Studies of granulocyte colony-stimulating factor (GCSF) in renal stem cells regeneration

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Abstract: All animal cells come from stem cells. Stem cell pluripotency means a stem cell having the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm. Pluripotent stem cells can be differentiated to any fetal or adult cell type. However, the pluripotent stem cells cannot develop into a fetal or adult organism alone because they are lack of the potential to contribute to extraembryonic tissue, such as the placenta. This article is a research protocol offered to the readers as the reference for their researches, and hope some scholars can get useful information in it.
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The overall aim of this project is to study the application of granulocyte colony-stimulating factor (GCSF) in stem cell regenerate and in repair of the damaged kidney. The research program is divided into 3 sections: (1) Clone GCSF in E-coli, and apply the purified recombinant GCSF in kidney damaged rats. (2) Observe the renal stem cell regeneration after the renal damaged rats treated by GCSF. (3) Get the renal recovery by stem cell through GCSF treatment.

This article is a research protocol offered to the readers as the reference for their researches, and hope some scholars can get useful information in it. And, thanks all the scholars whose work results and ideas used in this article and can be shared with other, for the scientific development.

1. Specific Aims

The scientific objective of this application is to establish a technique by using GCSF in the stimulating renal stem cell regeneration to repair and regenerate damaged kidney.

Aim 1. To clone GCSF in E-coli.

Aim 2. To apply the purified recombinant GCSF in rat bone marrow to stimulate the regeneration of stem cells.

Aim 3. To select renal stem cells from regenerated stem cells.

Aim 4. To apply the selected renal stem cells in kidney damaged rats.

Aim 5. To obtain the renal recovery by stem cell through GCSF application.

2. Background and Significance

Chronic kidney disease is increasing at the rate of 6-8% per year in the Unite States. At present, dialysis and transplantation are the common treatment options. However, it is possible to use stem cells and regenerative medicine as the additional choices for kidney disease treatment. Such new treatments might involve induction of repair using endogenous or exogenous stem cells or the reprogramming of the organ to reinitiate development (Hopkins et al. 2009).

In the 20th century, an efficient treatment was given to patients with renal failure through the development of kidney dialysis and transplantation. These techniques have been proved successful, but are marred by inflammation and limited organ availability and graft survival due to immune rejection. More recently, hope has been placed in the development of stem cell-based therapies, in which the function of the failing organs is restored by injected multipotent cells. Possible sources for these cells include differentiated embryonic stem (ES) cells, adult renal stem cells, and circulating multipotent cells, such as bone marrow-derived stem cells. Using the patient's own stem cells to repair kidney damage could circumvent the problems of immune rejection and organ availability.

Kidneys can regenerate, which varies among species. Some bony and cartilaginous fish continue to form new nephrons during adult life. Adult mammals cannot form new nephrons but, to a certain extent, tubules and glomeruli may recover structure and function after limited injury such as acute tubular necrosis. Severe or prolonged injury results in replacement of functional parenchyma by scar tissue, i.e. fibrosis, which correlates clinically with the development of renal failure.

An effective treatment of renal disease is renal cell regeneration, or replacement of damaged renal cells, that discourage fibrosis. The origins for renal parenchymal cells could be: (1) the re-entry into cell cycle of differentiated cells; (2) direct transdifferentiation of one cell type into another, such as tubular cells into interstitial cells or vice versa; (3)

differentiation from stem cells of the kidney or the bone marrow.

Embryonic stem cells are different from adult or tissue-specific stem cells. Embryonic stem cells are the stem cell that can be grown in large numbers in the laboratory and retain the ability to grow into any type of cells including renal, nerve, heart muscle, bone and insulin-producing cells. It is difficult for the tissue-specific adult stem cells to grow in a great number, hard to isolate and are difficult to grow outside the body. Adult stem cells, such as skin and bone marrow stem cells, normally grow into a limited number of cell types (Snykers et al. 2008).

The role of embryonic or adult stem cells, in particular bone marrow-derived stem cells, in regenerating the kidney after injury has been the subject of intensive investigation. Bone marrow-derived stem cells have been shown to give rise to small numbers of most renal cell types, including tubular cells, mesangial cells, podocytes, vascular cells and interstitial cells. Injections of bone marrow-derived cells do improve renal function in many animal models of renal disease. Many stages of nephrogenesis can be studied using cultured embryonic kidneys, but there is no efficient technique available to readily knockdown or overexpress transgenes for rapid evaluation of resulting phenotypes. Embryonic stem cells have unlimited developmental potential and can be manipulated at the molecular genetic level by a variety of methods. ES cell technology may achieve the objective of obtaining a versatile cell culture system in which molecular interventions can be used in vitro and consequences of these perturbations on the normal kidney development program in vivo can be studied (Steenhard et al. 2005).

Stem cells and progenitor cells are necessary for repair and regeneration of injured renal tissue. Infiltrating or resident stem cells can contribute to the replacement of lost or damaged tissue. However, the regulation of circulating progenitor cells is not well understood. Many factors influence the stem cell growth in damaged kidney. For example, low levels of erythropoietin induce mobilization and differentiation of endothelial progenitor cells and erythropoietin ameliorates tissue injury. Full regeneration of renal tissue demands the existence of stem cells and an adequate local milieu, a so-called stem cell niche. It was reported that in the regenerating zone of the shark kidney, stem cells exist that can be induced by loss of renal tissue to form new glomeruli. Stem cell may eventually contribute to novel therapies of the kidney disease (Perin et al. 2008).

Researchers used a rat model of chronic renal failure in which one kidney is excised so as to increase the load of the remaining kidney, thus causing a chronic deterioration that resembles the clinical situation of renal failure (Alexandre et al. 2008). In Alexandre's project, the rats were divided into 4 groups: Group 1 were sham operated and both kidneys left in place; Group 2 had a kidney removed but were not administered cells; Group 3 were administered $2x10^6$ lineage negative bone marrow cells on day 15 after one of the kidneys was removed; Group 4 were administered $2x10^6$ lineage negative bone marrow cells on days 15, 30, and 45 after one of the kidneys was removed. They found: (1) Expression of inflammatory cytokines was reduced on day 16 in the kidneys of rats receiving stem cells as compared to rats that were nephrectomized but did not receive cells. (2) On day 60 rats receiving stem cells had decreased proteinuria, glomerulosclerosis, anemia, renal infiltration of immune cells and protein expression of monocyte chemoattractant protein-1, as well as decreased interstitial area. (3) Injured rats had higher numbers of proliferating cells in the kidney, whereas rats receiving stem cells had less. (4) Protein expression of the cyclin-dependent kinase inhibitor p21 and of vascular endothelial growth factor increased after nephrectomy and decreased after stem cell treatment. (5) On day 120, renal function (inulin clearance) was improved in the rats which were administered bone marrow cells compared to controls. This study supports the possibility of using bone marrow cells for various aspects of kidney failure. Other studies have demonstrated that administered stem cells promote kidney repair by secretion of insulin growth factor-1 (Cornelissen et al. 2008).

Bone marrow stromal cells, also known as mesenchymal stem cells or fibroblastic colony-forming units, are multipotent non-hematopoietic stem cells adhering to culture plates (Abdallah and Kassem 2009). Mesenchymal stem cells of the bone marrow have the ability to renew and differentiate themselves into multiple lineages of conjunctive tissues, including bone, cartilage, adipose tissue, tendons, muscle, and bone marrow stroma. Those cells have been first described by Friedenstein et al., who found that mesenchymal stem cells adhere to culture plates, look like in vitro fibroblasts, and build up colonies (Friedenstein et al. 1987).

Bone marrow is the site of hematopoiesis and bone marrow transplant has been successfully used for decades as a means of treating various hematological malignancies in which the recipient hematopoietic compartment is replaced by donor-derived stem cells. Progenitor cells in bone marrow are capable to differentiate into other tissues, such as cardiac tissue. Clinical trials have been conducted demonstrating beneficial effects of bone marrow infusion in cardiac patients. It is believed that injured tissue, whether neural tissue after a stroke, or injured

cardiac tissue, has the ability to selectively attract bone marrow stem cells, perhaps to induce regeneration. Bone marrow has therapeutic effect in conditions ranging from liver failure, to peripheral artery disease, and the possibility of using bone marrow stem cells in kidney failure has been relatively understudied (Ma et al. 2009).

Mesenchymal stem cells have been brought to the attention of many researchers, because these cells are of great interest for treating various human diseases. Many studies have isolated mesenchymal stem cells and controlled, in vitro, its differentiation into cartilaginous tissue and bone using specific growth factors, with the objective of using this technology for repairing injured tissues of mesenchymal origin (Xian and Foster 2006; Kurdi and Booz 2007).

2.1 Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (GCSF) is a colony-stimulating factor hormone. GCSF is also known as colony-stimulating factor 3 (CSF 3). It is a glycoprotein, growth factor and cytokine produced by a number of different tissues to stimulate the bone marrow to produce granulocytes and stem cells. GCSF then stimulates the bone marrow to release them into the blood. GCSF also stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils. GCSF regulates them using Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signal transduction pathway.

Mouse GCSF was first recognised and purified in Walter and Eliza Hall Institute, Australia in 1983, and the human form was cloned by groups from Japan and the Germany/United States in 1986.

GCSF is produced by endothelium, macrophages, and a number of other immune cells. The natural human glycoprotein exists in two forms, a 174- and 180-amino-acid-long protein of molecular weight 19,600 dalton. The more-abundant and more-active 174-amino acid form has been used in the development of pharmaceutical products by recombinant DNA (rDNA) technology.

The GCSF-receptor is present on precursor cells in the bone marrow, and, in response to stimulation by GCSF, initiates proliferation and differentiation into mature granulocytes. GCSF is also a potent inducer of HSCs mobilization from the bone marrow into the bloodstream, although it has been shown that it does not directly affect the hematopoietic progenitors that are mobilized.

Beside the effect on the hematopoietic system, GCSF can also act on neuronal cells as a neurotrophic factor. Indeed, its receptor is expressed by neurons in the brain and spinal cord. The action of GCSF in the central nervous system is to induce neurogenesis, to increase the neuroplasticity and to counteract apoptosis. These properties are currently under investigations for the development of treatments of neurological diseases such as cerebral ischemia.

