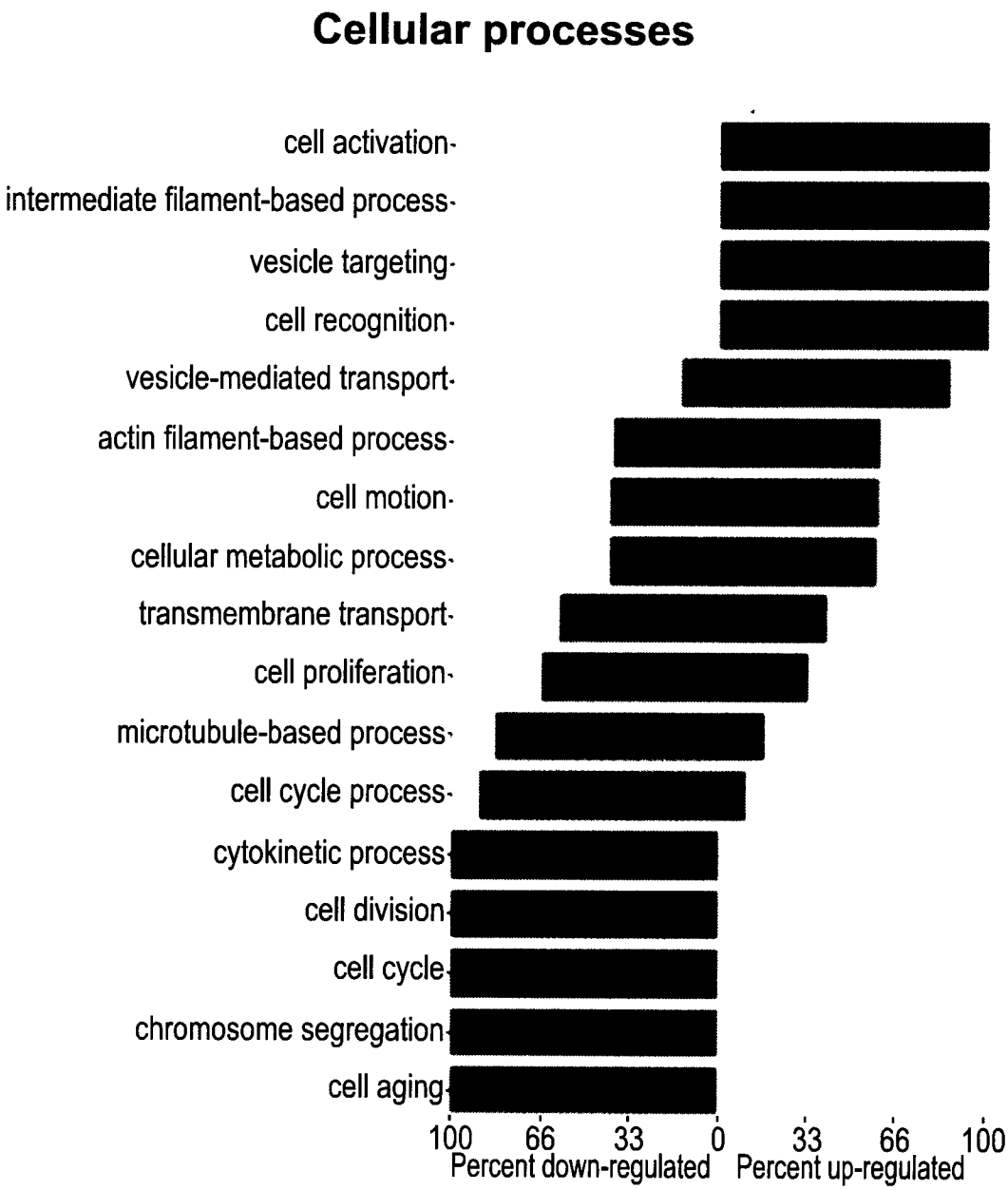

Cellular processes
cell activation
intermediate filament-based process
vesicle targeting
cell recognition
vesicle-mediated transport
actin filament-based process
cell motion
cellular metabolic process
transmembrane transport
cell proliferation
microtubule-based process
cell cycle process
cytokinetic process
cell division
cell cycle
chromosome segregation
cell aging
100 66 33 0 33 66 100
Percent down-regulated
Percent up-regulated

