Potential Protocols of Renal Stem Cell Research

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Abstract: Stem cell studies are important and useful in the current life science application. This article describes some protocols in stem cell studies. It is possible to introduce stem cells into a damaged adult kidney to aid in repair and regeneration. Transdifferentiation offers the possibility of avoiding complications from immunogenicity of introduced cells by obtaining the more easily accessible stem cells of another tissue type from the patient undergoing treatment, expanding them in vitro, and reintroducing them as a therapeutic agent. Adult stem cells may possess a considerable degree of plasticity in the differentiation. Immunoisolation of heterologous cells by encapsulation creates opportunities for their safe use as a component of implanted or ex vivo devices.
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Introduction

As the origin of an organism's life, stem cells have the potential to develop into many different types of cells in life bodies. Stem cells can be used in the clinical medicine to treat patients with a variety of diseases (Daar, 2003). Serving as a repair system for the living body, the stem cells can divide without limit to replenish other cells as long as the living body is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell situation or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, a bone cell, a nerve cell, or a brain cell. Stem cell research is a typical and important topic of life science (Ma, 2005).

Currently, stem cell medicine has become a practical method on many seriously diseases. The general techniques on stem cell application are to define, isolate and expand stem cells from target tissues so that they can be used to either repair or regenerate damaged organs. Normally, the research practical program on stem cell could be divided into 3 sections: (1) Isolation of stem cells from embryo and/or other resources (such as adipose and somatic tissue). (2) Embryonic stem (ES) cells differentiate into somatic stem cells and/or progenitor cells. (3) Somatic stem and/or progenitor cells differentiated from ES cells are used for body repair and/or regeneration.

Many contents and info in this article are collected from libraries and Internet to offer to the readers as the references to design potential projects, which may same as other articles.

1. Embryos

C57BL/6 female mice are injected with pregnant mare serum gonadotropin and human chorionic gonadotropin to collect oocytes. Spermatozoa are collected from the cauda epididymis of mice. In vitro fertilization is performed as described previously (Sugiyama et al., 1992). The fertilized embryos are frozen at the two-cell stage. The embryos developed to blastocyst stage in KSOM medium after thawing. Cell Culture and Chimera Production Brief exposure to acidic Tyrode's solution is performed to remove the zonae pellucidae from the cultured blastocysts. The denuded embryos are placed on a feeder layer of mitomycin C-inactivated confluent embryonic fibroblasts in four-well plates. The embryonic fibroblasts and blastocysts are cultured in Dulbecco's modified Eagle's medium supplemented with 15% KSR (Invitrogen, San Diego, CA), 0.1 mM 2-mercaptoethanol, 103 units/ml leukemia inhibitor factor, LIF (ESGRO), nonessential amino acids, and sodium pyruvate. The growing blastocysts attached to the feeder layer within 48 h. The inner cell mass (ICM) is apparent inside and extended above the flat trophoblast cells spreading from the attached blastocysts. The expanded colonies are dissociated, trypsinized, and seeded onto a new feeder layer 4 days after attachment. This process is repeated several times at intervals of 2 to 4 days; colonies are never allowed to become larger than 400 mm in diameter. The putative ES cells are stocked at passage 7 and 8. We used B6G-2 cells after passage 10 to examine a potential for pluripotency in vitro and in vivo. Host embryos are cultured from the two-cell stage to the morula and blastocyst stages after superovulation and natural mating. The ES-like cells are injected into the

cavity of the host blastocyst and the subzonal cavity of the host morula. After injection, the chimeric blastocysts are transferred to the uteri of pseudopregnant recipient mice at 2.5 days postcoitus.

2. ES cells isolation and cell line establishment

Renal stem cells are isolated from the blastocysts of mouse embryos using the culture conditions. Natural mating between 20 female superovulated and male mice are done to provide blastocysts. The blastocysts are flushed to form uterus by M2 media 5 days after mating. Mouse embryonic fibroblast in a natural cycle or in a superovulated cycle are prepared to form embryos in a midgestation age according to protocols described by Shimizukawa (Shimizukawa et al. 2005).

The blastocysts are transferred to 35 mm dishes on the mouse embryonic fibroblast feeder group or mouse embryonic fibroblast feeder layer group which previously inactivated with mitomycin C (Kyowa, Japan) 10µg/ml for 2 h in CO_2 incubator. The ES media containing DMEM high glucose (Sigma) + 20% FBS (Gibco) + LIF 1000 IU/ml (Sigma) + 2-mercaptoethanol 0.1 mMol (Sigma) + L–Glutamin 2 mMol (Sigma) and Penicillin/Streptomycin 100 mg/100 IU/ml (Sigma) for 3 days. Disaggregation is carried out according to method described by Bongso et al. (1994) with some modification. Briefly, the outgrowth ICM are disaggregated mechanically by hand pulled Pasteur pipette in different size in 50 µl DMEM media under mineral oil (Sigma). Then, the disaggregated ICM is transferred to one well of 96-well dish (NUNC) and cultured for 3 days. An alternative procedure for disaggregation of ICM is culturing of blastocysts on 96-well dish and trypsonizing the outgrowth ICM in situ with trypsin/EDTA 0.1% /1 mM (Sigma) in PBS. It is possible to trypsonize the cells in 96-well dishes up to 3 more passages every 3 days until the ES colony morphology appear in the expansion stage. It is essential to monitor microscopically the formation of ES colonies in this stage daily. The colony positive dishes have to subculture 2 times more in the colony formation stage until the cells became confluent enough for passage in 4-well dish (NUNC). Depending to doubling time the cells must be trypsonized up to 4 further passages every 3 days. Then the confluent cells are passaged into 35 mm dish (NUNC) as the passage number one. The first frozen cells are carried out in passage number two (60 mm dish) using DMSO 10%, FBS 20% and DMEM media. Alkalin phosphatase assessment The ES cells are cultured in 35 mm dish for growing, then the ES colonies are fixed by 4% formalin in PBS buffer and naphtol AS-MX (Sigma) is used according to manufactures instruction for alkalin phosphatase staining.