The gene for GCSF is located on chromosome 17, locus q11.2-q12. Nagata et al. found that the GCSF gene has 4 introns, and that 2 different polypeptides are synthesized from the same gene by differential splicing of mRNA.

The 2 polypeptides differ by the presence or absence of 3 amino acids. Expression studies indicate that both have authentic GCSF activity.

It is thought that stability of the GCSF mRNA is regulated by an RNA element called the GCSF factor stem-loop destabilising element.

GCSF stimulates the production of white blood cells (WBC). In oncology and hematology, a recombinant form of GCSF is used with certain cancer patients to accelerate recovery from neutropenia after chemotherapy, allowing higher-intensity treatment regimens. Chemotherapy can cause myelosuppression and unacceptably low levels of white blood cells, making patients prone to infections and sepsis. GCSF is also used to increase the number of hematopoietic stem cells in the blood of the donor before collection by leukapheresis for use in hematopoietic stem cell transplantation. It may also be given to the receiver, to compensate for conditioning regimens.

Itescu planned in 2004 to use GCSF to treat heart degeneration by injecting it into the blood-stream, plus stromal cell-derived factor (SDF) directly to the heart.

It was first marketed by Amgen with the brand name Neupogen. Several bio-generic versions are now also available in markets such as Europe and Australia.

The recombinant human GCSF synthesised in an *E. coli* expression system is called filgrastim. The structure of filgrastim differs slightly from the structure of the natural glycoprotein. Most published studies have used filgrastim. Filgrastim (Neupogen) and PEG-filgrastim (Neulasta) are two commercially-available forms of rhGCSF (recombinant human GCSF). The PEG (polyethylene glycol) form has a much longer half-life, reducing the necessity of daily injections.

Another form of recombinant human GCSF called lenograstim is synthesised in Chinese Hamster Ovary cells (CHO cells). As this is a mammalian cell expression system, lenograstim is indistinguishable from the 174-amino acid natural human GCSF. No

clinical or therapeutic consequences of the differences between filgrastim and lenograstim have yet been identified, but there are no formal comparative studies.

Filgrastim is a granulocyte colony-stimulating factor (GCSF) analog used to stimulate the proliferation and differentiation of granulocytes. It is produced by recombinant DNA technology. The gene for human granulocyte colony-stimulating factor is inserted into the genetic material of *Escherichia coli*. The GCSF then produced by *E. coli* is only slightly different from GCSF naturally made in humans.

It is marketed by Amgen under the brand name Neupogen, Dr. Reddy's Laboratories under the brand name Grafeel, Reliance Life Sciences under the brand name Religrast, Zenotech Laboratories Limited under the brand name Nugraf, Raichem Life Sciences under the brand name Shilgrast, Intas Biopharmaceuticals under the brand name Neukine and Emcure biopharmaceuticals under the brand name Emgrast.

Apricus Biosciences is currently developing and testing a product (under the brand name Nupen) which can deliver filgrastim through the skin to improve post-chemotherapy recovery of neutrophil counts.

Filgrastim is used to treat neutropenia (a low number of neutrophils), stimulating the bone marrow to increase production of neutrophils. Causes of neutropenia include chemotherapy and bone marrow transplantation. Filgrastim is also used to increase the number of hematopoietic stem cells in the blood before collection by leukapheresis for use in hematopoietic stem cell transplantation. It is produced by many companies worldwide.

The kidney has the ability to regenerate various injuries, and the potential use of stem cell therapy in accelerating such regenerative processes, thereby delaying the occurrence of end-stage renal failure. Research in recent years has identified the presence of both bone marrow-derived stem cells and quiescent stem cells residing in the kidney [Nephrol. Dial. Transplant. (2008) 23 (6): 1826-1830] (http://ndt.oxfordjournals.org/content/23/6/1826.full).

Bone marrow stem cells can differentiate into various renal cells including mesangial cells (Imasawa, et al., 2001), tubular epithelial cells (Gupta, et al, 2002) and podocytes (Poulsom, 2001). Moreover, bone marrow stem cell abnormalities have been shown to affect renal function, raising the possibility of the existence of a bone–kidney stem cell axis (Imasawa, et al., 1999; Terrier, et al., 2006). This possibility is further substantiated by the observation of Y chromosome-positive tubular epithelial cells in the transplanted kidney of a male patient who received a kidney transplant from a female donor. In general, bone marrow-derived stem cells can migrate towards a site of injury and differentiate under the appropriate microenvironment (Zhang, et al, 2004). The circulating precursor cells can not only transdifferentiate, but can also fuse with the neighbouring cells to repair damaged tissue (Ying, et al., 2002). The interaction of CD44 and its ligand hyaluronic acid has been shown to influence the exogenous mesenchymal stem cells to localize in the kidneys with experimentally induced acute renal failure to enhance renal repair (Herrera, et al., 2007). The extent and involvement of the bone marrow-derived stem cells in renal repair is, however, an unsolved issue, and an intense area of research.

Contraindications

Filgrastim should not be used in patients with known hypersensitivity to E. coli-derived proteins.

2.2 Embryonic Stem Cells (ES cells)

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 cells can be directed into forming renal progenitor cells, and eventually differentiated renal cells. Ureteric bud epithelial cells and metanephric mesenchymal cells that comprise the metanephric kidney primordium are capable of producing nephrons and collecting ducts through reciprocal inductive interaction. Once these cells are induced from pluripotent ES cells, they have the potential to become powerful tools in regeneration of kidney tissues. However, there is **a** risk to use stem cells in clinical practice. In vivo, injection of ES cells can give rise to teratomas, which are tumors containing cells of all three lineages (ectoderm, endoderm and mesoderm). ES cell-derived teratomas in vivo, renal primordial structures, can be detected histochemically. Genes involved in metanephrogenesis express the potential of ES cells to produce renal primordial duct structures and provide the insight into the regeneration of kidney tissues (Yamamoto et al. 2006). This same potential was reported when ES cells were injected into embryonic mouse kidneys in vitro, and gave rise to ES cell-derived tubules, in this case without forming teratomas (Steenhard et al. 2005). 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. Cultured Wnt4-EBs have an ability to differentiate into renal tubular cells; and second, that Wnt4, HGF, and activin A may promote the differentiation of ES cells to renal tubular cells (Kobayashi et al. 2005). The Wnt4-transfected cells

can be transplanted into mouse renal cortex, where they also express aquaporin-2 and formed tubular structures. According to Kim et al reported, murine ES cells primed in vitro with retinoic acid, activin-A and BMP-7 (Kim and Dressler 2005), activin-A alone (Vigneau et al. 2007), or BMP-4, differentiate into cells expressing markers of the intermediate mesoderm, early kidney development and/or renal tubule-specific markers (Bruce et al. 2007). 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 are injected in vivo into the kidneys of newborn mice, where they are integrated as proximal tubular cells, without teratoma formation (Vigneau et al. 2007). Human ES cells differentiate in vitro into WT1- and renin-expressing cells following treatment with a combination of specific growth factors (Schuldiner et al. 2000). However, research of the role for ES cells in renal regeneration is still in its infancy (Roufosse and Cook 2008).

2.3 Native Renal Stem Cells and Renal Regeneration

In the embryo, most types of renal parenchymal cells are derived from metanephric mesenchymal cells, which are multipotent and are in addition self-renewing, making them attractive candidates as the stem cells of the embryonic kidney.

In animal models, embryonic metanephroi transplanted into the abdominal cavity of adult animals are colonized by host vasculature, undergo nephrogenesis and produce urine, even if the operation is carried out across species barriers, and with a surprising lack of rejection (Little 2006). Human and porcine embryonic kidney progenitor cells have been isolated and, when injected into mice, can lead to the formation of miniature kidneys producing urine (Dekel et al. 2003), or protect against acute renal failure (Lazzeri et al. 2007). However, there are ethical issues to deal with human ES cells.

In adult mammals, a range of methods have been used to identify potential multipotent precursor cells, including label retention in slow cycling cells, identification of a side population, and expression of stem cell markers such as CD133. This has led to the identification of several candidate renal stem cells, which, depending on the study, are located amongst the tubular cell population (Dekel et al. 2006; Gupta et al. 2006), in the Bowman's capsule, papillary region or cortical interstitium (Bussolati et al. 2005; Sagrinati et al. 2006; Rad et al. 2008). Of note, other studies have not confirmed the presence of a large pool of precursor cells amongst the tubular population and, instead, argue that regeneration occurs through proliferation of differentiated tubular cells (Vogetseder et al. 2008; Witzgall 2008). Some of the candidate renal stem cells have been shown to enhance recovery after tubular injury, possibly by integration in the tubular epithelium (Rad et al. 2008).

2.4 Bone Marrow-Derived Stem Cells and Renal Regeneration

Bone marrow stem cells would be an ideal source of multipotent cells: they are easy to harvest and are in theory an unlimited source of expandable autologous cells. They display an unexpected plasticity which has been the subject of extensive research over the last few years. The plasticity has been observed both for the hematopoietic stem cell, which gives rise to all differentiated blood cell types, as well as for the bone marrow mesenchymal stem cells, which provide stromal support for haematopoietic stem cell in the bone marrow, and also give rise to various mesenchymal tissues, such as bone, cartilage and fat.

There are important discrepancies in the literature addressing the role of bone marrow cells in renal regeneration. The technique most commonly used to study bone marrow cell plasticity is bone marrow transplantation. The host bone marrow is replaced by donor bone marrow, and after bone marrow chimerism is established, donor cells are tracked down in the kidney. The donor bone marrow cells are distinguished from host cells by virtue of their chromosome content (male Y chromosome-positive cells in a female host), the expression of a reporter molecule (β -galactosidase, luciferase, enhanced green fluorescent protein), or the performance of a function (re-establishment of a function in a knockout mouse model). The type of host cell that the bone marrow-derived cell has given rise to (tubular, mesangial, etc.) is ascertained most often using immunohistochemistry.

Discrepancies between studies are attributable to several factors: (1) observations in different species (mouse, rat, human); (2) use of different models of renal damage (ischaemia/reperfusion, toxic, immunological); (3) different protocols for bone marrow transplantation (irradiation doses, quantity of cells injected); (4) injection of different subgroups of bone marrow cells (whole bone marrow, haematopoietic stem cell, mesenchymal stem cell); (5) sensitivity and specificity of the detection method for bone marrow cell origin (in situ hybridization for the Y chromosome, detection of reporter molecules, functional assays), and (6) sensitivity and specificity of the detection method of the renal cell type (immunohistochemistry for specific cell types such as tubular cell, mesangial cells, etc.).