FIG. 10C

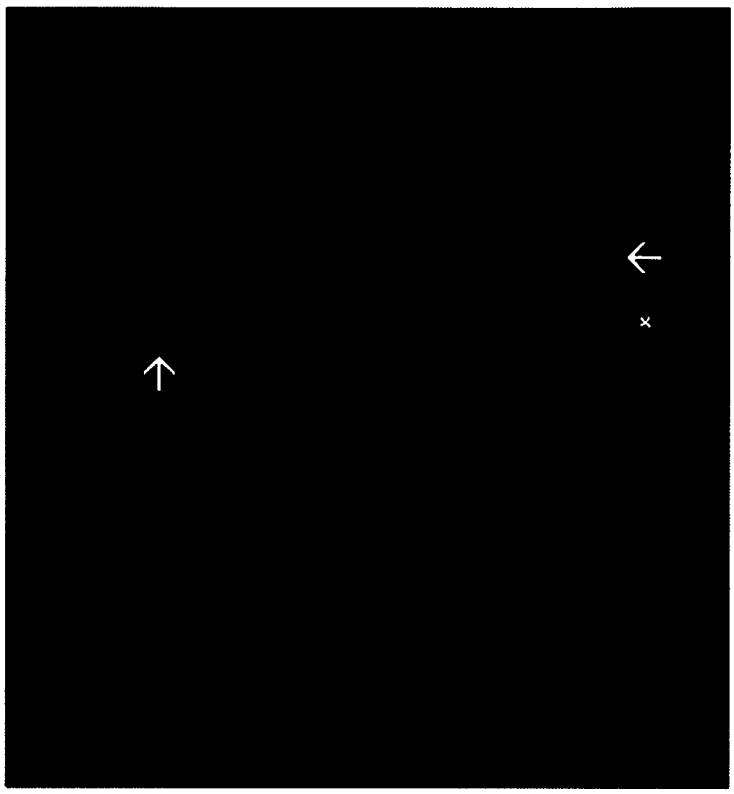


FIG. 11B

FIG. 11A

FIG. 12A

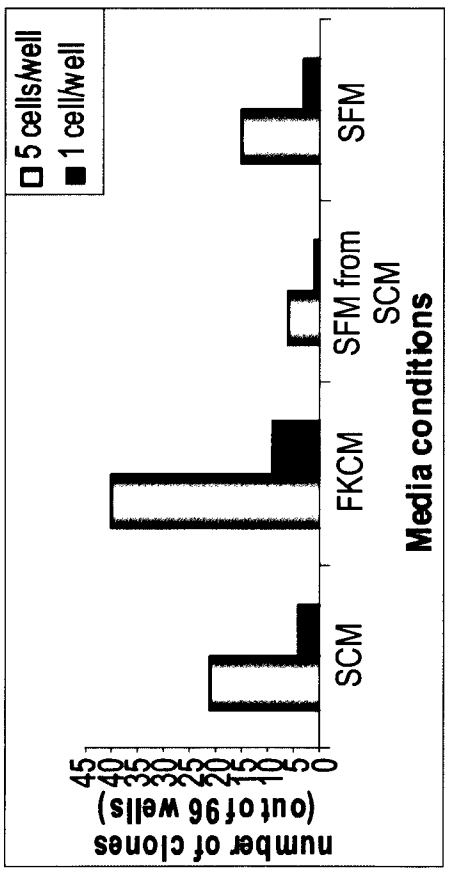


FIG. 12B

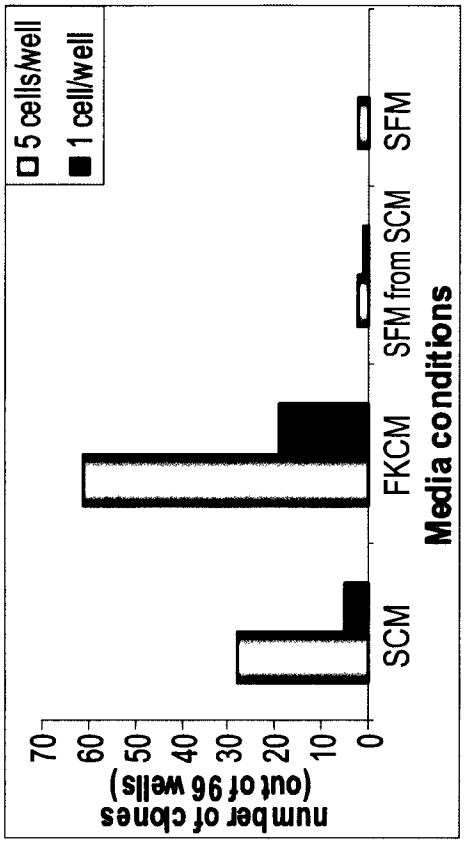
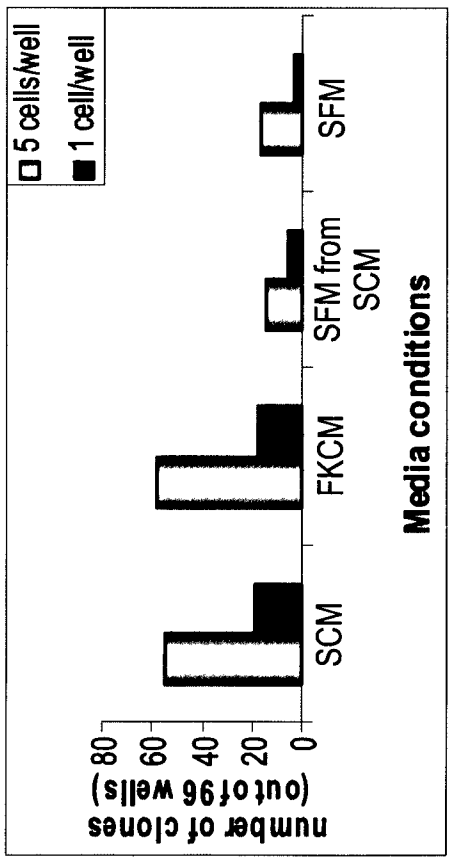