3. Isolation of renal stem cells or multipotent renal progenitor cells (MRPC)

Multipotent renal progenitor cells (MRPC) are isolated from adult mouse kidneys using culture conditions (Jiang et al. 2002).

Mouse kidneys are perfused *in vivo* through aorta with cold saline to flush the blood from the kidney, harvested, minced, and partially digested using collagenase in the presence of soybean trypsin inhibitor. The cell suspension is washed and plated in a medium that consisted of 60% DMEM-LG (Life Technologies-BRL, Grand Island, NY, USA), 40% MCDB-201 (Sigma Chemical Co., St. Louis, MO, USA), 1x insulin-transferrin-selenium (Invitrogen, USA), LA-BSA 1 mg/ml (Sigma, USA), 0.05 uM dexamethasone (Sigma) and 0.1 mM ascorbic acid 2-phosphate (Sigma), 100 U penicillin and 1000 U streptomycin (Life Technologies-BRL, USA) with 2% FCS (Hyclone Laboratories, Logan, UT, USA), 10 ng/ml EGF, 10 ng/ml PDGF-BB, and 10 ng/ml leukemia inhibitory factor (R&D Systems, Minneapolis, MN, USA). The cells are plated on fibronectin coated culture flasks at low density (300 cells/cm^2), to avoid cell–cell contact, and cultured at 37°C in the presence of 5% CO_2. Suppose that the cells could live up to 6 weeks. Single clones of cells are obtained by plating the cells at nontouching density and then using cloning rings to pick individual colonies of cells at the 5- to 10-cell stage.

4. Cell culture medium

- **Media for mouse embryonic feeder layer cells:** High-glucose Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 100 unit/l penicillin, 100 unit/l streptomycin, 1% nonessential amino acids. For 1 liter, mix 890 ml DMEM with 90 ml FBS, 10 ml penicillin and streptomycin solution, 10 ml nonessential amino acids.
- **Medium for mouse stem cell:** High-glucose DMEM, 10% FBS, 100 unit/l penicillin, 100 unit/l streptomycin, 1% nonessential amino acids, 5 ml nucleoside solution, 0.09 mg/l insulin, 1000 unit/ml LIF. For 1 liter, mix 890 ml DMEM with 90 ml FBS, 10 ml penicillin and streptomycin solution, 10 ml nonessential amino acids, 10 ul insulin solution and 1 ml LIF.
- **Differentiation medium:** RPMI 1640 supplemented with 10% FBS (heat inactivated), 1 mM L-glutamine, 100 unit/l penicillin, 100 unit/l streptomycin. For 1 liter, mix 890 ml RPMI with 90 ml FBS, 10 ml

penicillin and streptomycin solution, 10 ml L-glutomine.

- **Freezing medium**: 9 ml DMEM and 1 ml DMSO.

5. Mouse ES cells in culture

Isolated ES cells are added to 5 ml DMEM supplemented by 10% FBS (Fetal Bovine Serum, Gibco, UK), 100 U/ml penicillin (Sigma, USA) and 100 U/ml streptomycin (Sigma, USA) and washed by centrifugation at 1200 rpm for 5 min. The cell pellet is collected and cultured in a 75-cm^2 flask in a DMEM medium supplemented by 10% FBS and antibiotics. The cultures are incubated at 37°C in a 5% CO_2 environment. Four days after primary culture initiation, the culture medium are collected, centrifuged and the resultant cell pellet are replated in a fresh 75-cm^2 flask. These cultures (established from removed medium) are fed twice weekly and upon confluence, the cells are lifted by Tripsin/EDTA (Gibco, UK), counted and passaged at 1:3 ratios (about 1.5×10^6 cell/75-cm^2 flask). Cell passage is performed up to subculture 3 (it should be mentioned that the medium of each passaged culture are contained a few floating cells not attached on culture surface with replating due probably to their non mesenchymal nature). In parallel to the culture established from removed medium, the cultures of marrow, primarily adherent cells, are expanded by three successive passages at 1:3 split ratios. During the cultivation period, time needed by the culture (established either by primarily adherent cells or the cells floating in removed medium) to approach confluence, as an index of cell growth rate, are recorded. At the end, the passaged-3 cells from either group are evaluated in terms of their differentiation potential towards skeletal lineages as bone, cartilage and adipose cells (Saito et al. 2002; Winkler et al. 2008).

- Mytomycin C treatment of feeder layer cells: When the feeder layer cells reach confluency they are treated with mitomycin C to induce mitotic arrest. The cells are still capable of conditioning the media.
- Add 5 ml mitomycin C (10 ug/ml) to a 10 ml dish.
- Incubate the cells for 3 hours.
- Remove the mitomycin C solution, rinse the dish 5 times with PBS and add fresh medium.
- Gelatin coating of tissue culture plastic: Coat tissue culture dishes with 1% gelatin solution; Incubate for 4 hours; Wash 3 times with PBS.