Renal failure can be the result of an initial insult directed against the tubular epithelium, the glomerular cells or the vascular compartment. In the search for remedies for these varied renal diseases, studies have therefore addressed potential bone marrow origin for various renal cell types. It is useful to bear in mind these technical variations when analysing results reported in the literature (Roufosse and Cook 2008).

2.5 Tubular Epithelium

Although initial studies suggested a high contribution of bone marrow to tubular regeneration, the current view is that only a small proportion of tubular cells are bone marrow-derived, and there is disagreement over whether mesenchymal stem cells, haematopoietic stem cells or both are contributing (Humphreys and Bonventre 2008). The current consensus view is that the predominant source of tubular regeneration is through the proliferation of differentiated tubular cells (Lin et al. 2005). A few authors have not found any bone marrow cells engrafted in tubules, and propose that positive observations of bone marrow-derived tubular cells are the result of artifact (Bussolati et al. 2009). There may also be a progenitor slow-cycling cell population contributing to tubular repair.

Firstly, under certain circumstances, bone marrow engraftment in tubules can be dramatically increased. Held et al. made use of a transgenic fumarylacetoacetate (FAH)-/- mouse, in which discontinuation of the rescue drug NTBC leads to acute tubular necrosis (Held et al. 2006). After transplanting bone marrow from wild-type mice into FAH-/- mice, a few bone marrow-derived tubular cells are noted. In a subset of the FAH-/- mice, there is, in addition, loss of heterozygosity (LOH) in the liver for homogentistic acid hydrogenase, which induces a more severe, ongoing form of acute tubular necrosis. In FAH-/- animals with additional hepatic LOH, up to 50% of tubular cells are bone marrow-derived cells. Engraftment of these wild-type bone marrow-derived cells leads to morphological resolution of ATN and to disappearance of the aminoaciduria present in control mice. In this model, the bone marrow cells have a strong survival advantage over native tubular cells, due to their ability to metabolise toxic products. It is possible that this strong positive selective pressure is necessary for regeneration to occur through wild-type bone marrow cells. Interestingly, most of the bone marrow-derived tubular cells are derived from cell fusion between bone marrow cells and tubular cells. This is supported by a study by Li et al. in which fusion of bone marrow cells to tubular cells account for part of bone marrow-derived tubular cells after ischaemia/reperfusion (I/R) injury, but not all. In this model without selective pressure, the percentage of bone marrow-derived tubular cells is low (1.8%) (Li et al. 2007b).

Secondly, although there is disagreement concerning the underlying mechanism, injection of bone marrow cells, particularly mesenchymal stem cells, has repeatedly been shown to improve renal function in ATN, whether induced by toxins (cisplatin and glycerol) or I/R (Imai and Iwatani 2007). With the role of actual engraftment of bone marrow cells as tubular cells thought to be minimal or absent, mesenchymal stem cells may exert their beneficial effects through their antiapoptotic, mitogenic, immunomodulatory and angiogenic properties, or through the contribution of the bone marrow cells to endothelial cell replacement in the peritubular capillaries. It is important to know the nature of the mediators involved in these properties, and the mechanisms governing the homing of mesenchymal stem cells to the kidney (Imai and Iwatani 2007). Imberti et al. confirmed the importance of paracrine mechanisms using co-culture of mesenchymal stem cells with tubular cells in a Transwell® culture excluding contact between the two cell types, which led to less cisplatin-induced tubular cell death. mesenchymal stem cells have been shown to produce vascular endothelial growth factor, basic fibroblast growth factor, monocyte chemoattractant protein-1, hepatocyte growth factor, and insulin-like growth factor, as well as immunomodulators TGF-β and PGE_2 (Imai and Iwatani 2007; Imberti et al. 2007). In a recent study, administration of conditioned medium from cultured stromal cells provided the same renoprotective effects as injection of mesenchymal stem cells, suggesting that systemic administration of the beneficial mediators may be just as good as mesenchymal stem cell injection, and safer (Imberti et al. 2007). It is a concern that there have been a few observations of adipogenesis associated with fibrosis and osteogenesis after injection of mesenchymal stem cells (Imai and Iwatani 2007).

Mesenchymal stem cell homing to the kidney has been linked to interactions between molecules upregulated in the injured kidney (SDF-1, hyaluronic acid and PDGF) and ligands expressed on mesenchymal stem cells (respectively, CXCR4, CD44 and PDGF-R) (Imai and Iwatani 2007). Similar beneficial effects on renal function may be induced by mobilizing bone marrow cells from the patient's own bone marrow by administration of growth factors (GF) such as granulocyte colony-forming factor, granulocyte/monocyte colony-forming factor, monocyte colony-forming factor, and stem cell factor. Possible explanations for improved renal function include increased numbers of bone marrow-derived

tubular cells, a decrease in neutrophilic infiltrate, or increased cell proliferation and decreased apoptosis in kidneys of GF-treated mice (Roufosse and Cook 2008).

In summary, most but not all authors agree that a small proportion of tubular cells (at most a few percent) are bone marrow-derived after renal injury. The role these bone marrow-derived tubular cells play in improved renal function is probably insignificant, with intrinsic renal cells, either stem cells or differentiated, more likely to play the predominant role in regeneration. However, administration of bone marrow cells or mobilization of bone marrow cells using GF may be used to protect against renal injury. This may be due to paracrine / immunomodulatory effects or endothelial regeneration. In addition, there may be a therapeutic role for bone marrow-derived cells engineered to replace a defective gene, due to a local strong positive selective pressure. mesenchymal stem cells have emerged as the most promising candidate for stem cell therapy, and appear safe, such that phase I clinical trials of mesenchymal stem cell injection for the treatment of acute kidney injury are scheduled to begin shortly (Imai and Iwatani 2007).

2.6 Mesangial Cells

Mesangial cells are modified smooth muscle cells in the glomerular tuft, and provide structural support for the complex of glomerular capillaries. They may be injured by immune complex deposition, toxins and in diabetes. Although mesangial cells have regenerative potential, persistent mesangial damage can lead to glomerulosclerosis. In cell culture, bone marrow cells treated with PDGF-BB in the presence of collagen IV convert to cells with many mesangial characteristics (Suzuki et al. 2004). In rodent models of bone marrow transplantation, there is also support for partial bone marrow derivation of mesangial cells, whether glomeruli are injured or not. In models where mesangial damage has been induced, infusion of bone marrow cells may be associated with improved function, which has been attributed to mesangial and endothelial regeneration or, in the case of mesenchymal stem cells, paracrine mechanisms. Conversely, a deleterious mesangial phenotype responsible for mesangial sclerosis, such as in Os/- or db/db mice, can be induced by transplanting wild-type mice with transgenic mouse bone marrow. Some studies have further illustrated functionality of the bone marrow-derived mesangial cells by harvesting the bone marrow-derived mesangial cells, growing them in culture, and showing angiotensin-II induced contraction in vitro, a typical mesangial function (Kunter et al. 2006).

2.7 Podocytes

Podocytes are epithelial cells with complex interdigitating foot processes which create the slit diaphragm, and contribute to the synthesis of the glomerular basement membrane. Both the slit diaphragm and the glomerular basement membrane are implicated in creating a filtration barrier between blood and urine. Initial studies identified rare bone marrow-derived cells at the periphery of the glomerular tuft, in the location of podocytes. Two recent studies have suggested integration of bone marrow-derived cells as functional podocytes, with production of matrix protein. These studies involved the use of a mouse model of Alport's disease, in which the animals suffer from defective synthesis of the alpha-3 chain of collagen type IV, with glomerular basement membrane abnormalities and progression to glomerulosclerosis and renal failure. Using whole bone marrow transplantation from wild-type animals, both Prodromidi et al. and Sugimoto et al. showed the presence of bone marrow-derived podocytes and mesangial cells, accompanied by re-expression of the defective collagen chains, and improved renal histology and function. Although the bone marrow-derived cells were not numerous, their presence was sufficient to re-establish synthesis of the defective collagen chain. However, the improvement in renal function was substantial raising the possibility that there may be mechanisms involved other than replacement of podocytes. A similar experiment using mesenchymal stem cells only rather than whole bone marrow also led to a reduction in interstitial fibrosis, but without engraftment of bone marrow cells in the kidney, and with no beneficial effect on survival or renal function (Sugimoto et al. 2006).

2.8 Vascular Cells

Endothelial cells are present in the glomerular capillaries, in large vessels and in the abundant network of peritubular capillaries. Endothelial cells are attractive candidates for progeny of bone marrow-derived cells in view of their immediate contact with circulating cells, the existence of known circulating endothelial precursors, and the existence of a known endothelial precursor in the bone marrow: the haemangioblast.

In a rat model of glomerulonephritis, where glomerular endothelial cells are injured, culture-modified bone marrow mononuclear cells injected into the renal artery boosted renal regeneration. This was attributed both to incorporation of bone marrow-derived cells into the endothelial lining and to production of angiogenic factors by the injected cells. Similarly, following acute tubular necrosis, the peritubular capillaries are damaged. The return of blood flow, which depends on endothelial cell integrity, is essential for renal recovery. Duffield et al.

contend that bone marrow cells boost renal function after I/R by participating in endothelial cell regeneration. Li et al. observed bone marrow-derived vWF+ and CD31+ endothelial cells in a mouse model of adriamycin-induced nephrosis with subsequent renal fibrosis (Li et al. 2007a).

2.9 Interstitial Cells

The kidney contains a complex population of interstitial cells serving several functions, such as providing a scaffold for renal structure and producing several hormonal substances such as erythropoietin. It may even contain a population of adult native renal stem cells which play a role in renal regeneration. There is also evidence that bone marrow-derived cells could be a source for up to 30% of α-SMA-positive interstitial myofibroblasts, which have been incriminated in the production of extracellular matrix in renal fibrosis. If the bone marrow is indeed a source for such cells, the use of bone marrow cell injections for the treatment of renal failure would run the risk of enhancing fibrosis (Broekema et al. 2007).

