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CHAPTER

13

Induced Pluripotent Stem Cells

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OUTLINE

INTRODUCTION

An increasing number of patients with end-stage renal failure are undergoing dialysis therapy worldwide. It causes both medical and medicoeconomic problems. Renal transplantation has proven a successful therapy for most patients with end-stage renal failure, as the therapy results in a significant improvement in the patient's quality of life, prolongs survival and is considered cost-effective [1]. However, the annual increase in the number of new patients with end-stage renal disease who need a renal transplant, and the widening gap between the demand for and the supply of donor kidneys have led to a progressive shortage of donor organs for transplant. This has become a serious issue and is worsened by the problem of limited graft survival due to immune rejection [1].

Among the strategies to overcome these problems is kidney regeneration using stem cells. Stem cells may be divided into two large categories: organ-specific or somatic stem cells and pluripotent stem cells. In contrast to organ-specific stem cells that generally have a limited potential for growth and differentiation, pluripotent stem cells, such as embryonic stem cells (ESCs) cells

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[2–4] and induced pluripotent stem (iPS) cells [5–7], have a virtually unlimited replicative capacity on culture dishes and are theoretically able to give rise to any cell type in the body. Stem cells have increasingly been used as a model system for understanding developmental mechanisms. In addition, in vitro culture and differentiation of stem cells offer unique opportunities for disease modeling, drug discovery, toxicology and cell replacement therapy [8]. The generation of specific functional cell types from ESCs has been demonstrated, including neural cells (several kinds of neuron and glia), vascular endothelia and smooth muscle, cardiomyocytes, hematopoietic cells, pancreatic insulin-producing cells and hepatocyte-like cells [8]. However, the protocol for in vitro differentiation of pluripotent stem cells into renal lineage cells has not been fully established.

Other approaches to regenerate kidney have also been investigated using organ-specific local stem cells within the kidney and bone marrow-derived hematopoietic stem cells [9]. Kidney regeneration using mesenchymal stem cells localized in bone marrow has also been investigated [10]. However, the approaches are still being developed and the role of these stem cells in kidney regeneration remains to be well defined.

Therapeutic approaches using human ESCs face two major problems. One is the ethical issue derived from the use of human fertilized eggs, and the other is immune rejection in any cell or tissue transplantation due to histocompatibility antigenic differences between ESCs and patients. These problems have been overcome by a breakthrough experiment by Takahashi and Yamanaka. They identified four factors normally found in ESCs, Oct3/4, Sox2, c-Myc and Klf4, that were sufficient to reprogram both mouse and human somatic cells to closely resemble mouse and human ESCs [5–7]. They named these iPS cells. Since iPS cells can be generated from somatic cells of patients, clinical approaches using iPS cells are not associated with the two above problems (use of human fertilized egg and immune rejection). In the next natural step after iPS cell creation, significant progress has been made in redifferentiating iPS cells into somatic cells. As is the case with ESCs, iPS cells have been redifferentiated into several somatic tissues, including active motor neurons [11], insulin-secreting islet-like clusters [12], hepatocyte-like cells [13,14] and a number of cardiovascular cells (arterial endothelium, venous endothelium, lymphatic endothelium, cardiomyocytes), but not kidney [15,16].

This chapter first summarizes the mechanisms of kidney development and the research on the directed differentiation of ESCs into renal lineages based on the knowledge of kidney development. In vitro generation of kidney using the undifferentiated cell mass in amphibian eggs, similar to mammalian pluripotent stem cells in that the cell mass can differentiate into various organs in vitro, is also described as a reference to kidney regeneration in mammals. Recent advances in the iPS cell research and technology are then reviewed, and finally the future direction of iPS cells in the field of regenerative nephrology is described.