6. Mouse renal stem cells isolation protocol

Kidneys of adult C57BL/6 mice (10 to 12 weeks old) are washed extensively with sterile PBS to remove contaminating debris and red blood cells (RBC). Kidneys then are diced and treated with 0.075% collagenase (type D; Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS for 10 min at 37°C with gentle agitation. The collagenase is inactivated with an equal volume of culture medium (DMEM/10% FCS/1% penicillin streptomycin), and the dissolved tissue is minced further and centrifuged for 10 min at low speed. The cellular pellet is resuspended in culture medium and sequentially filtered through 70 and 40 m mesh filters to remove debris and cell segments. Cell suspensions are treated with cold ACK buffer (0.15 M potassium-ammonium chloride buffer) to remove remaining RBC. A comparison of kidney cells, obtained through mincing and filtration, with or without collagenase, yielded similar results. Enrichment of Sca-1^+ cells is achieved by incubating cells with anti–Sca-1 microbeads (Miltenyi Biotec, Auburn, CA, USA) and purification by at least two cycles of magnetic selection. Sorted populations are reanalyzed by flow cytometry, and the purity of Sca-1^+ cells is confirmed before use. FACS analysis is performed using a modified FACS method (BD Biosciences, Mountain View, CA, USA). Fluorescence data are collected using three-decade logarithmic amplification, as determined by forward light scatter intensity.

Cells are labeled with Sca-1-PE, Sca-1-APC, Sca-1-Biotin; CD45, B220, Mac-1 (CD11b), NK, TER119, CD11c, CD29, I-AD, Fas (CD95), and H-2b-FITC (BD Pharmingen, San Diego, CA, USA); CD34, CD31, CD25, Gr-1, CXCR4, CD62L, CD49e, CD44, CD90, Flk-1, EpCAM (Pharmingen, USA), and c-Kit-PE (SBA, Birmingham, AL); CD4 and CD8-PerCp (Pharmingen, USA). Biotinylated B7.1 (CD80), B7.2 (CD86; SBA), and 1B2 antibodies are detected with streptavidin-PerCp or streptavidin-APC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and nonviable cells are detected with propidium iodide (PI). PE-rat IgG2a, FITC-hamster IgG (Serotec, Oxford, UK), and PerCp- and APC-streptavidin are used as controls. FACS sorting is performed using BD FACSAria, a high-speed sorter (acquisition rates of up to 70,000 events/s) with fixed-alignment cuvette flow cell and up to three aircooled lasers at 488, 633, and 407 nm wavelengths. Cells are sorted in a cold and sterile environment for high and low PE staining (Sca-1). Sca-1^{bright} and Sca-1^{dim} cells are collected into different cold glass FACS tubes, and after cells are centrifuged, they are transferred to culture medium and incubated at 37°C, 7% CO_2 on plastic plates (Dekel et al. 2006).

7. Maintenance of mouse ES cells (Nichols and Ying 2006)

Mouse embryo stem cells are isolated from the inner cell mass of 5-day old blastocysts, derived from pregnant rats, and isolated mouse ES cells are grown on mitomycin C-treated feeder layer or on gelatin-coated dishes:

- Culture mouse embryo stem cells at a moderate density and subculture by splitting no more than 1/10.
- Routinely, subculture mouse embryo stem cells every 3 days. To prevent differentiation, the rat embryo stem cells should be dissociated into single cells after subculturing.
- Change the media every day.

8. Tetraploid ES cell aggregates

Two-cell embryos are prepared from C57BL/6 females that are superovulated and mated naturally. The fusion of blastomeres of the two-cell stage is performed using an ET-3 Embryonic Cell Fusion System (Fujihira, Tokyo) in accordance with the manufacturer's instructions to produce tetraploid embryos. The tetraploid embryos are then cultured in KSOM medium until aggregation. The zonae pellucidae from tetraploid embryos are removed with acidic Tyrode's solution. Two tetraploid embryos are aggregated with ES cells at the eight-cell stage (Nagy et al., 1990, 1993) and transferred to the uteri of pseudopregnant recipient mice at 2.5 days postcoitus.

9. Characterization of stem cells and multipotent renal progenitor cells (Gupta et al. 2006; Lazzeri et al. 2007)

- **Cell surface marker analysis:** All staining reactions are performed using 10^5 cells in 100 ul of staining buffer. Rat ES cells for stage-specific embryonic antigen-1 (SSEA-1) or freshly isolated rat bone marrow cells are used as positive control. Unstained cells and corresponding isotype antibodies are used as negative control. Primary antibodies are used. Dead cells are excluded and doublets are excluded on the basis of three hierarchical gates (forward/side scatter area, forward scatter height/width, and side scatter height/width). Antibodies of mouse anti-rat CD90-PerCP, CD11b-FITC, CD45-PE, CD106-PE, CD44H-FITC, RT1B-biotin, RT1A-biotin, CD31-biotin (Becton Dickinson, San Diego, CA, USA) and purified anti-mouse SSEA-1 (MAB4301; Chemicon, Temecula, CA, USA) are used.
- **Telomere Length and Telomerase Enzyme Assay:** For measurement of telomere length, DNA is prepared from cells by standard methods of proteinase K digestion followed by salt precipitation and digested overnight with Hinf III and RsaI. Fragments are run on a 0.6% agarose gel and vacuum blotted to positively charged nylon. The blot is probed overnight with a digoxigenin-labeled hexamer (TTAGGG) and then incubated with anti–digoxigenin-alkaline phosphatase-labeled antibody for 30 min. Telomere fragments are detected by chemiluminescence. The TRAP protocol adapted by Roche Applied Science (Indianapolis, IN, USA) is used to assay for telomerase activity.
- **DNA analysis by FACS:** MRPC are fixed in ice-cold 70% ethanol for 10 min and treated with 1 mg/ml ribonuclease for 5 min at room temperature. Propidium iodide (50 ug/ml) is added to the cell suspension and analyzed using 488 nm excitation, gating out doublets and clumps, using pulse processing and collecting fluorescence above 620 nm on a FACS Calibur (BD Bioscience, San Jose, CA, USA). Data are analyzed using Modfit LT software (Verity Software House, Topsham, ME, USA).