FIG. 12C



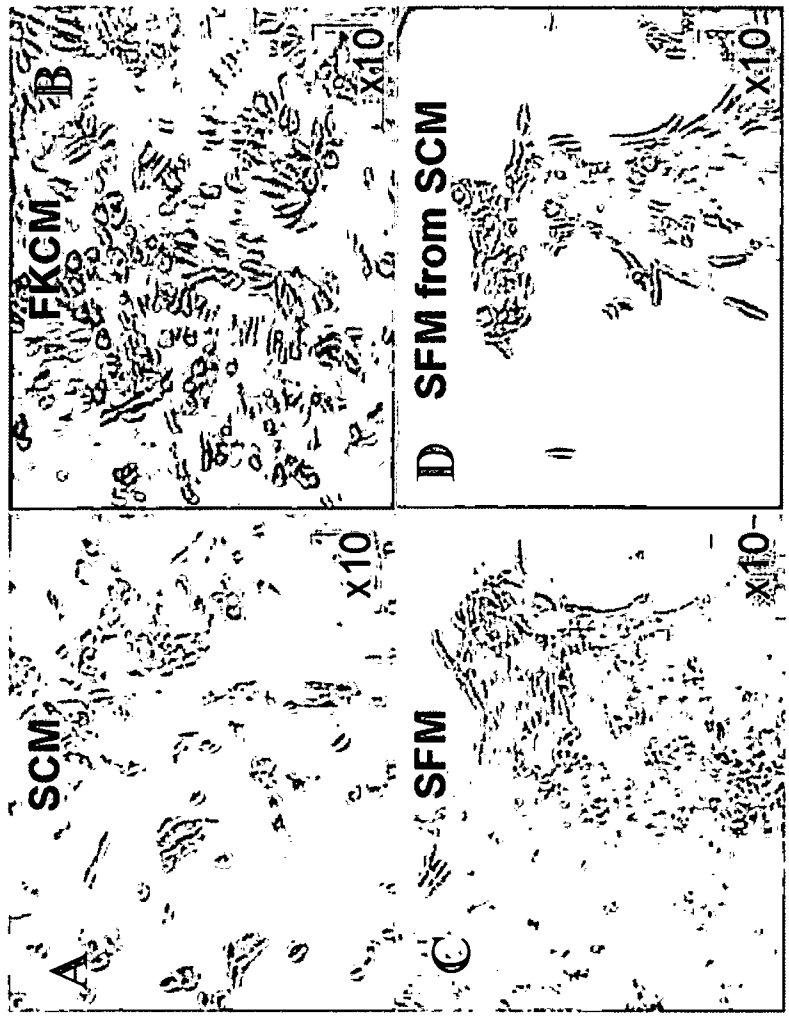


FIG. 13A FIG. 13B

FIG. 13C FIG. 13D

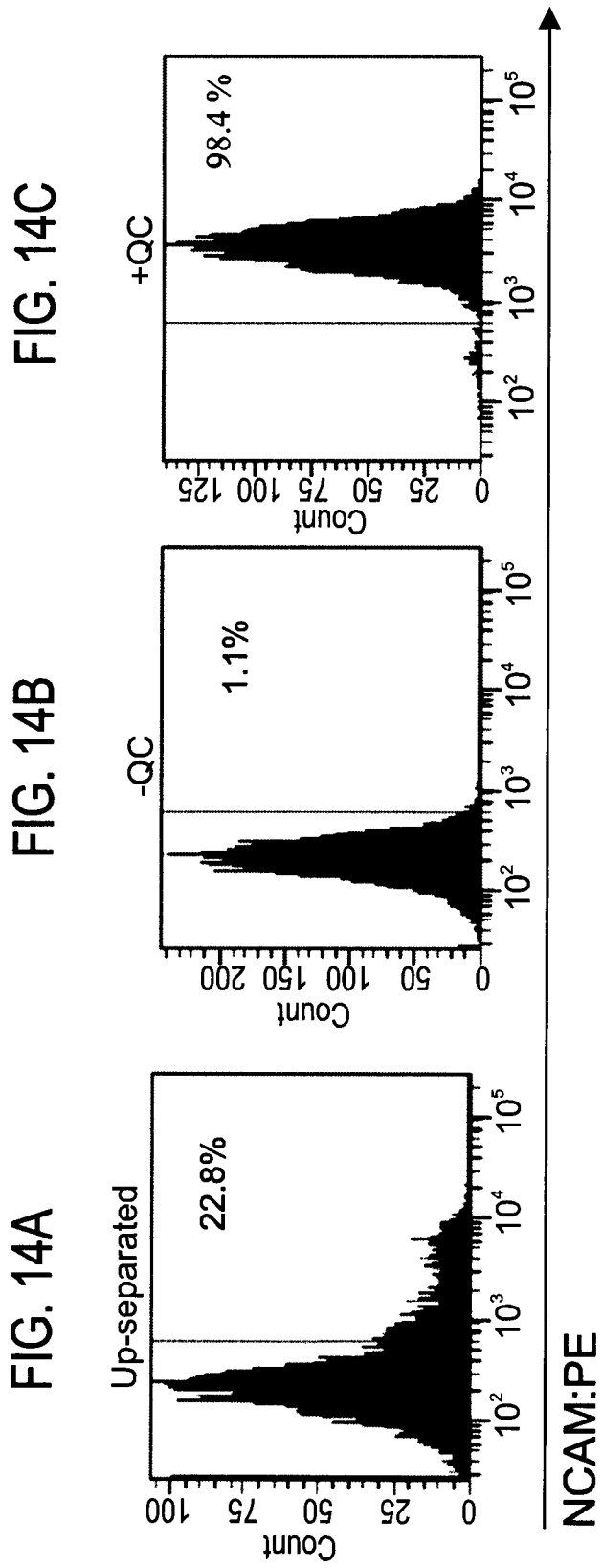


FIG. 14A
FIG. 14B
FIG. 14C
Up-separated
22.8%
-QC
1.1%
+QC
98.4 %
Count
NCAM:PE

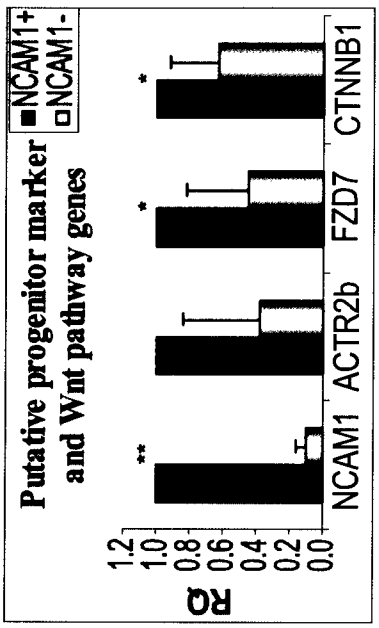


FIG. 15B

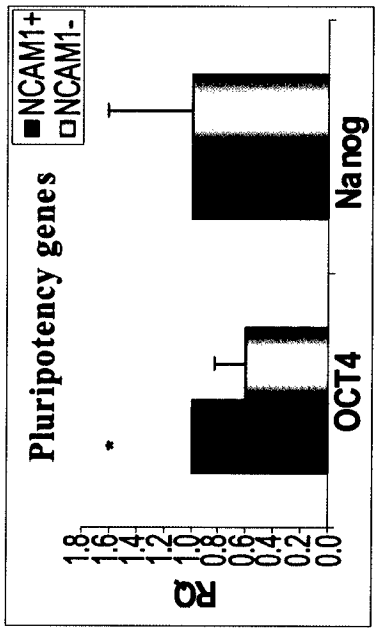


FIG. 15D

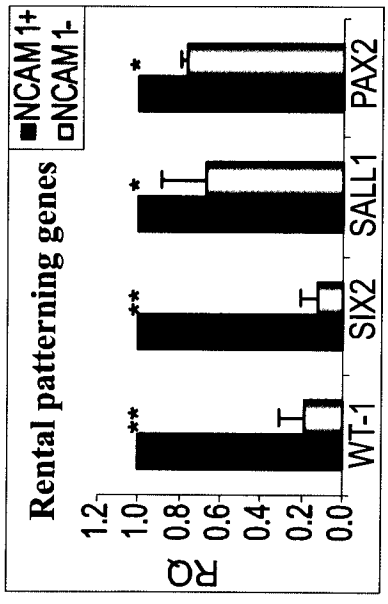


FIG. 15A

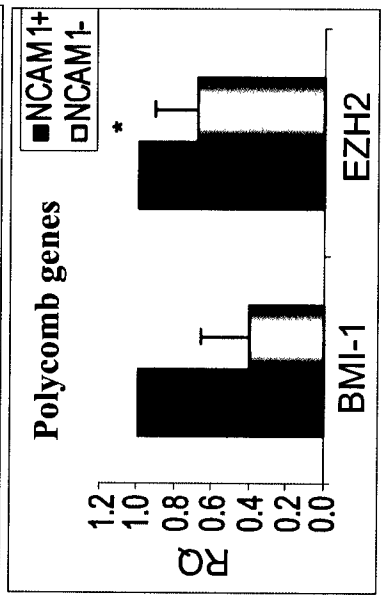


FIG. 15C

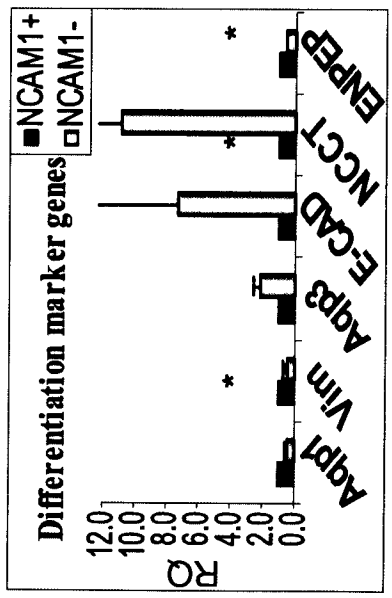


FIG. 15E

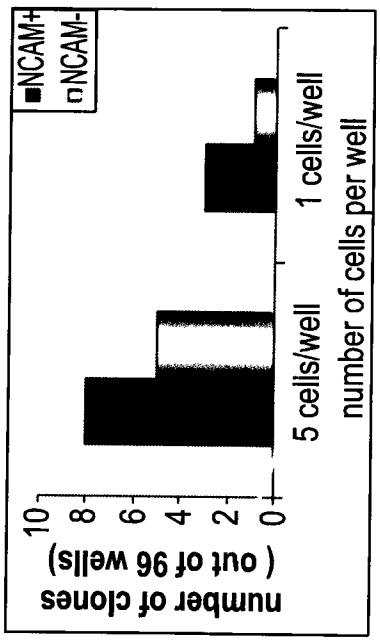


FIG. 16A

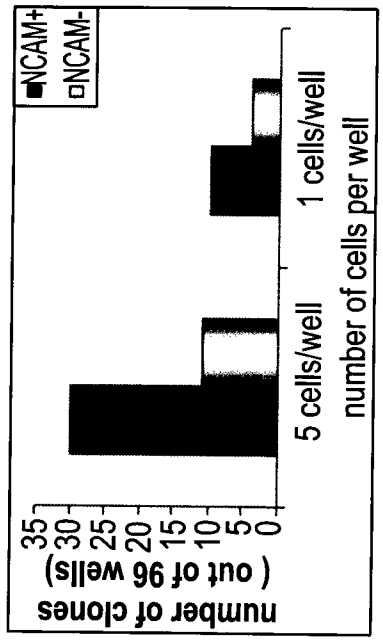


FIG. 16B

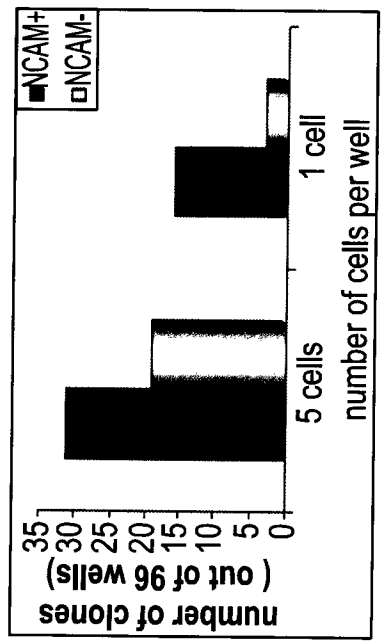


FIG. 16C

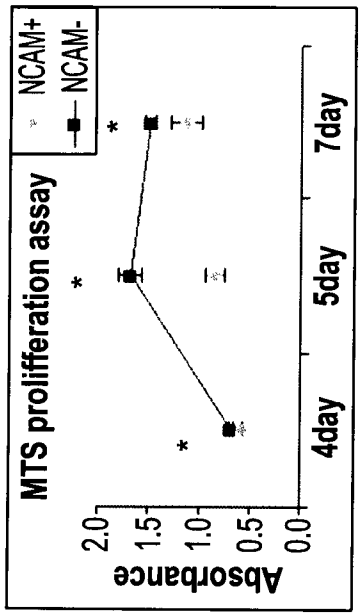


FIG. 16D