MECHANISMS OF KIDNEY DEVELOPMENT

Vertebrates develop successively three kidneys: pronephros, mesonephros and metanephros. The three kidneys consist of a basic functional unit, the nephron, although the number of nephrons differs among kidneys [17]. The kidneys are derived from a portion of the early embryonic germ layer, the intermediate mesoderm, that is located between the lateral and paraxial mesoderms [18]. A lineage tracing experiment has demonstrated that intermediate mesodermal cells expressing *Odd-skipped related* (*Osr*)*-1*, an essential transcriptional factor for intermediate mesoderm and kidney development, give rise to all components consisting of mammalian adult kidney metanephros [18]. Metanephros is formed by the reciprocal interaction between two tissues derived from the intermediate mesoderm: the metanephric mesenchyme and the ureteric bud [17]. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules. By creating a novel culture system which uses the coculture with the cell line expressing a renal-epithelializing factor *Wnt4*, it was demonstrated that mouse metanephric mesenchyme contains multipotent progenitor cells that can give rise to several kinds of epithelial cells found in adult kidney, such as glomerular podocytes and epithelia of proximal and distal renal tubules and the loop of Henle [19]. The progenitor cells are contained only in the cell population strongly expressing *Sall1*, a zinc finger transcriptional factor that is essential for kidney development. It was also shown that the progenitor cell population can reconstitute a three-dimensional kidney structure in vitro, which contains glomerulus- and tubule-like components in an organ culture setting (Fig. 13.1A–C). The presence of progenitor cells was also demonstrated in vivo using a lineage tracing experiment for *Six2*, which is also an essential transcriptional factor for the formation of kidney [20].

The progenitors in the metanephric mesenchyme differentiate into epithelia after Wnt stimulation, but a cell fate decision is required for further differentiation towards glomerular podocytes, proximal or distal renal tubules, or the loop of Henle. The molecular mechanisms of the cell fate decision are largely unknown,

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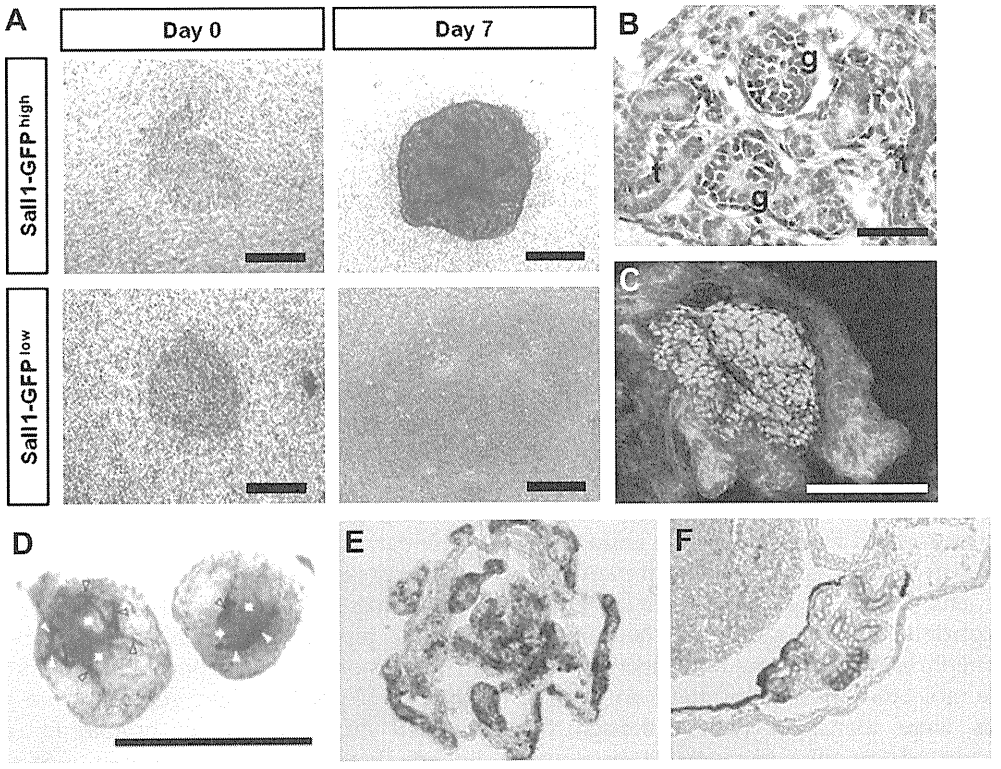