2.10 Adipose Stem Cells

Adipose, also known as fat tissue, is the richest and most accessible known source of stem cells. It contains a specialized class of stem cells comprised of multiple cell types that promote healing and repair. Adipose stem cells have been shown to differentiate into multiple cell types including muscle, bone, fat, cartilage and nerve, etc. Beyond differentiation, regenerative cells may provide therapeutic benefit through the release of growth factors and other therapeutic healing mechanisms. The major advantages of adipose tissue as a source of regenerative cells, which distinguish it from alternative cell sources, include: **(1) Yield:** A therapeutic dose of regenerative cells can be isolated in approximately one hour without cell culture. **(2) Safety:** Patients receive their own cells (autologous-use) so there is no risk of immune rejection or transmission. **(3) Versatility:** Stem cells from adipose tissue benefit from multiple mechanisms-of-action.

There is crucial disagreement on the issue of functionality of these cells. Do the bone marrow-derived cells actively participate in extracellular matrix synthesis? Roufosse et al. in a mouse model of unilateral ureteric obstruction, detected bone marrow-derived α-SMA-positive cells. Using two reporter molecules under the control of the promoter and enhancer elements of the collagen I α2 chain gene, we did not however observe any functional bone marrow-derived fibroblasts or myofibroblasts producing collagen I. On the other hand, Iwano et al. in a mouse model of unilateral ureteric obstruction, and Broekema et al. in a rat model of unilateral I/R injury, demonstrated double immunostaining positivity of α-SMA-positive interstitial cells with pro-collagen I protein (Broekema et al. 2007).

In this project, with the mouse model, we aim to find the practical conditions to induce ES cells differentiating into renal stem cells and to find the ways using the renal stem cells to repair and regenerate obstructed kidney. For these, we will explore the techniques to induce ES cells to adopt a renal fate using co-culture with cell lines, metanephroi and novel growth factors. Meantime, we will characterize the expression profile of different renal subcompartments so as to identify the secreted proteins involved in renal differentiation and to isolate the specific cell surface markers identifying renal stem cells.

The theoretical background justifying the pursuit of the potential of bone marrow cells to participate in renal regeneration has been laid. Stem cells, both embryonic and from the adult bone marrow, in the right conditions, can express renal markers in vitro and give rise to renal cells in vivo.

In addition, injection of stem cells into the kidney or the bloodstream can lead to an improvement of renal function, although this does not always seem to be mediated by transdifferentiation into renal cells. Current views favour a predominant role for the delivery of a cocktail of angiogenic and immunomodulatory mediators as the main means by which bone marrow cells enhance epithelial and endothelial cell survival. As far as engraftment of bone marrow cells as renal parenchymal cells is concerned, proving functionality of the engrafted bone marrow-derived cells is crucial in order to assign to them a role in improved renal function, rather than relying on morphological observations alone.

The kidney is a complex organ with over 30 different cell types, and present technology does not envisage constructing a whole kidney from stem cells. However, within existing kidneys where the basic scaffolding is intact, stem cells may contribute to a variety of specialised cell types, either promoting more efficient repair or correcting genetic defects. These would include: (1) acute tubular necrosis (ATN) caused by toxins or ischaemia/reperfusion (associated with kidney transplantation); (2) mesangial damage, often associated with immune complex deposition and diabetes; (3) defective podocyte function (Alport's disease); (4) vascular endothelial damage (e.g. in glomerulonephritis) (Alison et al. 2007; Alison 2009).

Whether stem cell injections will ever be used for the treatment of renal failure is at this stage still unknown. There is certainly some hope to be found in the numerous animal models that have been developed and analysed over the last few years.

To reach the goal, we propose the following basic research objectives: (1) Use expression profiling to further dissect the processes of commitment to a metanephric fate during normal development. (2) Identify novel renal progenitor cell markers and growth factors to assist in the identification, isolation and/or reactivation of renal stem cells. (3) Examine the potential for ES cells to be differentiated into the lineages necessary for renal regeneration or endogenous repair.

3. Preliminary Studies

In this laboratory, we have used the obstructed rat kidney model in renal function project. The similar model of obstructed mouse kidney will be used in the stem cell research plan (Ma, et al, 2008; Nair, et al, 2008).

3.1 Pentoxifylline (PTF) study with rat kidney obstruction model (Ma, et al, 2008)

Experiments were performed on 40 male Sprague-Dawley rats (200-250 g) from maintained on regular rat chow or on the same diet with pentoxifylline (PTF) (400 mg/liter) added to the drinking water.

Studies were performed in 7 groups of rats as follows:

(1) Group 1 Normal (n=4)
(2) Group 2 (14 days sham-PUO + vehicle) (n=8). Sham-PUO rats receiving vehicle (drinking water) were studied 14 days after right nephrectomy.
(3) Group 3 (14-day PUO + vehicle) (n=8). Rats with PUO receiving vehicle in drinking water were studied 14 days after right nephrectomy and PUO.
(4) Group 4 (14-day PUO + PTF) (n=8). Rats with PUO receiving PTF (Sigma, St. Louis, MO, USA) (400 mg/l in drinking water) in drinking water were studied 14 days after right nephrectomy and PUO.
(5) Group 5 (30-day sham-PUO + vehicle) (n=8). Rats of sham-PUO receiving vehicle (drinking water) were studied 30 days after right nephrectomy.
(6) Group 6 (30-day PUO + vehicle) (n=8). Rats with PUO receiving vehicle in drinking water were studied 30 days after right nephrectomy and PUO.
(7) Group 7 (30-day PUO + PTF) (n=8). Rats with PUO receiving PTF in drinking water were studied 30 days after right nephrectomy and PUO.

3.1.1 Partial ureteral obstruction

The rats were anesthetized with sodium pentobarbital (50 mg/kg I.P.) and a low midline abdominal incision was made. Right nephrectomy was performed first. The right kidney was mobilized with minimal dissection. Two 2-0 silk ties were placed around the hilar vessels and the kidney was removed. The left ureter was then traced to its insertion in the bladder, mobilized with minimal dissection to preserve the surrounding neurovasculature and retracted with vessel loops. The psoas muscle was split by blunt dissection to create a space which would accommodate two-thirds of the length of the ureter. The left ureter was moved into that interstice, after which the muscle was reapproximated with three interrupted 5-0 silk sutures. The abdominal wound was then closed with sutures. For the sham operation, the rats underwent the same surgical procedure, including right nephrectomy, but the left ureter was diverted into the psoas muscle.

3.1.2 C1earance studies

Renal clearance experiments were carried out in rats with sham-PUO or with PUO (Chou et al. 2003). The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a heating pad to maintain body temperature. A tracheostomy was performed. Then the jugular vein and carotid artery were 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 was 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) was made and the bladder was cannulated for urine collection with a PE-240 catheter which had a mushroom-shaped cap. This was secured in the bladder with two 3-0 silk sutures.

Following the surgical preparation, the inulin/PAH were allowed to equilibrate in the rat for 60 minutes before blood and urine samples were obtained. Thereafter (starting at t=0), urine was separately collected from the bladder for three 30 minute periods. Blood samples were taken at t=45 minutes and t=105 minutes. GFR and renal plasma flow were measured with the standard methods of inulin and PAH clearance, respectively. At the end of the clearance experiments, the rats were sacrificed with an intravenous injection of sodium pentobarbital.

Urine volume was measured gravimetrically. Blood cells were separated from the plasma by centrifugation. Inulin concentrations in urine and plasma were measured by standard spectrophotometry. GFR was calculated by standard inulin clearance techniques.

3.1.3 Western Blot analysis of alpha-smooth muscle actin (alpha-SMA)

Accumulation of alpha-SMA, an indicator of tubulointerstitial fibrosis (Bohle A. Strutz F), was measured in the cortex and medulla by Western blotting. Immunoblot analysis of alpha-SMA protein in the cortex and medulla were performed in the sham-operated rats and rats with PUO in 7 groups of rats as described above. After the left kidney was removed through a midline abdominal incision, the cortex and medulla were separated and glass homogenized in lysis buffer on ice for total protein extraction. The homogenates were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were stored at -80°C in aliquots until analysis.

3.1.4 Histological studies

The kidney specimens were embedded in paraffin after overnight fixation in 10% neutral buffered formalin. Sections (5-μm thick) were 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 were graded on a scale from 0-4 under a micrometric ocular grid in accordance with the methods previously described (Remuzzi et al. 1999).

3.1.5 Radiological studies.

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

3.1.6 Statistical analysis.

All results are expressed as mean ± standard error. Experimental data were subjected to one-tailed analysis of variance and the statistical significance for multiple comparisons was determined by Tukey's *post hoc* test. $P<0.05$ is considered statistically significant.

3.1.7. Results and discussion of pentoxifylline study

At day 14 of PUO, pyelogram showed hydronephrosis, which became more pronounced at day 30 of PUO. Histological studies at day 14 of PUO revealed tubulointerstitial fibrosis, which became more intense at day 30 of PUO. Histological examination of the renal tissue showed that PTF treatment significantly reduced renal fibrosis at 30 days of PUO, findings supported by a decrease in the expression of α-SMA by Western blot.

In 6 normal rats, GFR was 1.23 ± 0.18 ml/min/g. In 6 rats with sham-PUO at 14 days fed on a regular diet, GFR was 1.10±0.07 ml/min/g, which was not significant different from the normal rats. At 14 days of PUO, GFR in 4 rats on a regular diet (control) was 0.41 ± 0.13, no significant difference from the 0.48 ± 0.05 ml/min/g in 4 rats treated with PTF, and the GFR of 14-day PUO of both control and PFT treated was significant lower that 14-day sham-PUO. In 6 rats with sham-PUO at 30 days fed on a regular diet, GFR was 1.19 ± 0.20 ml/min/g, which is not significant from the normal rats. At 30 days of PUO in 4 rats on a regular diet (control), GFR was 0.24 ± 0.12 ml/min/g that was significant lower than sham-PUO at 30 days fed on a regular diet ($p<0.005$). For the 30-day group, GFR was 0.89 ± 0.06 ml/min/g in 4 PUO rats treated with PTF, significantly higher than the 30-day rats on a regular diet ($P<0.001$). Treatment with PTF preserved renal function for the 30-day PUO rats.