10. In vitro differentiation

Differentiation into cells of the neuronal lineage in vitro is performed according to Strubring et al.'s (1995) protocol. The putative ES cells are prepared at 400 cells / 20 ml in DMEM medium containing 20% FCS (Hyclone, Logan, UT), 10-7 all-trans retinoic acid (Sigma), 2 mM glutamine (Invitrogen), nonessential amino acids, and 50 mM 2-mercaptoethanol. The cells are cultured by the hanging drop method for 2 days to form embryo-like aggregates. The embryoid bodies are then collected, washed carefully, and plated into dishes coated with gelatin in the above medium without RA to allow them to attach and differentiate.

For differentiation of MRPC toward a renal cell lineage, cells are grown to confluence on fibronectin-coated four-well chamber slides and incubated with a "nephrogenic cocktail" that contained fibroblast growth factor 2 (FGF2; 50 ng/ml), TGF-beta (4 ng/ml), and leukemia inhibitory factor (20 ng/ml). All differentiation cultures are maintained for 2 weeks except where stated, and medium is renewed every 48 hours. For determination of whether MRPC could differentiate into cells of other germ cell layers, cells are incubated under conditions that promoted differentiation into endothelium (mesoderm), neurons (ectoderm), and hepatocytes (endoderm). Endothelial differentiation is induced by growing MRPC on fibronectin-coated wells (15,000 cells/cm^2)

in the presence of 10 ng/ml vascular endothelial growth factor (VEGF). Neuronal differentiation is induced by growing MRPC on fibronectin-coated wells (5000 cells/cm^2) in the presence of 100 ng/ml basic FGF. Hepatocyte differentiation is induced by growing MRPC on Matrigel (20,000 cells/cm^2) in the presence of 10 ng/ml FGF-4 and 20 ng/ml hepatocyte growth factor. Cells are characterized by reverse transcriptase–PCR (RT-PCR) and immunofluorescence as described in the RT-PCR section. For the MRPC that are differentiated into endothelial cells, LDL uptake is examined by incubating the cells with Dil-Ac-LDL (10 ug/ml) at 37°C for 60 min. Undifferentiated MRPC are used as a control (Chen et al. 2008; Wong et al. 2008).

11. In vivo differentiation

- **Ischemia reperfusion experiment:** For these experiments, MRPC are transduced using a mouse stem cell virus–enhanced green fluorescence protein (eGFP) retrovirus. These cells expressed eGFP and are referred to as eMRPC. Mice are anesthetized with pentobarbital (50 mg/kg intraperitoneally) and prepared, and using a midline incision, nontraumatic vascular clamps are applied across both renal pedicles for 35 min. Immediately after ischemia, 100 ul (10^6 cells) of an eMRPC cell suspension in PBS is injected directly into the abdominal aorta, above the renal arteries, after application of a vascular clamp to the abdominal aorta below the renal arteries to direct the flow of the injected cells. The kidneys are harvested 10 days later to examine *in vivo* differentiation of the injected cells.
- **Subcapsular injection experiment:** Mice are anesthetized, the kidneys exposed, and eMRPC (10^6 cells) are injected under the renal capsule. Mice are killed 3 weeks later, and kidneys are harvested for tissue analysis.
- **Effect of MRPC on renal function after ischemia-reperfusion:** For determination of whether MRPC injection facilitates renal functional recovery, mice undergo 30 min of ischemia induced by bilateral renal artery clamps followed immediately by injection of MRPC. As controls, mice are treated identically except that they receive either the saline vehicle or an MRPC cell suspension (10^6 cells) that have been preincubated for 12 hours with actinomycin D (1 ug/ml) to block transcription in the injected cells. For determination of whether injected MRPC have a deleterious effect on renal function, experiments are performed injecting saline vehicle (n=2) or an MRPC cell suspension (10^6 cells; n=2) after sham operation. Renal function is assessed by serial measurement of serum creatinine and 24-hour creatinine clearance (Hishikawa and Fujita 2008).

12. Signs of differentiation

Cell surrounding the characteristic colonies, with a flattened morphology and a dark and spiky appearance, are typical for different treated cells. Cells with a clearly visible nucleus and growing within flat colonies are more likely to have undergone differentiation. For AP staining of embryo stem cells, use the following protocol:

- Rinse cells thoroughly with PBS.
- Fix the cells in 10 ml ice-cold methanol for 10 min.
- Rinse with aqua dest and incubate in fresh distilled water for 1 min.
- Freshly prepare AP substrate.
- Incubate for 45 min at room temperature, then rinse with aqua dest.
- Counter stain nuclei with Hemalum for 5 min.
- Mount the cells with Kaiser's glycerin gelatin and cover with cover slips.

13. Formation of EBs and spontaneously differentiation

The ES colonies are cultured for 5 days on 24-well dish (Cellstar) in suspension state by adding 1% trypsin to ES media and removing LIF. Then, the EBs are trypsonized with mild 0.5% /0.5mM trypsin/EDTA (Sigma) in PBS and then the media removed and transfer into centrifuge tube for a few minutes. The sedimentary EBs are transferred on the collagen coated 4-well dish and cultured for 20 days to induce the spontaneously differentiation. For detection of hematopoeitic cells, the differentiated cells are fixed by carrnoy's fixative (glacial acetic acid and metanol 1:3) and stained by Wright-Gimsa method.