FIGURE 13.1 In vitro generated kidneys. (A–C) Metanephric kidney formed in vitro from multipotent progenitor cells in embryonic mouse kidney. (A) Metanephric cells strongly expressing *Sall1* (Sall1-GFP^{high} cells; upper panels) that contain multipotent progenitors differentiate into kidney structure in an organ culture setting, while those weakly expressing *Sall1* (Sall1-GFP^{low} cells; lower) disappear. (B) Hematoxylin & eosin staining of sections of kidney structure formed in vitro from Sall1-GFP^{high} cells. Tubule- (t) and glomerulus-like structures (g) are seen. (C) Double staining with WT1 (red, podocyte marker) and *Lotus tetragonolobus* lectin (LTL, green, proximal tubule marker) of kidney formed from Sall1-GFP^{high} cells. (D–F) Pronephric kidney formed in vitro from an undifferentiated cell mass in amphibian eggs (animal cap). Double immunostaining with pronephric tubule-specific antibody 3G8 (red) and pronephric duct-specific antibody 4A6 (blue) of kidney structure formed in animal cap treated with activin A and retinoic acid (D, E) and a stage 40 *Xenopus* larva (F). (D) Whole-mount staining. (E, F) Section staining. Scale bars: (A) 500 μm, (B, C) 25 μm, (D) 1 mm. Please see color plate at the back of the book.

although it was demonstrated that *Notch2* is required for the differentiation of proximal nephron structures, such as podocytes and proximal renal tubules, as *Notch2* deletion leads to the impaired formation of these proximal structures [21]. There may be many other molecules involved in the lineage commitment, and they remain to be elucidated. Metanephric mesenchyme may contain at least two other precursor populations in addition to the epithelial progenitors: (i) vascular precursor cells that can give rise to vascular and glomerular endothelial cells, vascular smooth muscle cells (pericytes) and mesangial cells, and (ii) stromal precursors eventually differentiating into interstitial cells within the adult kidney. The ureteric bud is known to elaborate the lower urinary tract system, from collecting ducts through renal pelvis and ureters to a part of the urinary bladder [17]. Most of the mechanisms of the lineage commitment, by which the intermediate mesoderm gives rise to the ureteric bud, stromal and vascular cells, are unknown. Further investigations are required to elucidate the mechanisms, which will eventually help us to completely understand the commitment of multiple cell lineages within a kidney and reproduce it in vitro from pluripotent stem cells.

ANIMAL CAP IN FERTILIZED EGGS OF AMPHIBIANS

Amphibians have been used as experimental animals for research into developmental biology mainly because of the ease with which they can be handled and observed. In amphibian eggs, the ectodermal cell mass of mid-blastula embryos, called the animal cap, is similar to ESCs and iPS cells in mammals in that it possesses a potential of multipotent differentiation. The animal cap can be easily excised from fertilized eggs and cultured as explants in vitro in simple saline solution. After treatment with differentiation-inducing factors, the animal cap can differentiate into various tissues and organs in vitro [22]. The pronephros is a simple excretory organ of *Xenopus* larvae with only

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one nephron, consisting of the glomus, a filtering unit equivalent to the glomerulus in metanephros, tubule and duct. When animal caps were treated with a combination of activin A, a protein inducer belonging to transforming growth factor-β (TGF-β) superfamily, and retinoic acid, differentiation into pronephric tubules was observed [23]. In the same differentiation system, the marker gene expression for pronephric glomus was also detected [24]. It was demonstrated that the pronephric duct, a third component of the pronephric kidney, can be generated in the explants, in addition to the pronephric tubule and glomus, and that the pronephros formed in vitro is similar to that in *Xenopus* embryos both histologically and in gene expression [25] (Fig. 13.1D–F). The pronephric tubule and duct also show similar ultrastructure to those of *Xenopus* embryos by electron microscopy.