Western blot showed that the α-smooth muscle actin expression (an indicator of renal fibrosis) in the medulla was 1.16±0.40 (densitometry unit) in control 30-day rats, significantly greater than 0.37±0.03 in PTF-treated 30-day rats. Histological examination of the renal tissue revealed that PTF treatment significantly reduced renal fibrosis in PUO rats, findings supported by a decrease in the expression of α-SMA by Western blot. In conclusion, a rodent model of PUO allowed chronic studies of morphological and histological changes of the partially obstructed kidney and demonstrated progressive tubulointerstitial fibrosis and a decline in filtration function. PTF treatment ameliorated renal fibrosis and helped preserve filtration function of the kidney.

3.2 Study of diabetic rats with streptozotocin (STZ)

Previously, we and others have shown that renal lipid metabolism may play important roles in renal inflammation, glomerulosclerosis and tubulointerstitial injury in diabetic nephropathy. We were also able to show that disturbance of renal expression of SREBP is involved in the development of nephropathy. Nuclear transcription factor SREBP-2 directly controls the gene transcription of 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGCR), the later participates *de novo* cholesterol biosynthesis and serves as the working target for statins inhibition. Thus far, the mechanism of statin-mediated renal protection remains elusive, and the modulation of renal lipid metabolism pathway by atorvastatin hasn't been documented to our best knowledge. In current report, we studied the therapeutic effect of atorvastatin in streptozotocin (STZ)-induced diabetic rat and its impact on renal SREBP expression. We have found marked improvement of proteinuria in diabetic rat

with 30 day atorvastatin treatment. In addition, the diabetic animals treated with atorvastatin also showed better blood glucose profile compared with the untreated STZ rat. To further explore the molecular mechanism underlying the observed atorvastatin-associated renal protection, we performed real time PCR and Western blot to assess the expression profile of SREBP. We found that in STZ diabetic rat kidney cortex, SREBP-1 was significant augmented by the introduction of diabetes; whereas SREBP-2 was slightly down-regulated compared to sham rat. Upon the administration of atorvastatin, the renal SREBP-1 was further induced by the agent ($p<0.05$), while renal SREBP-2 was dramatically down-regulated by atorvastatin treatment ($p<0.005$), compared with untreated diabetic group. Our results were further confirmed by PCR gene analysis. Thus, we demonstrated that renal cortex SREBP-1 and SREBP-2 were differentially modulated by STZ-induced diabetes or atorvastatin treatment. Based on our observation, we conclude that 1) atorvastatin attenuates proteinuria in STZ diabetic rat; 2) atorvastatin improves overall glucose homeostasis profile in STZ diabetic rat; 3) SREBP-1 and SREBP-2 are differentially expressed in STZ diabetic kidney cortex; 4) atorvastatin predominantly suppresses renal cortex SREBP-2; taken together, our results suggest that atorvastatin-associated renal protective effect may be partially mediated by suppression of renal SREBP-2 dependent lipid metabolic pathway and attenuation of hyperglycemia by atorvastatin may also contribute to its beneficial effect observed in STZ-induced rat diabetic nephropathy.

3.3 Studies of $P2Y_{12}$ in dialysis patients (Raval, et al, 2008)

Purinergic receptor is the molecular receptor for adenine, guanine, or other purines, or their nucleosides or nucleotides, including cyclic nucleotides other than cAMP. $P2Y_1$ and $P2Y_{12}$ are the two most important ADP receptors in platelet. This study sought to evaluate the effect of renal hemodialysis on $P2Y_{12}$ mRNA expression using real-time PCR (RT-PCR) and $P2Y_{12}$ level detected by Western Blot. ESRD patients underwent regular renal hemodialysis two times per week, and 20 ml of blood was drawn from each patient pre- and post-dialysis. Platelets were isolated by Ficoll-Pague method. Total platelet RNA was extracted using TRIzol reagent. Primers and probes were designed as follows: $P2Y_{12}$-sense: TCC ATT TTG CCC GAA TTC C; $P2Y_{12}$-antisense: CAG AGT ATT TTC AGC AGT GCA GTC A; $P2Y_{12}$-probe: 6 (FAM) CCT GAG CCA AAC CCG GGA TGT CT (TAMRA); GAPDH-sense: CAA GGC GCC CAA TAC G; GAPDH-antisense: CCA GGC GCC CAA TAC G; GAPDH-probe: (FAM) AAG GTG AAG GTC GGA GTC AAC GGA TTT G (TAMRA). $P2Y_{12}$ mRNA was detected by RT-PCR with TaqMan One-Step RT-PCR Master Mix Reagents Kit and the RT-PCR was performed using an ABI PRISM 7000 Sequence Detector. Western Blot was used to detect $P2Y_{12}$ receptor in platelet. $P2Y_{12}$ mRNA expression normalized to GAPDH was significantly decreased after hemodialysis (post-dialysis $2^{-\Delta\Delta Ct}=1.86\pm1.47$ vs. pre-dialysis $2^{-\Delta\Delta Ct}=5.61\pm1.87$, $n=14$, $p<0.01$). $P2Y_{12}$ level decreased 38% in platelet post-dialysis (Western Blot comparative band density post-dialysis 0.88 ± 0.23 vs. pre-dialysis 1.21 ± 0.17, $p<0.05$).

4. Research Design and Methods

4.1 GCSF gene primer design and cDNA synthesis

A. Construction of GCSF

cDNA is produced by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) using total RNAs isolated from human monocytes. A modified hGCSF cDNA (mhGCSF) lacking signal sequence, G+C content at 5' coding region of which is reduced without altering the predicted amino acids sequence and the codon TGC (C17A) is replaced with codon GCC, is produced by PCR by three steps based on the template as described above. These alterations respectively facilitate its translation in E coli and inhibit the formation of disulfide-linked oligomers to stabilize hGCSF. In first step, 5' 55 bp fragment is made by forward primer 5' CAT ATG ACA CCC CTA GGC CCT GCC 3' and reverse primer 5'C TTG CTC TAA **GGC** CTT GAG CAG G3'. 3' 484 bp fragment is made by forward primer 5'C TTA GAG CAA GTG3' and reverse primer 5'TGAATTCA TTA GGG CTG GGC AAG GT A3'. Both of fragments are denatured, annealed and elongated with DNA polymerase at 96°C for 10 minutes and at 72°C for 20 minutes. Finally, the modified 540 bp GCSF (mhGCSF) is made based with the following: CAT ATG ACA CCC CTA GGC CCT GCC as forward; primer; and TGAAT TCA TTA GGG CTG GGC AAG GT as reverse primer (Figure 1). The amplified products are fractionated on agarose gels. The GCSF cDNA eluted from the agarose gel is digested by Bam HI and EcoR I, and ligated to pBluescript II SK that has been cut with the same restriction enzymes. The ligation mixture is transformed into Escherichia coli DH5α competent cells for characterization of the cloned cDNA, and is confirmed by a thorough DNA sequencing analysis.

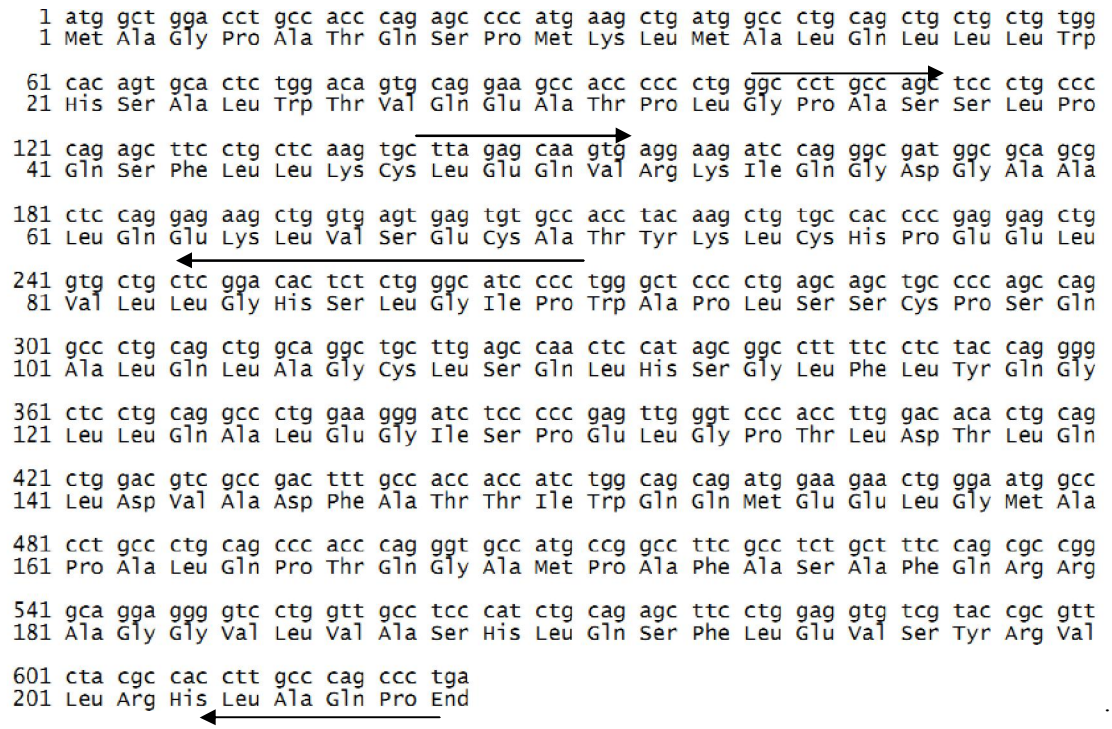

Figure 1. mhGCSF protein sequence and gene sequence (Gene Bank)

B. Expression in E coli

a. E coli strain and vector system:

E. coli BL21 (DE3) (Novagen, Inc.) is used as the host for rh-GCSF expression. This strain is transformed with a commonly available plasmid, pET23a inducible expression vector (Novagen, Inc.), in which the rh-GCSF gene (Biotehnology Research Center, Tehran, Iran) is inserted into the NotI and NdeI sites. Host cells are transformed with the plasmid using the calcium chloride procedure. Transformed cells are spread on several LB agar plates containing 100 mg/l ampicilin.

b. Media and solutions:

LB (Luria-Bertani) medium is used for plate cultivation of E. coli strain BL21 (DE3) and M9 medium is used for preparation of seed culture. M9 medium is used for preparation of seed culture. The M9 modified medium consisted of 10 g glucose, 15 g K_2HPO_4, 7.5 g KH_2PO_4, 2 g Citric acid, 2.5 g $(NH_4)2SO_4$, 2 g $MgSO_4.7H_2O$, and 1 ml trace element solution per liter. The trace element solution contained 2.8 g $FeSO_4.7H_2O$, 2g $MnCl_2$ $4H_2O$, 2.8 g $CoSO_4.7H_2O$, 1.5 g $CaCl_2.2H_2O$, 0.2 g $CuCl_2.2H_2O$, and 0.3 g $ZnSO_4.7H_2O$ g per liter in 1 M HCl. Batch cultivations are simultaneously carried out in two 2 l bench-top bioreactors with the working volume of 1 l.