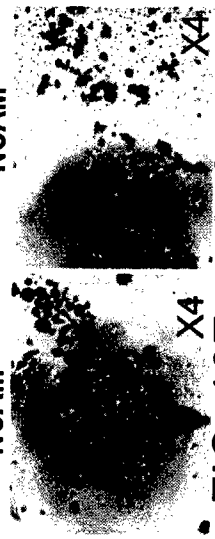


FIG. 16E

FIG. 16F

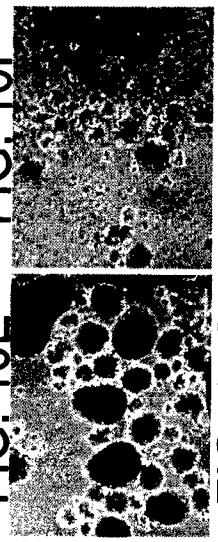


FIG. 16G

FIG. 16H

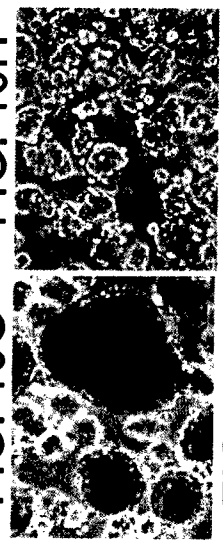


FIG. 16I

FIG. 16J

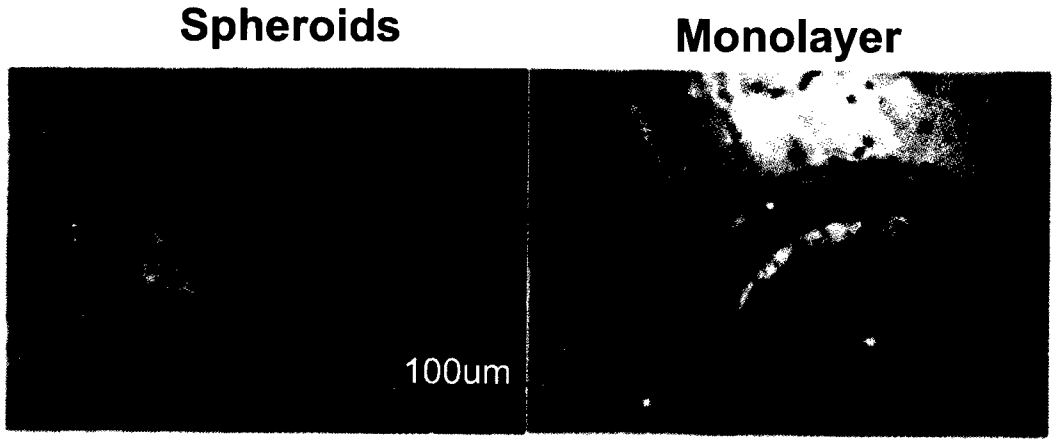


FIG. 17A FIG. 17B

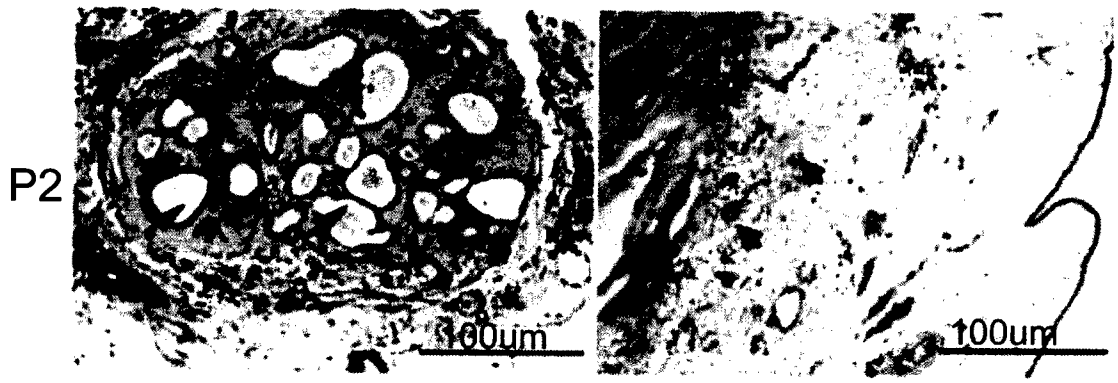


FIG. 17C FIG. 17D

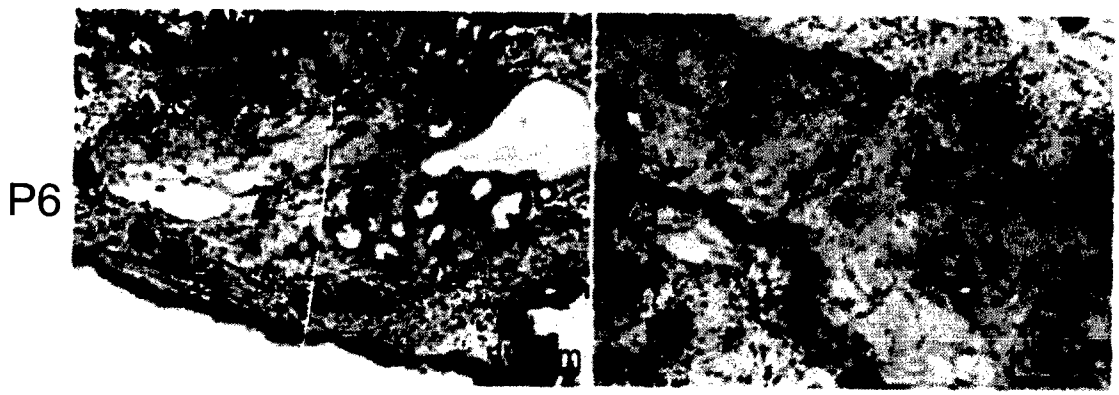


FIG. 17E FIG. 17F

FIG. 17G

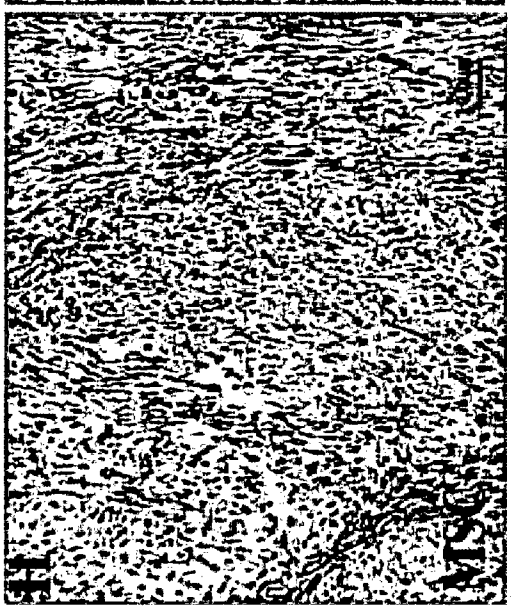


FIG. 17H

FIG. 17I

FIG. 17J

FIG. 17K

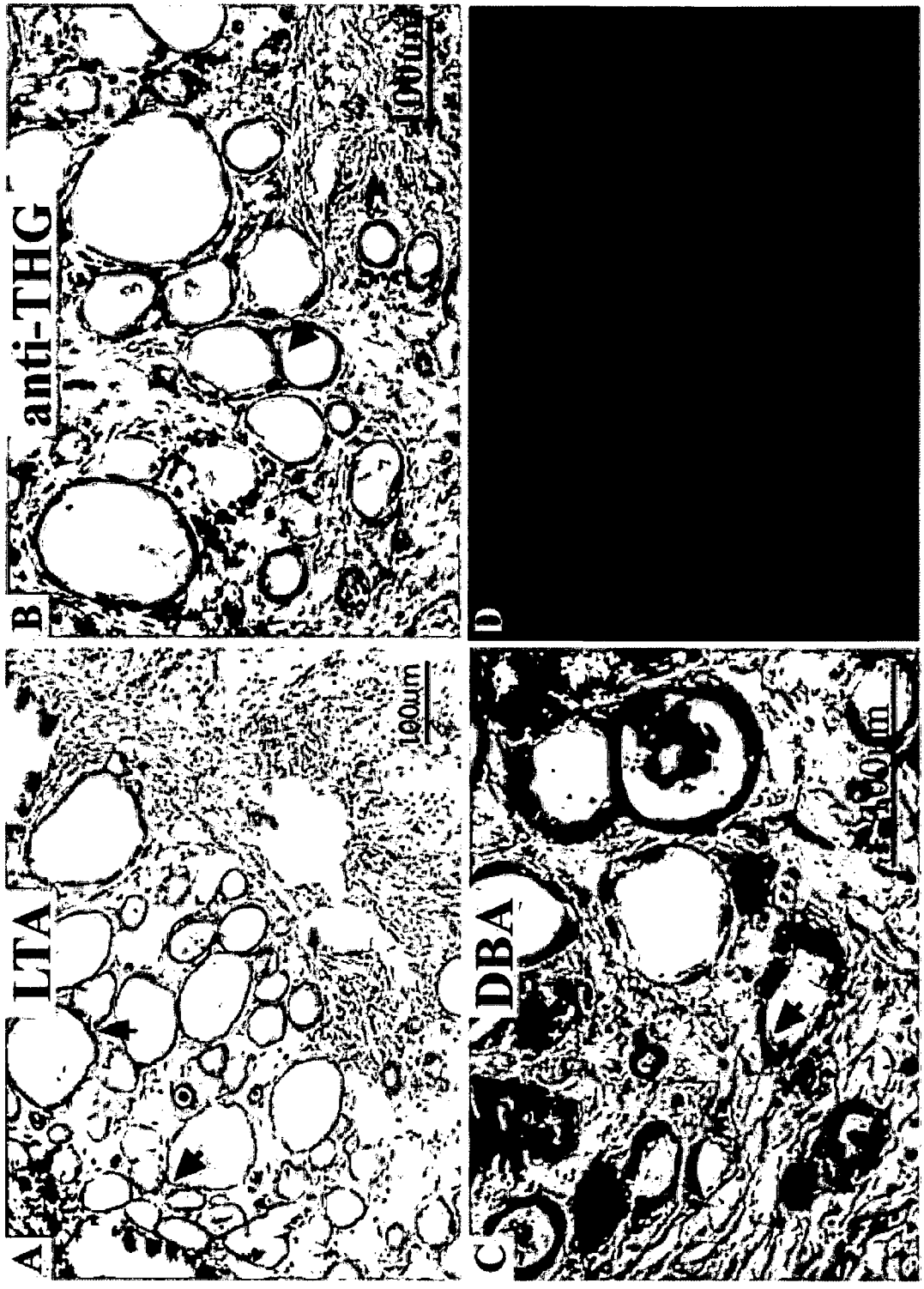


FIG. 18A

FIG. 18B

FIG. 18C

FIG. 18D

FIG. 19B

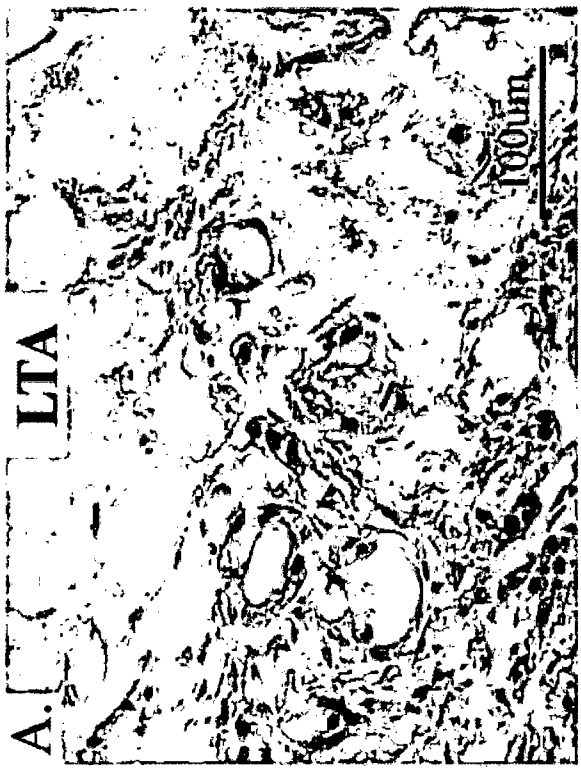


FIG. 19A

FIG. 19C

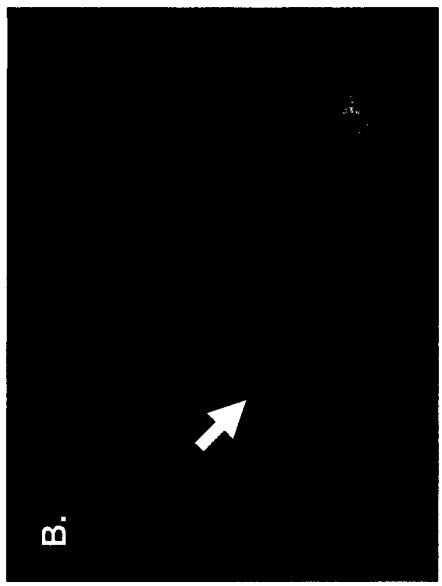


FIG. 20B

FIG. 20A