The pronephros formed in vitro from the animal cap shows a temporarily similar gene expression pattern to that in *Xenopus* embryos. Using the in vitro system to generate pronephric tissue, the molecular mechanisms underlying pronephros development have been investigated and some molecules involved in the formation of the pronephros were identified [22]. Although the pronephros produced in vitro from the animal cap cannot be directly translated to clinical applications, this system should facilitate the study of kidney regeneration and may promote a shift from tissue engineering to clinical applications. Indeed, this induction method established for the animal cap using activin A and retinoic acid has been applied in a differentiation experiment from mouse ESCs into renal lineages [26].

EMBRYONIC STEM CELLS

ESCs are pluripotent stem cells derived from the inner cell mass of fertilized eggs in mammals. The first derivation of mouse ESCs by Evans and Martin was described in 1981 [2,3] and human ESCs was established by Thomson about 20 years later [4]. Both mouse and human ESCs have a virtually unlimited replicative capacity and are theoretically able to give rise to any cell type in the body. Since their derivation, mouse ESCs have mainly been used as a research tool to generate experimental mouse models by combination with gene targeting techniques. However, following successes in human ESC derivation, the regenerative medical strategy has been considered closer to clinical applications, which aim at accomplishing the functional recovery of dysfunctional organs by transplanting organ-specific cell types generated from ESCs in vitro. The in vitro differentiation of ESCs into specific cell types of various organs has since been more vigorously investigated to develop cell transplantation therapies.

In vivo injection of ESCs into immunocompromised mice can produce teratoma, which are tumors containing cells of all three embryonic germ layers (ectoderm, mesoderm and endoderm). Glomerulus- and tubule-like structures can be formed in teratomas derived from mouse [27] and human ESCs [4], which demonstrates that mouse and human ESCs do have a potential to differentiate into kidney lineage cells. Furthermore, fundamental research aiming to establish methods to efficiently induce mouse ESCs into renal progenitors and fully differentiated renal cell types have already been carried out [26,28–31]. Mouse ESCs treated with the combination of hepatocyte growth factor (HGF) and activin A in addition to the transfection of a renal-epithelializing factor Wnt4 [28], the combination of activin A, bone morphogenetic protein (BMP)-7 and retinoic acid [26], activin A alone [29], BMP-4 alone [30], or the combination of four chemical compounds [31] produced cells expressing markers for intermediate mesoderm, developing kidney and fully differentiated renal cells. In some reports, the induced cells formed tubule-like structures or were incorporated into developing mouse kidneys [26,28,29]. Few reports have described the directed differentiation of human ESCs into a renal lineage. It was shown that human ESCs differentiated in vitro into cells expressing WT1 and rennin, marker genes for glomeruli, following treatment with growth factors [32]. Combinational treatment with activin A, retinoic acid and BMP-4 or BMP-7 also induces marker gene expression for intermediate mesoderm and developing kidney from human ESCs [33].

In summary, these data suggest that ESCs are a potential source for kidney regeneration, although the efficiency of generation and the induction rate of renal lineage cells from ESCs are unknown in many cases. It remains to be determined whether renal lineage cells produced from ESCs have excretory or endocrinological functions as in the kidney. It is also unknown whether tubule-like structures or cells integrated into the kidney are metanephric or of earlier kidneys (pronephros and mesonephros). The research on kidney regeneration using ESCs is in its infancy and further studies are required to develop this research field.

CREATION OF INDUCED PLURIPOTENT STEM CELLS

Development involves two distinct cellular processes: division and differentiation. Division is the means by which one cell gives rise to two daughter cells, and is indispensable for the growth of an organism and the renewal of fully developed tissues. Differentiation refers to the process by which a cell specializes to perform a particular biological function in an adult.

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Differentiation usually occurs through a combination of cell–cell interactions, exposure to diffusible factors and other positional cues that ultimately alter gene expression, conferring a specific cellular identity and function.

One of the more remarkable observations made in the past century was that differentiation is not a unidirectional process. Instead, it can be turned back much like the hands of a clock. This rewinding of the developmental clock is termed nuclear reprogramming and is often defined as the process whereby an adult somatic nucleus has a developmental potential restored to it [34]. Nuclear reprogramming has been accomplished in three ways: (i) somatic cell nuclear transfer (SCNT) or cloning, (ii) cell fusion, and (iii) factor-based reprogramming to produce iPS cells.