c. Batch Cultivation

Batch culture is started by adding 100 ml of an overnight-incubated seed culture (OD600=0.7-1.0) into the bioreactor containing 900 ml of medium. The pH is controlled at 7±0.05 by the addition of 25% (w/v) NH_4OH or 3 M H_3PO_4. Dissolved oxygen is controlled at 30-40% of air saturation by controlling both the inlet air and agitation rate. Foaming is controlled by adding silicon-antifoaming reagent. In batch culture, cells are induced by the addition of IPTG (1 mmol/l) when initial DCW (2.2 g DCW/l) reached a considered level. Then, the production phase continued until the growth ceased. All batch fermentations are performed twice.

d. Rh-GCSF purification:

Cell lysis and IB recovery: The fermented broth is centrifuged at 4°C and 8000 g for 30 min and the obtained pellet is washed twice with 50 mM phosphate buffer pH7.4. The wet cells (50 g) are suspended in 200 ml of lysis buffer. The lysis buffer composition is 50 mM Tris–HCl containing 1 mM EDTA, 1 mM PMSF. The cells are broken by passing the medium through a homogenizer three times (NIRO-SOAVI) at 800 bar. The cells are cooled to 4°C between each pass. The cell homogenate is centrifuged for 30 min at 6000 g at 4°C, the supernatant is discarded and the inclusion bodies recovered. IBs washing: The IBs pellet obtained in

previous step is resuspended in wash buffer and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g. The washing protocol is 2.5 g/l Triton X-100 in 50 mM Tris–HCl pH 8.0 containing 5 mM EDTA and 1 mM PMSF. In the second washing, the IBs pellet is resuspended in wash buffer and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g. The washing protocol is 1 M urea. IB solubilization and refolding: Washed inclusion bodies are dissolved in 30m M Tris–HCl, pH=8, containing 6 M urea, 1 mM EDTA and 100 mM GSH, The solution is incubated at 25–28°C for 45 min and spun down at 10,000 g for 30 min to get rid of insoluble cell debris and recovered by centrifugation at 25–28°C for 30 min at 8000 g. and then solubilized inclusion bodies is refolded by refolding buffer that the refolding buffer protocol is 30 mM Tris–HCl (pH=7.5), 2 mM GSSG, 20 mM GSH, 1 mM EDTA, 3M Urea and incubated 12 hours at 4°C. After completion of refolding, the protein pH is adjusted with 2 M citric acid and centrifuged at 10,000 g for 20 min at 4°C. Anion Exchange Chromatography: The pH of Refolded protein is adjusted to 5-6 by adding 2 M Acetic acid and then loaded in mono Q column in FPLC (SYKAM-S2100). The column temperature and flow rate are maintained at 20°C and 1 ml/min respectively throughout the process. The column is equilibrated with 3 bed vol. of 25 mM sodium acetate buffer (pH=4.5). The refolded protein sample is directly loaded on to the column at the same flow rate. The column is extensively washed with 3 beds vol. of the same buffer but with 1 M NaCl.

C. Expression in yeast

a. Yeast and vector:

Pichia pastoris strain GS115 (Invitrogen) is used for mhGCSF protein expression. The hG-CSF cDNA is digested with XhoI and EcoRI restriction enzymes and ligated into the pPIC9 vector (Invitrogen), under the control of AOX1 promoter. The resultant recombinant vector is named as pPIC9/hG-CSF (Figure 2). Transformation of P. pastoris with pPIC9/hG-CSF: P. pastoris strain GS115 cells are made electro competent following manufacturer's instructions (Invitrogen, CA, USA). Approximately 10 µg of recombinant expression plasmid pPIC9/hG-CSF is linearized by digestion with the BglII enzyme, resulting in P. pastoris GS115 transformants carrying the His+ Mut+ and His+ Muts phenotypes. Electroporation is carried out by a Gene Pulser (Eppendorf Germany). Transformants are plated onto MD plates and incubated at 30ºC for 4 days. The parent pPIC9 without the insert, linearized with BglII is also transformed into P. pastoris and used as a negative control. Transformants carrying the methanol utilization plus (Mut+) phenotype are selected by growing on minimal methanol medium (MM) and minimal dextrose medium (MD) plates. Transformants bearing the chromosomally integrated copies of the pPIC9/hG-CSF are then detected by a genomic PCR assay using the 5′ and 3′ AOX1 primers.

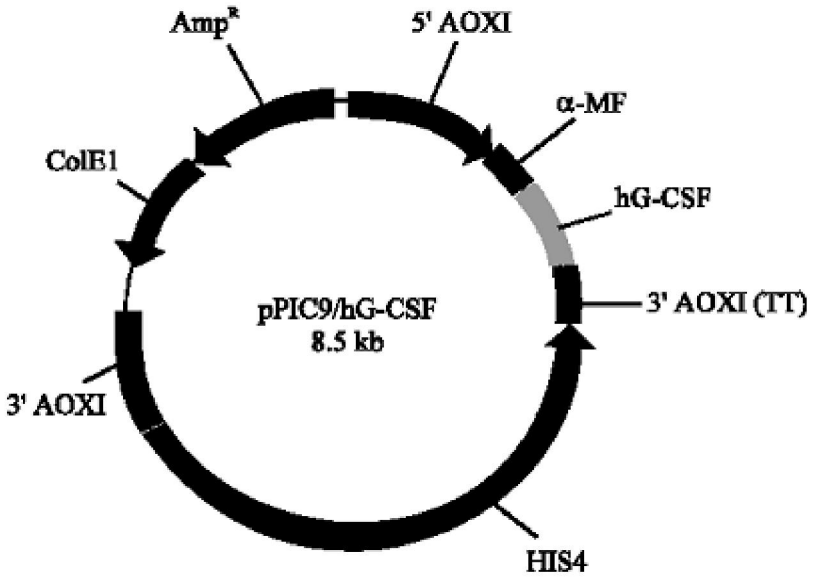

Figure 2. Vector pPIC9/hG-CSF

b. Small scale culture conditions:

Cells are grown in 10 ml BMGY medium at 30°C. After 48 hours of cultivation, the cells are pelleted by centrifugation at 3000 g for 5 minutes and re-suspended in BMMY medium. Induction is maintained for another 48 hours by spiking the cultures twice daily with 100 µl of 100% methanol (1% final concentration). Subsequently, the cultures were harvested by centrifugation (3000 g for 10 minutes) and the supernatants were frozen at -20°C until further use.

c. Ferment setup:

Ferment action is carried out in 5-liter BioFlo III or 3000 bioreactors (New Brunswick Scientific) containing two liters of FM22 medium supplemented with 8.7 ml PTM1 trace salts (added after autoclaving). The temperature is maintained at 30°C and the pH at 5.0 (controlled with concentrated ammonium hydroxide). The dissolved oxygen (DO) is set to 40% and controlled by an agitation/O_2 cascade. Pure oxygen is supplemented as needed to maintain the DO setpoint. A part of the o ff- gas is diverted to an MC-168 methanol monitor and controller (PTI Instruments) equipped with a TGS822 methanol sensor (Figaro Engineering), which are used to maintain a constant level of methanol in the broth. A methanol feed pump (Mo del 101 U/R, Watson-Marlow), balance (Model PR1203, Mettler Toledo) and the MC-168 controller are interfaced with the NBS-Bio Command supervisory control and data acquisition software to make a closed-loop feed control system. The amount of methanol delivered is measured u sing a balance as the difference between the initial mass in the methanol tank and the current mass. The P. pastoris strain mhGCSF is grown in a 1 liter shake flask with 300 ml B MGY medium for 20-24 h at 30°C and 300 rpm to an optical density (at 600 nm) of 4-8. Following microscopic exam in at ion for contamination, 100 ml of this pre-culture is used as inoculum. To increase cell mass and simultaneously prepare the cells for induction, a glycerol fed-batch phase is per-formed. A 1-hour fed-batch period is used at a feed rate of 20 g 50% w/w glycerol (containing 12 m l P TM1 trace salts per liter) per hour per liter of broth. Following the fed-batch phase, a transition phase is applied to shorten t he time required for t he cells to fully adapt to methanol. The transition phase is initiated by the addition o f 1.5 g/l methanol. Simultaneously, the glycerol feed rate was ramped down linearly from 20 g/l/h to 0 over a 3-h period. By the end of the transition phase, the cells are fully adapted to methanol, which is confirmed by a sharp drop in DO (Zhang, et al, 2000). Once the cells are fully adapted to methanol, the methanol fed -batch phase is started. Methanol supplemented with 12 ml of PTM1 per liter is used. Two feeding strategies are applied. The first is a methanol - excess feed strategy in which the methanol concentration is kept constant at ~2 g/l. This allowed the maxi mum specific growth rate (µ max) to be determined. The second strategy use d methanol-limited fee ding to maintain the growth r ate below µ max at a constant p re-determined value. This is achieved by varying the methanol feed rate (F) exponentially, using the following equation (Zhang, et al, 2000):

$$F=V_{MeOH}(X_0V_0)e^{\mu t} \qquad (1)$$

X_0 is the c ell density and V_0 the broth volume at the start of the feeding profile (t=0), µ is the desired specific growth rate (h^{-1}), and ν MeOH is the specific methanol consumption rate (g/g/h). The latter can be calculated once µ max and ν MeOH, max have been deter mined empirically under methanol excess conditions. For a desired µ, ν_{MeOH} is estimated by the following equation:

$$\nu_{MeOH} = \mu \ \nu_{MeOH,\ max}/\ \mu_{max} \qquad (2)$$

Equation (2) is base d on the as sumption that the yield of biomass t o substrate $Y_{X/S} = \mu_{max} / \nu_{MeOH,\ max}$ and is independent of µ and that the maintenance coefficient is negligible (Zhang, et al, 2000).

d. Protein purification:

All purification steps are conducted at 4°C. A GS115 hG-GCSF secreting clone is grown in BMGY-containing shake flasks at 30°C. After 24 hours of cultivation the cells are harvested by centrifugation and GCSF protein expression is induced by suspending the cell pellet in BMMY medium containing 2% methanol. At 24, 36 and 48 hours of induction, 2% methanol is added to maintain induction. After 60 hours, five liter of cultivation medium is then obtained upon centrifugation at 18,000 g for 30 minutes. Ammonium sulphate is added to 80% saturation and the suspension w as incubated overnight to precipitate the protein fraction. The protein pellet is isolated by centrifugation at 18,000 g for 30 minutes and re-dissolved in 90 ml of 25 mM sodium acetate pH 4.5, 0.1% CHAPS. After centrifugation at 40,000g for 30 minutes to remove a minor non-soluble fraction, this solution is desalted on a Sephadex G25 column of 475 ml (XK26×90 cm, GE Healthcare) to 25 mM sodium acetate pH 4.5, 0.1% CHAPS. The desalted protein fraction was loaded on a 20 ml Source 15 S column (XK 16×10 cm, GE Healthcare) to remove contaminants and potential endotoxins. After equilibration, GCSF is eluted by a linear gradient over 20 column volumes of NaCl from 0 to 1 M in 25 mM sodium acetate pH 4.5, 0.1% CHAPS. The obtained fractions are analyzed by SDS-PAGE. The major m GM-CSF-containing fractions

are pooled and loaded onto a HiLoad 26/60 Superdex 75 prep-grade size exclusion column (GE Healthcare) with PBS as the elution buffer. Following concentration of the product peak by ultrafiltration (Vivaspin 20, 3000 MWCO; Sartorius Stedim Biotech).

D. Analytical Methods

a. SDS-PAGE

In the reduced SDS-PAGE 15% gel and three standards of rh-GCSF (Neupogen®, Roche, Germany), (P Dgrastim, Pooyesh Darou, Iran) and a molecular weight marker (#SM0431, Fermentas) are used. Sample buffer [0.5 M Tris pH 6.8, 50% (v/v) glycerol, 100 g/l SDS, 20 g/l bromophenol blue and 50 g/l 2-mercapto ethanol (2-ME)] is added to three samples and standards before boiling for 5 min. The samples are loaded on to the gel and ran at a constant voltage of 120 V for 100 min. Gels are stained with Coomassie brilliant blue R250.

b. Western blotting

For confirmation of rh-GCSF band in gel, western blotting with polyclonal human GCSF antibody is performed. Separated proteins on the SDS-PAGE gels are transferred into a poly-vinylidene fluoride (PVDF) membrane (Roche Diagnostic, Germany) for recognizing the exact existence of rh-GCSF. PVDF sheet is blocked with 3% BSA in TBS-T solution (20 mM Tris-HCl, pH 7.5, 150 mm NaCl, and 0.05% Tween 20). Then, anti-GCSF polyclonal antibody is added at a dilution of 1:500 in TBS-T solution for 1 h. A second incubation with HRP anti-rabbit (1:1000) in TBS-T is carried out and the third incubation of 5-10 min is done with Diaminobenzine (DAB) solution. (0.5 mg/ml DAB, 0.1% H_2O_2) (Barreda, et al., 2004).

c. Endo-toxin assay:

The GCSF concentration is determined using the BCA assay (Pierce) and endotoxin levels are determined with the Toxin Sensor Chromogenic LAL Endotoxin Assay Kit (GenScript USA). The process generated 92 mg GCSF from 5 liters of supernatant (18 mg /l) with a purity >95% based on SDS-PAGE and an endotoxin level of 0.15 EU/mg.

E. Assay of GCSF bioactivity

a. In vitro bioactivity assay:

In vitro activity of recombinant GCSF is determined using the murine yelobalstic cell line NFS-60 as originally described by Shirafuji. We also employed this bioassay method as described above for measuring the activity of human G-CS Fusing NFS - 60 cells. Recombinant GCSF made in house as well as commercial one are used as positive controls. The concentrations that stimulate 50% cell proliferation rate (ED50) are obtained for each cytokine by a serial doses (10 pg, 20 pg, 100 pg, 200 pg, 1μg, and 20 μg per milliliter, respectively).

Animal neutropenia models: BALB/CICR C57 male mice with an average w eight of 22.5±1.2 g are irradiated with cesium-137 (4 Gy) for 5 days using Gammacell-40 apparatus (Nurolion, Canada) to induce leukopenia (Or animals are intravenously administered with cyclopho-sphamide at a dose of 50 mg/kg/day for 2 days). The mouse with neutropenia is subcutaneously administered with 1 mg/kg GCSFa or Neupogen. To determine the effect of the hGCSF, the samples are collected from the tail vein 24, 48, and 72 hr after administration. Peripheral white blood cells and neutrophils are counted using an automated hematology cell counter (Biochem Immunosystem). To get the percentage of poly-morphonuclear leukocytes, blood samples are analyzed by flowcytometry after stained with anti-CD11.

b. Plasma levels of the test proteins

Plasma levels of the test proteins are quantitated using Quantikine human GCSF ELISA kits. Blood samples are drawn at 1, 2, 4, 8, 12, 16, 24 hours post-injection, and are centrifuged. Then, the plasma is frozen at -80℃. A predose blood sample is drawn 1 day prior to injection of test compounds.

F. Application of GCSF in renal therapy

GSCF is applied to stimulate increasing of bone marrow-derived stem cells in kidney damaged rats. Bone marrow stem cells will be applied to differentiate into various renal cells (Imasawa, et al., 2001) to improve the mouse renal function. This will be considered as the exploration of the treatment on renal failure to enhance renal repair. The extent and involvement of the bone marrow-derived stem cells stimulated by GSCF in renal repair will be studied. The effects of GCSF on embryonic stem cells are also studied in this project.

G. Statistical analysis:

Data obtained from mouse studies are subjected to statistical analysis using a Q test. The results are considered statistically significant when P value is less than 0.05.

4.1 Animals and husbandry

C57BL/6 mice are obtained from Charles River Laboratories, Inc (Boston, MA, USA) and kept at a controlled temperature of 23°C, under a 12:12-h light:dark cycle. Mice have free access to commercial chow. 40 female mice and 40 male mice (20-25 g, labeling #1-40 for the pair) are used for mating

separately. After the first birth, baby mice are grown with the clearly labeling of the mother/father numbers and operated with PUO to induce renal disfunction in the age about 60 days. Meantime, the mother/father mice mate for ES cell isolation from embryos. The ES cells are isolated from embryos after the 5 days of the pregnant. The ES cells are induced differentiate into renal stem cells, and the renal stem cells are administrated to the damaged mouse kidneys come from same mother/father (sisters/brothers). 300 mice are used in the project (Table 1). The experiments are conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All animal studies are approved by the Institutional Animal Care and Use Committee at the Brookdale University Hospital and Medical Center.

Table 1. Mice used in the project

Mouse groups (total n=300)	Treatment and purpose
Group 1, mating, female, n=40	Normal, mating for pregnant to get ES cells
Group 2, mating, male, n=40	Normal, mating with female for ES cell
Group 3, sham, male, n=20	Normal, as sham
Group 4, control, male, n=100	PUO, control, no stem cell treatment
Group 5, stem cell treated, male, n=100	PUO, treated with stem cell

Total animals used: 300.

4.2 Partial Ureteral Obstruction (PUO)

Male mice weighing 25 to 30 g are used in this experiment. The mice are housed in individual metabolic cages, have free access to water, and observed for intake of food and water. After the induction of general anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, intraperitoneally) and a low midline abdominemal incision is made. All mice are operated the both side proximal ureteral ligation. The ureters are identified through a small suprapubic incision and are traced to insertion in the bladder individually then isolated from the surrounding tissues via an abdominal incision, and mobilized with minimal dissection to preserve the surrounding neurovasculature and retracted with vessel loops. The psoas muscle is split by blunt dissection to create a space which will accommodate two-thirds of the length of the ureter. The ureter is moved into that interstice, after which the muscle is reapproximated with 2 interrupted 4-0 silk sutures at the ureterovesical junction. The abdominal wound is then closed with sutures under aseptic conditions, and the ligation is released after 24 h and the animals, referred to as unilateral ureteral obstruction-treated, allowed to recover. After recovery from anesthesia, the mice are housed individually in plastic cages and given buprenorphine hydrochloride (0.02 mg/kg body weight, i.m.) to relieve postoperative discomfort. Before the surgery and clearance experiments, the mice fast but have free access to water. The sham operation consists of a similar suprapubic incision and identification of the left ureter, but ligation of the ureter is not performed (Ma, et al, 2008). The animals are conducted in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. The study is approved by the Institutional Animal Care and Use Committee.

4.3 Stem cell isolation and cell culture

The technique of magnetic selection using antibody binding will be used for the stem cell separation (e.g., labeled anti-CD34 antibody with magnetic nanoparticles binding to CD34+ cells).

4.3.1 Isolation of 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, USA) and 0.1 mM ascorbic acid 2-phosphate (Sigma, USA), 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.

4.3.2 Embryos treatment

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 (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, USA), 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.

4.3.3 Embryo stem 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 is done to provide blastocysts. The blastocysts are flushed to form uterus by M2 media 5 days after mating. Mouse embryonic fibroblasts in a natural cycle or in a superovulated cycle are prepared to form embryos in a midgestation age according to protocols discribed by Shimizukawa (Shimizukawa et al. 2005).

The blastocysts are transfered to 35 mm dishs on the mouse embryonic fibroblast feeder group or mouse embryonic fibroblast feeder layer group which perviously inactivated with mitomycin C (kyowa, Japan) 10 µg/ml for 2 h in CO_2 incubator. The ES media containg DMEM high glucose (Sigma, USA) + 20% FBS (Gibco, USA) + LIF 1000 IU/ml (Sigma, USA) + 2-mercaptoethanol 0.1 mMol (Sigma, USA) + L–Glutamin 2 mMol (Sigma, USA) and Penicillin/Streptomycin 100 mg/100 IU/ml (Sigma, USA) for 3 days. Disaggregation is carried out according to method discribed by Bongso et al. (1994) with some modification. Briefely, the outhgrowth ICM are disaggregated mechanically by hand pulled Pasteur pipette in different size in 50 µl DMEM media under mineral oil (Sigma, USA). Then, the disaggregated ICM is transfered 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, USA) in PBS. It is possible to trypsonize the cells in 96-well dishs 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 untill the cells became confluent enough for passage in 4-well dish (NUNC). Depending to doubelling 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, USA) is used according to manufactures instraction for alkalin phosphatase staining.