14. Damaged renal repairing and renal regeneration by stem cells

Cultured renal stem cells or MRPC are introduced to the obstructed kidneys of mice. The use of human embryonic ES cells for the treatment of organ dysfunction is associated with legal and ethical issues which society as a whole has yet to decide on. In the meantime, fundamental research aiming to prove that ES cells can be directed into forming renal progenitor cells, and eventually differentiated renal cells, is underway. ES cells are pluripotent cells derived from the inner cell mass of blastocysts, and are in theory able to give rise to all the cell types of

the body. ES cell lines have been derived from mice, non-human primates and humans. In vivo injection of ES cells can give rise to teratomas, which are tumors containing cells of all three lineages (ectoderm, endoderm and mesoderm). This tumorigenesis may limit the clinical use of ES cells to treat organ dysfunction. Nevertheless, in murine ES cell-derived teratomas in vivo, renal primordial structures can be detected histochemically, and genes involved in metanephrogenesis are expressed. This same potential is noted when ES cells are injected into embryonic mouse kidneys in vitro, and gave rise to ES cell-derived tubules, in this case without forming teratomas. In vitro, transfection of murine ES cells with renal developmental gene Wnt4, as well as the addition of hepatocyte growth factor and activin-A, both promote the formation of renal tubule-like structures, with expression of tubular marker aquaporin-2. The Wnt4-transfected cells are transplanted into mouse renal cortex, where they also expressed aquaporin-2 and formed tubular structures. Similarly, murine ES cells primed in vitro with retinoic acid, activin-A and BMP-7, activin-A alone, or BMP-4, differentiate into cells expressing markers of the intermediate mesoderm, early kidney development and/or renal tubule-specific markers. After injection of these primed murine ES cells into embryonic kidney cultures, ES cells are incorporated into developing renal tubules, without cell fusion, or into the nephrogenic zone. The primed cells are enriched for renal progenitor cells by FACS and injected in vivo into the kidneys of newborn mice, where they are integrated as proximal tubular cells, without teratoma formation (Wu et al. 2008).

15. Analysis of the cultured renal stem cells

15.1 RT-PCR

Total RNA is isolated using the TRIzol Reagent (Invitrogen, USA). The RNA is DNase 1 treated, and cDNA is synthesized using the Taqman Reverse Transcription Kit (BioRad, USA). Aliquots of 5 mg of total RNA are used for cDNA synthesis using the SuperScript II first-strand synthesis system with oligo(dT) (Invitrogen). The forward and reverse primers used are listed in Table 1. For *Pax2*, the RT2 PCR primer set is used for rat (LOC293992; Superarray Bioscience Corp., Frederick, MD, USA). The BD rat universal reference total RNA is used as a positive control for this reaction (BD Biosciences, USA). Quantitative real-time PCR is performed on a Bio-Rad RT-PCR equipment. Reaction conditions for amplification are as follows: 40 cycles of a two-step PCR (95°C for 15 seconds and 60°C for 60 seconds) after initial denaturation (95°C for 10 minutes) with 1 ul of a cDNA reaction in 1x SYBR Green PCR Master Mix (BioRab, USA).

cDNAs are amplified with Taq DNA polymerase (Takara, Tokyo, Japan). The PCR reaction consisted of 25–40 cycles. The sequences of the upstream and downstream primer pairs and amplimer lengths (bp) for each gene are as follows:

Pou5f1 (GGCGTTCTCTTTGGAAAGGTGTTC, CTCGAACCACATCCTTCTCTAG, 313 bp),
Pecam1 (TGCGATGGTGTATAACGTCA, GCTTGGCAGCGAAACACTAA, 384 bp),
Utf1 (GCCAACTCATGGGGCTATTG, CGTGGAAGAACTGAATCTGAGC, 204 bp),
Cd9 (CAGTGCTTGCTATTGGACTATG, GCCACAGCAGTCCAACGCCATA, 424 bp),
Zfp42 (CGTGTAACATACACCATCCG, GAAATCCTCTTCCAGAATGG, 123 bp),
Spp1 (GCAGACACTTTCACTCCAATCG, GCCCTTTCCGTTGTTGTCCTG, 243 bp).

15.2 Immunohistochemistry

Kidney tissue sections are fixed in 4% paraformaldehyde and permeabilized with Triton X-100. After blocking with 1% BSA/PBS for 1 hour, sections are incubated with primary antibodies diluted in 0.3% BSA/PBS overnight at 4°C. Slides subsequently are washed in PBS and incubated with secondary fluorochrome-conjugated antibodies for 45 minutes. The following antibodies are used in 1:100 dilution: Anti–von Willebrand factor (anft-vWF; F-3220; Sigma, USA), anti-albumin (55442; ICN/Cappel, Costa Mesa, CA, USA), FITC-conjugated anti-pan cytokeratin (F0397; Sigma, USA), anti-neurofilament 200 (N0142; Sigma, USA), Texas red–conjugated anti-GFP (600-109-215; Rockland, Gilbertsville, PA, USA), anti–zona occludens-1 (anti–ZO-1; 61-7300; Zymed, San Francisco, CA, USA), anti–MHC I (12-5321-81; eBioscience, San Diego, CA, USA), anti–MHC II (12-5999-81; eBioscience, USA), TRITC-conjugated anti-PCNA (SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-THP (CL-1032-A; Cedarlane, Burlington, NC, USA), and anti-vimentin (V4630; Sigma, USA). The following lectins are used in 1:500 dilutions for 45 minutes at room temperature: Rhodamine Peanut Agglutinin (RL-1072; Vector Laboratories, Burlingame, CA, USA) and Rhodamine Phaseolus Vulgaris Erythroagglutinin (RL-1122; Vector Laboratories, USA). For detection of Oct4, 8-um-thick formalin-fixed, paraffin-embedded sections of rat kidney are deparaffinized in xylene for 10 min, followed by hydration through graded ethanol. Endogenous peroxidase is injected for cells. The kidneys are harvested 10 days later to examine *in vivo* differentiation of the injected cells.