SCNT, a procedure in which the nucleus of an adult cell is injected into an unfertilized egg whose chromosomes have been removed, has demonstrated that the genome of adult cells can be reset to an embryonic state [35,36]. Using this strategy researchers have generated cloned embryos that possess the potential to develop into an adult animal or become an ESC line that is genetically identical to that of the donor nucleus [37,38]. These experiments established that no irreversible changes are made to the genome during development and further showed that animal oocytes harbored factors that could accomplish nuclear reprogramming.

In a related series of experiments, several researchers have shown that when somatic cells are fused with ESCs, the resulting tetraploid hybrid cells silence the expression of somatic genes and establish a program of transcription indistinguishable from ESCs, indicating that ESCs contain the necessary reprogramming activities to accomplish this transformation [39,40]. Thus, the cytoplasm of the enucleated oocyte and the ESC is able to re-establish the pluripotent state via a mechanism dependent on global epigenetic and transcriptional changes.

The mechanism by which this transformation occurred and the mediators of nuclear reprogramming were largely undefined until Takahashi and Yamanaka identified four factors normally found in ESCs that could reprogram mouse somatic cells (see Introduction).

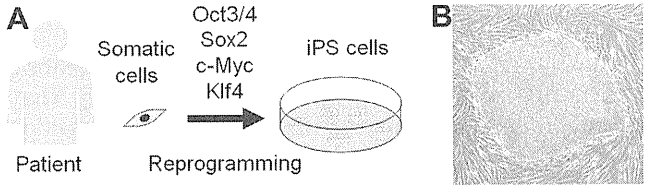

FIGURE 13.2 (A) Induced pluripotent stem (iPS) cells can be established by introducing genes encoding four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into fibroblasts. (B) An iPS cell colony generated from adult human dermal fibroblasts by introducing the four factors using retroviral vectors. Please see color plate at the back of the book.

They called these iPS cells [5] and these stem cells were also derived from human somatic cells 1 year later (Fig. 13.2A, B) [6,7]. Since this first report, the technique has been rapidly confirmed, improved and subsequently applied to successfully reprogram somatic cells. This technology has since given birth to an entire research field that has progressed at a phenomenal pace.

CELLS AND ANIMALS TO USE FOR THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS

The rate at which iPS cell lines are being created is rapidly increasing. While most iPS cells were created from fibroblasts in the beginning, cell lines have also been created from adult liver and stomach cells [41], adult neural stem cells [42], keratinocytes [43], hematopoietic cells [44–46], fetal cells harvested during both amniotic fluid and chorionic villus sampling, and several other somatic cell types [44]. It appears that the reprogramming is possible in almost any cell type, although the efficiency of the reprogramming process varies among cell types. Finding the most suitable somatic cell types to accomplish reprogramming remains a main challenge for iPS cell research.

The derivation of iPS cell lines from species other than human or mouse cells expands the research potential of iPS cell in additional animal models. The generation of iPS cells was reported from adult rhesus monkey fibroblasts [47]. Two separate groups also created iPS cells from adult rat cells [48,49].

In addition, iPS cells have been derived from somatic pig cells by two other groups. One group used tetracycline-inducible human Oct4/Sox2/Klf4/c-Myc/Nanog/Lin28 delivered via lentiviruses to transduce primary pig ear fibroblasts and primary bone marrow cells into iPS cells [50]. The other group transduced porcine fetal fibroblasts into iPS cells using human Oct4/Sox2/Klf4/c-Myc delivered with lentiviruses [51]. It has been reported that iPS cells was generated from canine cells by the combination of retroviral transduction and chemical inhibitors [52]. It is possible that culture conditions for the derivation or maintenance of iPS cells from species other than human or mouse have not been fully established. However, the availability of model animal iPS cells offers a new and potentially powerful model for therapeutic applications.