4.3.4 Cell culture medium

1. **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.
2. **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.
3. **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.

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

4.3.5 Mouse embryo stem 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 confluency, 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).

1. 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.
2. Add 5 ml mitomycin C (10 ug/ml) to a 10 ml dish.
3. Incubate the cells for 3 hours.
4. Remove the mitomycin C solution, rinse the dish 5 times with PBS and add fresh medium.
5. Gelatin coating of tissue culture plastic: Coat tissue culture dishses with 1% gelatin solution; Incubate for 4 hours; Wash 3 times with PBS.

4.3.6 Mouse renal stem cells isolation protocol detail

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. 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 FACScan (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, USA); 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).

4.3.7 Maintenance of mouse embryo stem cells (Nichols and Ying 2006)

Mouse embryo stem cells are isolated from the inner cell mass of 5-day old blastocysts, derived

from pregnant mice, and isolated mouse ES cells are grown on mitomycin C-treated feeder layer or on gelatin-coated dishes:

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

4.3.8 Tetraploid embryo stem 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.

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

1. **Cell surface marker analysis:** All staining reactions are performed using 10^5 cells in 100 ul of staining buffer. Mouset ES cells for stage-specific embryonic antigen-1 (SSEA-1) or freshly isolated mouse 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.
2. **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.
3. **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).

4.3.10 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).

4.3.11 In vivo differentiation

1. **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.
2. **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.
3. **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 or an MRPC cell suspension (10^6 cells) after sham operation. Renal function is assessed by serial measurement of serum creatinine and 24-hour creatinine clearance (Hishikawa and Fujita 2008).

4.3.12 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:

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

4.3.13 Formation of EBs and spontaneously differentiation

The ES colonies are cultured for 5 days on 24-well dish (Cellstar) in suspention state by adding 1% trypsine to ES media and removing LIF. Then, the EBs are trypsonized with mild 0.5% /0.5mM trypsin/EDTA (Sigma, USA) 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 spontanously 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.

4.4 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 tumours 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).

4.5 Analysis of the cultured renal stem cells

4.5.1 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, USA). The forward and reverse primers used are listed in Table 1. For *Pax2*, the RT-PCR primer set is used for mouse (LOC293992; Superarray Bioscience Corp., Frederick, MD, USA). The BD mouse 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 (Table 2):

Table 2. The sequences of primers used in RT-PCR

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).

4.5.2 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 mouse 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.

4.5.3 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.

4.5.4 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.

4.5.5 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.

4.5.6 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.

4.5.7 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. 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 hybridisation, 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.

4.5.8 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.

4.5.9 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.

4.5.10 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.

4.5.11 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 temprature 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.

4.5.12 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.

4.5.13 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.

4.5.14 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.

4.5.15 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.

4.5.16 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.

4.6 Analysis of renal repair effects by stem cells

4.6.1 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 mouse 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 mouse 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 mice 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.

4.6.2 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 mice and mice with PUO in 3 groups of mice 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, USA) 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, USA) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) 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, USA) 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, USA). The data for alpha-SMA immunoblot signals are normalized to the corresponding ß-actin band signals.

4.6.3 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, USA) 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).

4.6.4 Radiological studies:

Sham-operated mice and mice 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 mice are captured at 5 minutes by a portable x-ray machine (General Electric). A nuclear renal scan is also performed.

4.6.5 Blood biochemistry.

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

4.6.6 Kidney lipid contents.

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

4.6.7 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.

4.6.8 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).

4.6.9 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).

4.6.10 Blood and urine chemistries:

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

4.6.11 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).

4.6.12 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.

4.6.13 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 [18] with minor modifications as we have previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b).

4.6.14 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).

4.6.15 Lipid extraction and measurement of lipid composition

Lipids from the renal cortex are extracted by the method of Bligh and Dyer [19]. **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 [20-21].

4.6.16 Perfusion fixation of kidneys

Mice are anesthetized and perfused through the abdominal aorta as previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b)[20-21].

4.6.17 Periodic acid Schiff (PAS) staining

Paraffin sections are stained for PAS. The stained kidney sections are imaged with an Olympus

microscope and semi quantitatively scored in a blinded manner by the renal pathologist (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b).

4.6.18 Immunofluorescence microscopy

Staining for type IV collagen and fibronectin are performed in frozen kidney sections as previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b)[20-21]. For adipophilin imaging, paraffin-embedded sections are used and adipophilin is detected by incubating with antibodies to adipophilin (1:500) (Roche Biochemicals, Indianapolis, IN, USA), and then with Alexa-488- (Molecular Probes Inc., Eugene, OR, USA) labeled secondary antibody. Lipid droplets and nuclei are stained with Nile red and 4', 6-diamidino-2-phenylindol [22-23].

4.6.19 Electron Microscopy

Tissue is fixed in 3% paraformaldehyde in a 6:4 mixture of cacodylate buffer (pH 7.4; adjusted to 300 mosmol with sucrose) and 10% hydroxyethyl starch. The tissue is then post fixed in 1% buffered osmium tetraoxide. The sample is dehydrated in a graded series of ethanol, and embedded in an epoxy resin. Tissue is surveyed with a series of one micrometer sections for a representative sample. The selected specimens are thin sectioned, viewed and photographed with a Phillips electron microscope 201 (Phillips Electron Optics, Inc., Mahwah, NJ, USA). The sections are read by the renal pathologist in a blinded fashion for determination of basement membrane thickness and podocyte morphology (Jiang et al. 2005b)[23].

4.6.20 Synapse

The therapeutic efficacy of HMG-CoA reductase inhibitors in various renal diseases has been shown extensively in both experimental settings and clinical renal patient. However, the precise molecular mechanism underlying statin-mediated renal protective effect remains unclear. Pharmacologically, statin is a class of drugs inhibiting HMG-CoA reductase, a direct target gene of transcription factor sterol response element binding protein-2 (SREBP-2), subsequently suppressing de novo cholesterol biosynthesis and lowering serum cholesterol level. It is proposed that statin- mediated renal protective effect in diabetic animal models is direct suppression of renal inflammation process and possibly independent of its systemic cholesterol lowering effect, although the hypothesis hasn't been experimentally validated. Further, renal in situ cholesterol/lipid lowering effect mediated by statins treatment hasn't been examined yet. Previously, we and others have shown that renal lipid metabolism may play important roles in renal inflammation, glomerulosclerosis and tubulointerstitial injury in diabetic nephropathy. Furthermore, the facts that lipids serve as primary inflammation mediators participating in multiple disease processes have been very well recognized. Thus, we surmise that the disturbance of renal lipid metabolism may be involved in STZ associated renal injury, and the normalization of lipid metabolism abnormality contributes to statins-mediated renal protection. Based on the above mentioned rationales, we attempt to examine:

1. If renal SREBP-1, 2 expression/activation is modified in STZ mice. SREBP-2 is the principal transcription factor directly controlling the expression of HMG-CoA reductase gene expression. It is possible that SREBP-2 activity is altered and modified by statin treatment.
2. If HMG-CoA reductase expression is altered with or without the modulation of SREBP-2. Alternatively, HMG-CoA reductase could be modified post-transcriptionally.
3. If renal cholesterol or triglycerol content is altered in STZ mouse kidney and normalized by statin treatment.
4. If certain other important lipid metabolism pathways altered, such as lipid transport, lipid oxidation pathways, in addition to *de novo* biosynthesis namely SREBP-2/HMG-CoA Reductase pathway in STZ mouse and corrected by statin.
5. The statin may regulate certain key fibrosis associated factors such as VEGF, TGFb.

4.6.21 Parameters need to be analyzed

1. **Blood biochemistry.** Body/organ weight, serum lipids, BUN/Cr, e-GFR, cytokines (Interleukin 1b, TNFa, check one of them if possible, since these cytokines are elevated in patient with chronic kidney disease and suppressed by statin according to recent literature).
2. **Kidney lipid contents.** Determine cholesterol/triglycerol content from kidney tissue. (n=4-5 from each group)
3. **Protein expression in kidney. Use 1/2 kidney.** Western blot for HMG-CoA reductase. Save the rest for future use, n=3-4 from each group. VEGF or TGF level can be re-blotted with same filter.
4. **Nuclear extract for SREBP-2 protein. Use ½ kidney.** Including both nuclear and cytosol lysate in western blot and compare the abundance of nuclear form and cytosol form SREBP-2. Antibody can be obtained from Santa Cruz. (n=3-4 from each group).

5. **Kidney RNA for RT-PCR analysis. Use ½ kidney.** Ideally extract and keep RNA in 80% ETOH. Determine gene expression for SREBP-2, SREBP-1, HMG-CoA reductase, ABCA-1, IL-1, TNFa, podocyte markers nephrin, and podocin. (n=4-5 from each group)
6. **Histology study. Use 1/3 kidney.** PAS staining for fibrosis. Microphage infiltration/MCP-1 expression (n=3-4 from each group). May include some other markers later, depends on the result.

4.6.22 Data Analysis

The data are expressed as the mean±SD. The statistical significance of the results between samples obtained from four groups is determined by one-way analysis of variance with Student-Newman-Keuls analysis for multiple comparisons. Significance is accepted at the $p<0.05$ level.

5. Human Subjects

Not Applicable.

6. Vertebrate Animals

Three hundred C57BL/6 mice (Charles River Laboratories, Inc., Boston, MA, USA) will be used in this project. Mice are housed according to NIH guidelines and the study is conducted according to Brookdale Hospital's Animal Care and Use Committee approved protocol. Animal housing, maintenance, operating and post-operative facilities are available in the Animal Lab of Brookdale Hospital's.

7. Statistical Analysis

With Microsoft Office Excel and Jandel Scientific program SigmaStat (Sigma Chemical Co., St. Louis, Missouri, USA) will be used for data statistical analysis of transfected gene expression data. $P<0.05$ is considered statistically significant difference. Measured data are reported as mean±SD. The student t-test is used for comparison.

8. Expected Significant

9. Ethical Aspects of the Proposed Research

There will be no ethical conflict for this project.

10. Consortium/Contractual Arrangements

Not Applicable.

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