15.3 Characterization of markers for and regulators of renal progenitors

To define a renal stem cell or drive an ES cell towards a renal fate, it is first critical to define the populations required. Individual projects within Section 1 will involve a series of expression profiling experiments carried out to record the temporal transcriptional program of metanephric development. Each project is aimed at both defining the expression pattern of renal development and identifying secreted proteins and cell surface markers that may be of value to Section 2.

15.4 Identification of secreted factors involved in the induction of renal development

Identification of UB tip-specific novel growth factors which induce nephron induction and novel UB tip specific receptors that transduce branching signals from the MM. The former may be useful in experimental induction of stem cells towards a renal fate. The latter may assist in the isolation of UB progenitors. The aim of this project will be to profile ureteric tree versus renal mesenchyme. Profiling will also be performed on tree branch versus tip. These studies will identify growth factors being produced by the tree that may be important in instructing the mesenchyme to form nephrons. Hox7b-GFP transgenic mice will be sued to allow the separation of tree from mesenchyme using FACs and laser microcapture. c-ret antibodies will be used to identify tip from branch of the ureteric tree. Growth factors from mesenchyme that may direct branching will also be identified. Novel factors will be ectopically expressed and their ability to drive mesenchymal differentiation in explants or induce tubule formation from mIMCD3 cells in vitro will be assessed.

15.5 Identification of renal progenitor markers to assist in the identification and isolation of renal stem cell populations

In this project, a complete temporal expression analysis of the developing kidney from 10.5 dpc to postnatal will be examined. Upon this temporal framework, spatial information from Project 1 and 3 can be placed. Specific A versus B profiling will also be performed between 10.5 dpc renal mesenchyme and adjacent intermediate mesoderm that will not become kidney. This project particularly seeks to identify transmembrane markers of potential progenitor cell populations in the kidney. As describe previously, the membrane organization of all mammalian genes will be assessed computationally. Genes believed to encode trans-membrane proteins and are found to be expressed in the kidney, based on expression data obtained in projects 1 and 2, will be further analyzed. In situ hybridization will be used to assess the spatial expression pattern of these potential cell surface markers in the kidney. Antibodies will be made to lead markers and these will be used to isolate different cell populations. Finally, the potential of these populations to repair damaged kidneys will be assessed in a variety of explant and engrafting assays.

15.6 Expression profiling of renal sub-compartments, including the interstitial cells, tissue macrophages and podocytes, to identify specific markers of the endpoints of renal differentiation

In addition to the temporal expression profiling of kidney development, a series of profiling experiments are to be undertaken to define expression markers of specific cell types and regions of the kidney. Specific cell types are to be generated by primary culture methods (renal interstital cells) and cell sorting of cell specific GFP-tagged cells form the kidneys of different transgenic mice (renal macrophages, cap condensates, podocytes). This data will provide an expanded set of expression markers for specific cell types and differentiation states for cells that make up the mammalian kidney. It will also provide an excellent recourse for cell specific expression markers that can be used in Project 4.

15.7 Examination of the potential for ES cells to be differentiated into the necessary lineages for renal de novo generation or repair

In this project we will attempt to direct murine or human embryonal stem cells towards a renal fate using a variety of inducing conditions. In the case of murine ES cells, this will be an adaptation of the mesodermal induction process used in embryoid body (EB) formation (Figure 5). As tagged murine ES cells can be generated, this will be the most insightful approach. Information from Section 1 will provide i) markers for which progress towards a renal fate can be monitored using wholemount in situ hybridization (Figure 6), ii) growth factors which may assist in the process and iii) cell surface markers which can facilitate the isolation and enrichment of the desired cell types from mixed progenitor populations. We will also use human embryonal cells to more crudely assess this renal potential. Both of these can be used to test novel growth factors isolated in Projects 1 and 2.

15.8 Measuring the mesenchymal stem cells dimensions

Since the cell size can influence the time in which the culture become confluence, we measured the mesenchymal stem cells size from both cultures. For this, the length and width (the broadest part of the cells) of the fibroblastic mesenchymal stem cells from unconfluenced culture are measured using the

objective micrometer mounted on the phase contrast inverted microscope.

15.9 Adipogenesis

Confluenced passaged-3 cells in 6-well culture plates are used to evaluate the adipogenic ability of the isolated cells. The proliferation medium of the cells is replaced by adipogenic DMEM medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA). The cultures are then incubated for 21 days in 37ºC, 5%CO_2. The medium is changed 3 times a week. Occurrence of adipogenic differentiation is evaluated by Oil red staining as well as RT-PCR analysis.

15.10 Oil red staining

The culture is fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red solution in 99% isopropanol for 15 minute. At the end, the stain solution is removed and the cultures are washed with 70% ethanol before they are observed by light microscopy.

15.11 Osteogenesis

Confluenced passaged-3 cells in 6-well plates are used to induce bone differentiation. The proliferation medium of the cultures is replaced by osteogenic medium that is consisted of DMEM supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM ßglycerole phosphate (Sigma, USA). The cultures are incubated at 37°C temperature and 5% CO_2 environment for 21 days with medium replacement of three times a week. Occurrence of differentiation is examined by alizarin red staining and RT-PCR analysis.

15.12 Alizarin red staining

Alizarin red staining is used to detect wheatear the mineralized matrix is formed in the cultures. For staining, the cultures are first fixed by methanol for 10 minutes, then subjected to alizarin red solution for 2 minutes, washed by distilled water and observed with light microscope.