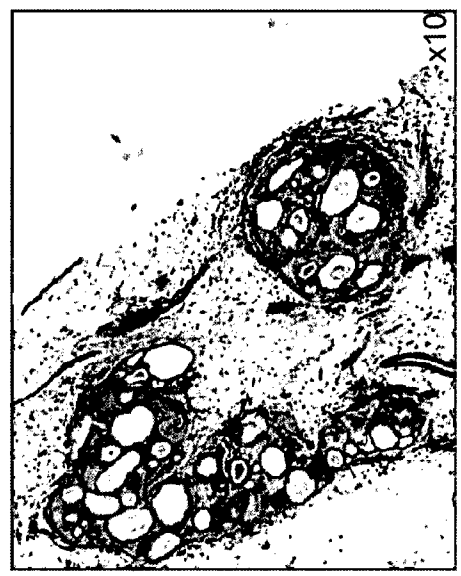


FIG. 20C

FIG. 20D FIG. 20E FIG. 20F

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2011/000376

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/07
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PODE-SHAKKED NAOMI ET AL: "Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, UNIVERSITY PRESS CAROL DAVILA, BUCHAREST, RO, vol. 13, no. 8B, 1 August 2009 (2009-08-01), pages 1792-1808, XP002581529, ISSN: 1582-1838, DOI: 10.1111/J.1582-4934.2008.00607.X [retrieved on 2008-12-16] page 1795, right-hand column; figure 1 ----- -/--	1-24

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2011	26/09/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Trommsdorff, Marion