MECHANISTIC UNDERSTANDING OF REPROGRAMMING PROCESSES

One of the major questions in iPS cell research seeks to define the underlying mechanism by which nuclear

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reprogramming is accomplished. This includes identifying the molecular players, the key cellular and molecular events and the likely ways in which this process might fail. The goal is to make the iPS induction process safer and more efficient, and one day to manipulate the underlying cellular state of any cell, thereby generating specific cell types at will. Indeed, it was demonstrated that in vivo expression of the combination of three defined factors, Ngn3 (also known as Neurog3), Pdx1 and Mafa, which are key developmental regulators of the pancreas, can reprogram differentiated exocrine cells in adult mice into cells that closely resemble beta-cells [53]. It has also been shown that the combination of three factors, Ascl1, Brn2 (also known as Pou3f2) and Myt1l, rapidly and efficiently converted mouse embryonic and postnatal fibroblasts into functional neurons in vitro [54]. The neurons generated were named induced neuronal (iN) cells.

The nature of the factors required for the reprogramming of somatic cells into iPS cells has been elucidated. In particular, Oct4 has emerged as a central molecule in iPS cell reprogramming; for example, neural stem cells can be reprogrammed to iPS cells with Oct4 alone, which was not unexpected as they express high levels of endogenous Sox2 [42]. Until recently, no cell line had been reprogrammed without Oct4. However, it has been shown that the orphan nuclear receptor Nr5a2 (also known as Lrh-1) can replace Oct4 in the derivation of iPS cells from mouse somatic cells in part through activating Nanog expression [55]. While Yamanaka's original four-factor combination remains the most widely used, several other combinations have been shown to generate iPS cells. One of the earliest alternative combinations was developed by the Thomson group. They successfully used Oct4/Sox2/Nanog/Lin28 to create iPS cell lines [7]. Recently, it has been shown that mouse embryonic fibroblasts (MEFs) were able to reprogram using Esrrb, which is an orphan nuclear receptor, in combination with Oct4, Sox2 and c-Myc [56].

Several reports have shown that some of the reprogramming factors can be replaced with chemicals. In these cases, chemicals either directly activate the expression of reprogramming factors or in some way compensate for their activity. A Harvard group added valproic acid (VPA), known to be a histone deacetylase (HDAC) inhibitor and a widely used antiepileptic drug, to newborn human skin (fibroblast) cells in culture and was able to create iPS cells with only two reprogramming factors, Oct4 and Sox2 [57], eliminating the need for two potent cancer-promoting genes, *c-Myc* and *Klf4*. Another group used a combination of the small molecules BIX-01294 and BayK8644 to generate iPS cells from MEFs that were transfected with only Oct4 and Klf4 [58]. It has also been demonstrated that a small-molecule inhibitor of TGF-β signaling can replace Sox2 by inducing Nanog expression [59]. The promise of these approaches is to generate iPS cells using chemicals alone. However, great care should be taken over the toxicity of chemicals, including their carcinogenicity.

IMPROVING METHODS FOR INDUCED PLURIPOTENT STEM CELL INDUCTION

The original method to generate iPS cells uses genome-integrating viral vector, retroviral or lentiviral vectors [5–7], and it causes the problem of potential carcinogenesis. To overcome this issue, several groups have recently developed alternative iPS cell production methods. The iPS cells produced in each new method appear to be very similar to those produced in the traditional method. Each new method has its own advantages and disadvantages compared with the original one, and each provides insight into how scientists may be able to develop iPS cells that are safe for use in clinical trials.

Adenoviruses were used to deliver reprogramming factors into adult mouse liver cells [60]. Newborn mouse fibroblasts were also transduced; however, these were transgenic and required doxycycline induction of Oct4 expression for iPS generation. In addition, three human iPS cell lines were established from fibroblasts using adenoviruses [61]. The adenoviral approach is advantageous because it avoids integrating exogenous genes into the genome, avoiding the potential of insertional mutagenesis. The virus needs to be present for only a short time to accomplish reprogramming. However, the technique is inefficient compared with iPS cell transduction with retroviruses, it still uses cancer-promoting genes, and the adenovirus may still integrate into the host DNA at low frequencies. Recently, a Japanese group succeeded in the generation of human iPS cells by introducing reprogramming factors with Sendai viral vectors [62]. Sendai virus is an RNA virus and carries no risk of altering the host genome. They also showed that Sendai virus-derived transgenes were decreased during cell division. This approach may provide a solution for insertional mutagenesis.