15.13 Chondrogenesis

To induce the cartilage differentiation, micro mass culture system is used. For this purpose, 2.5×10^5 passaged-3 cells are pelleted under 1200 g for 5 minute and cultured in a chondrogenic medium containing DMEM supplemented by 10 ng/ml transforming growth factor-ß (Sigma, USA), 10 ng/ml bone morphogenetic protein-6 (Sigma, USA), 50 mg/ml insulin/ transferin/selenium+ premix (Sigma, USA) and 1.25 mg bovine serum albumin (Sigma, USA) and 1% fetal bovine serum (Gibco, UK). The chondrogenic culture is maintained at 37ºC, 5% CO_2 for 21 days with a medium replacement of three times a week. At the end of this period, the cultures are evaluated for cartilage differentiation by specific straining of toloidin blue and RT-PCR analysis.

15.14 Toluidin blue staining

To examine cartilage differentiation, the pellets are subjected to the following: fixing in 10% formalin; dehydrating in an ascending ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5 µ by microtome. The sections are then stained in toluidin blue for 30 second at room temperature and viewed by light microscope.

15.15 RNA extraction and RT-PCR analysis of gene expression

Total RNA is collected from the cells having been induced to differentiate into osteoblastic, chondrocytic and adipocytic lineages as detailed above, using RNXPlusTM solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples are digested with DNase I (Fermentas) to remove contaminating genomic DNA. The standard reversetranscription reaction is performed with 5 µg total RNA using Oligo (dT) 18 as a primer and RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR is as follows: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 are utilized to detect differentiations. Amplification conditions are as follows: initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of denaturation at 94˚C for 45 minutes; annealing at 65 (insulin), 57 (GLUT1), 55 (GLUT2), 56 (glucagon), 65 (Oct4) and 60˚C (β-actin) for 45 minutes; extension at 72˚C for 30 minutes; and a final polymerization at 72˚C for 10 minutes. Each PCR is performed in triplicate and under linear conditions. The products are analyzed on 2% agarose gel and visualized by ethidium bromide staining.

15.16 Alkaline phosphatase analysis

Activity of the putative ES cells is stained for alkaline phosphatase activity in the cytoplasm using an alkaline phosphatase staining kit (Sigma, St. Louis, MO, USA). Fixation and staining are performed according to the protocol supplied by the manufacturer.

6 Analysis of renal repair effects by stem cells

16.1 C1earance Studies

The mice are anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a heating

pad to maintain body temperature. A tracheostomy is performed. Then the jugular vein and carotid artery are cannulated (with PE-50 catheters) for infusion of inulin/PAH and monitoring of arterial blood pressure/blood sampling, respectively. 10% inulin/20% PAH (in normal saline) infusion is started at the rate of 30 ul/min for measurement of GFR and effective RPF (starting at t=minus 60 min). A small low midline incision (just large enough to slip the bladder through) is made and the bladder is cannulated for urine collection with a PE-240 catheter which has a mushroom-shaped cap. This is secured in the bladder with two 3-0 silk sutures (Chou et al. 2003). Following the surgical preparation, the inulin/PAH are allowed to equilibrate in the rat for 60 minutes before blood and urine samples are obtained. Thereafter (starting at t=0), urine is separately collected from the bladder for three 30 minute periods. Blood samples are taken at t=45 minutes and t=105 minutes. GFR and renal plasma flow are measured with the standard methods of inulin and PAH clearance, respectively. At the end of the clearance experiments, the rats are sacrificed with an intravenous injection of sodium pentobarbital. Urine volume is measured gravimetrically. Blood cells are separated from the plasma by centrifugation. Inulin concentrations in urine and plasma are measured by standard spectrophotometry. GFR is calculated by standard inulin clearance techniques.

16.2 Western Blot Analysis of Alpha-smooth Muscle Actin (alpha-SMA)

Accumulation of alpha-SMA, an indicator of tubulointerstitial fibrosis (Bohle A. Strutz F), is measured in the cortex and medulla by Western blotting. Immunoblot analysis of alpha-SMA protein in the cortex and medulla are performed in the sham-operated rats and rats with PUO in 3 groups of rats as described above. After the left kidney is removed through a midline abdominal incision, the cortex and medulla are separated and glass homogenized in lysis buffer on ice for total protein extraction. The homogenates are centrifuged at 12,000 r.p.m. for 20 min at 4°C. The supernatants are stored at -80°C in aliquots until analysis. The total protein concentrations of the samples are determined using BCA Protein Assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Protein extracts containing 100 μg of total protein are used. Western blot analysis is performed according to the procedures previously reported from our laboratory (Chou et al. 2003). Mouse monoclonal anti-alpha-SMA (Sigma Chemical, Ann Arbor, MI) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) are used as the primary and secondary antibody at a dilution of 1:1000. The alpha-SMA immunoblot signals are normalized to the corresponding beta-actin band signals (Miyajima et al. 2000). A monoclonal mouse antibody for the structural protein β-actin (Sigma Chemical, St. Louis, MO) is used as a loading control. Membranes are stripped prior to β -actin analysis with buffer containing 0.2% sodium dodecyl sulphate and 50 mM glycine, adjusted to pH 2.6 with HCl, at room temperature for 2 min. After washing three times for 5 minutes each in 0.1% TBS, steps 3 to 9 above are repeated for β -actin. The immunoblot films are scanned and analyzed using imaging densitometry software (Bio-Rad, Hercules, CA). The data for alpha-SMA immunoblot signals are normalized to the corresponding ß-actin band signals.

16.3 Histological Studies:

The kidney specimens are embedded in paraffin after overnight fixation in 10% neutral buffered formalin. Sections (5-μm thick) are stained by H&E method and trichrome method with Gomori trichrome kit (Richard-Allen Scientific, Kalamazoo, MI) to demonstrate collagen deposition. Tubular and interstitial changes in each group are graded on a scale from 0-4 under a micrometric ocular grid in accordance with the methods previously described (Remuzzi et al. 1999).