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2011/000376

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BUSSOLATI B ET AL: "Isolation of renal progenitor cells from adult human kidney", AMERICAN JOURNAL OF PATHOLOGY, AMERICAN SOCIETY FOR INVESTIGATIVE PATHOLOGY, US, vol. 166, no. 2, 1 February 2005 (2005-02-01), pages 545-555, XP002454238, ISSN: 0002-9440 the whole document	1,2,6,9, 13
Y	LUSIS M ET AL: "Isolation of clonogenic, long-term self renewing embryonic renal stem cells", STEM CELL RESEARCH, ELSEVIER, NL, vol. 5, no. 1, 27 March 2010 (2010-03-27), pages 23-39, XP027106369, ISSN: 1873-5061 [retrieved on 2010-06-24] page 27	1,2,6,9, 13
Y	LIU X M ET AL: "Suspended aggregates as an immobilization mode for high-density perfusion culture of HEK 293 cells in a stirred tank bioreactor", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 72, no. 6, 28 March 2006 (2006-03-28) , pages 1144-1151, XP019441688, ISSN: 1432-0614, DOI: 10.1007/S00253-006-0409-3 page 1147; table 1	1,2,6,9, 13
A	MAESHIMA AKITO ET AL: "Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney", JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 17, no. 1, 1 January 2006 (2006-01-01), pages 188-198, XP002471868, ISSN: 1046-6673, DOI: 10.1681/ASN.2005040370 the whole document	1-24

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2011/000376

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	METSUYANIM SALLY ET AL: "Accumulation of malignant renal stem cells is associated with epigenetic changes in normal renal progenitor genes", STEM CELLS (MIAMISBURG), vol. 26, no. 7, 2008, pages 1808-1817, XP000002658726, ISSN: 1066-5099 the whole document --- ELLA BUZHOR ET AL: "Kidney Spheroids Recapitulate Tubular Organoids Leading to Enhanced Tubulogenic Potency of Human Kidney-Derived Cells", TISSUE ENGINEERING PART A, vol. 17, no. 17-18, 1 September 2011 (2011-09-01), pages 2305-2319, XP55006582, ISSN: 1937-3341, DOI: 10.1089/ten.tea.2010.0595 the whole document ---	1-24
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