16.4 Radiological studies:

Sham-operated rats and rats with PUO are anesthetized with pentobarbital sodium (50 mg/kg i.p.) and a tail vein is cannulated with a 24-gauge catheter. Ioversol (Optiray 300, Mallinckrodt Inc, St. Louis, MO, USA) is injected intravenously at 2 ml/kg BW and x-ray images of the rats are captured at 5 minutes by a portable x-ray machine (General Electric). A nuclear renal scan is also performed.

16.5 Blood biochemistry.

Body/organ weight, serum lipids, serum glucose and urine glucose are measured.

16.6 Kidney lipid contents.

Determine cholesterol/triglycerol content from kidney tissue (n=4-5 from each group).

16.7 Protein expression in kidney.

Use ½ kidney. Western blot for HMGCR, PPAR, SREBP-1, SREBP-2, TNF-α, TGF-β1, TGF-β2, HMG-CoA, PAI-1, nephrin, podocin, ABCA1, α-actin, VEGF, COX-2, and HIF expressions are performed.

16.8 Kidney RNA for RT-PCR analysis.

Use the other ½ kidney. Total RNA is ideally extracted with TRIzol and kept in 80% ethanol until PCR. Gene expression (mRNA) is determined for SREBP-1, SREBP-2, TGF-1, TGF-2, HMGCR, ABC-1, ABCA-1, PAI-1, nephrin and podocin (n=4-5 from each group).

16.9 Histology study.

¼ of the kidney is used. TRI and HE staining for fibrosis. Microphage infiltration/MCP-1 expression (n=3-4 from each group).

16.10 Blood and urine chemistries:

Serum glucose, urine glucose, total cholesterol, and triglycerides are determined by kits (Wako Chemicals USA, Inc., Richmond, VA, USA).

16.11 Serum creatinine and BUN:

Serum creatinine and BUN are determined using Autoanalyzer (Beckman Instruments Inc., Fullerton, California, USA). Urine albumin concentration is determined by competitive ELISA via the Albuwell M kit (Exocell, Philadelphia, PA, USA). Urine creatinine concentration is determined by Jaffe's reaction of alkaline picrate with creatinine via the Creatinine Companion kit (Exocell, Philadelphia, PA, USA, catalog number 1012).

16.12 RNA isolation and quantitative real-time PCR:

Total RNA is isolated from the cortex of kidney by using TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA is synthesized by using reverse transcript reagents (Bio-Rad iScript cDNA synthesis kit) after DNase treatment (Invitrogen, Carlsbad, CA, USA). The mRNA level is quantified by using Bio-Rad iCyCler Real Time PCR system. 36B4 is used as internal control and the amount of RNA is calculated by the comparative CT method. All the data are calculated from duplicate reactions. The primer sequences used are indicated in Table 1.

16.13 Homogenate, nuclei and membrane isolation:

Kidneys are homogenized at 4°C in homogenization buffer (20 mM Tris-Cl, pH 7.4, 75 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol), supplemented with a protease inhibitor cocktail consisting 10 mM AEBSF, 0.08 mM Aprotinin, 2 mM Leupeptin, 4 mM Bestatin, 1.5 mM pepstatin A, 1.4 mM E-64 (Sigma-Aldrich, St. Louis, MO, USA). Nuclear extracts are prepared according to the method of Morooka et al with minor modifications as we have previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b).

16.14 Protein electrophoresis and Western blotting of nuclear extracts and cortical homogenates:

Equal amount of protein samples are subjected to SDSPAGE (10% wt/vol) and they are then transferred to nitrocellulose membranes. After blockage with 5% fat-free milk powder with 1% Triton X-100 in Tris-buffered saline (20 mM Tris-Cl, 150 mM NaCl, pH 7.4), blots are incubated with antibodies against SREBP-1 (Santa Cruz, 1:1000), SREBP-2 (Santa Cruz, 1:1000), PPAR-β (ABR: 1:1000), TGF-1 (Santa Cruz, 1:1,000), TGF-2, plasminogen activator inhibitor-1 (PAI-1, Santa Cruz, 1:1,000), VEGF (Santa Cruz, 1:1,000), TGFb-1 (Santa Cruz, 1:1,000), type IV collagen (Santa Cruz, 1:1,000), or fibronectin (Sigma, 1:2,000). Corresponding secondary antibodies are visualized using enhanced chemiluminescence (Pierce, Bradford, IL, USA). The signals are quantified with a Phosphor Imager with chemiluminescence detector and the accompanying densitometry software (Bio-Rad, Richmond, CA, USA).

16.15 Lipid extraction and measurement of lipid composition

Lipids from the renal cortex are extracted by the method of Bligh and Dyer. **A) To determine triglyceride and total cholesterol content:** Totals lipids are extracted from the renal cortex and triglyceride and cholesterol content is measured as we have previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b). **B) To determine the glycosphingolipid composition:** an aliquot of the lipid extract is evaporated to dryness and subjected to alkaline methanolysis. The lipids are chromatographed on high performance thin layer chromatography plates (HPTLC, E. Merck 5641). Glucosylceramide and ganglioside GM3 are separated with a solvent system consisting of chloroform: methanol: water (65:25:4) on plates which are pretreated with 2.5% borax in methanol: water (1:1). The lipid bands are visualized by impregnating the plates with a modified charring reagent (100 g of $CuSO_45H_2O$ in conc. H_3PO_4: water: methanol (100:750:400). The charred TLC plates are scanned with a video densitometer. Comparing the density of each spot with the density of the corresponding standard curve is used to quantify the glucosylceramide and ganglioside GM3 bands.

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