The Yamanaka group reported success in generating murine iPS cells without using any viruses [63]. They successfully reprogrammed mouse cells by transfection with two plasmid constructs carrying the reprogramming factors; the first plasmid expressed c-Myc while a second, polycistronic plasmid expressed Oct3/4, Klf4 and Sox2. In a related approach, another group used one polycistronic construct expressing all four factors to achieve nucleofection in MEFs and induced iPS cell formation [64]. These methods avoid viruses entirely

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but still require cancer-promoting genes to accomplish reprogramming. As with the adenoviral strategies, plasmid-based approaches are much less efficient than retroviral methods and begin with embryonic skin cells, which may be more amenable to reprogramming than adult skin cells. Moreover, transfected plasmids have been shown to integrate into the host genome and therefore pose a risk of insertional mutagenesis [65].

Three separate research groups addressed the low efficiency of non-retroviral approaches to iPS cell induction by using the piggyBac transposon system to deliver the Oct4/Sox2/Klf4/c-Myc reprogramming factors to MEFs [66–68]. piggyBac is unusual among transposon systems because upon re-excision of the exogenous genes, no footprint mutations are left in the host cell genome.

Fibroblasts from Parkinson's disease patients have been reprogrammed using floxed doxycycline-inducible lentiviral vectors that can be excised using Cre-recombinase [69]. While this strategy yielded human iPS cells with global transcriptomes that more closely resembled those of human ESCs, a genomic footprint (the loxP site) was left behind, so the mutagenicity of the retroviral approaches remains.

One report described the use of the episomal vector oriP/EBNA1 to generate iPS cells from human foreskin fibroblasts [70]. This vector is duplicated as an extrachromosomal episome once per cell cycle and is stable as long as drug selection is used. In the absence of drug selection, the episomal vector is lost at a rate of 5% per iPS cell generation. After a few generations, iPS cells that do not carry the vector can be isolated. The major disadvantage to this approach is its low efficiency.

It was reported that iPS cells were successfully generated using recombinant proteins [71]. The protein reprogramming factors were delivered into MEFs by conjugating the proteins to polyarginine, a short peptide that mediates protein transduction. A parallel approach was shown to work in human fibroblasts by fusing the Oct4/Sox2/Klf4/c-Myc factors to cell-penetrating peptide sequences [72]. The major advantage of these protein-based strategies is that exogenous genes are not introduced. However, the strategy is again rather inefficient. Improved and efficient methods to derive iPS cells need to be developed without the integration of transgenes.

INCREASING EFFICIENCY OF INDUCED PLURIPOTENT STEM CELL INDUCTION

The efficiency of reprogramming adult fibroblasts remains low (< 0.1%). Whether this frequency reflects the need for the precise timing, balance and absolute levels of expression of the reprogramming genes, or selection for rare genetic/epigenetic changes either initially present in the somatic cell population or acquired during prolonged culture remains unsolved. Although considerable advances have been made in identifying the complex networks involved, it is not yet understood how these factors maintain pluripotency, how growth factors control and stabilize these networks, or how these cells respond so precisely to differentiation cues. Certain small molecules seem to improve the efficiency of the iPS cell generation process, including VPA [73], 5-aza-cytidine [74] and BIX01294 [75]. More chemicals that improve the efficiency of iPS cell reprogramming are expected to be identified. It has also been shown that mouse ESC-specific microRNAs (miRNAs), miR-291-3p, miR-294 and miR-295, can replace c-Myc and enhance the efficiency of three factor (Oct4/Sox2/Klf4)-induced reprogramming from MEFs [76]. It may be possible that other small RNAs can replace additional factors, and further study of the targets of these miRNA may offer insights into the mechanisms of reprogramming. Ultimately, the goal is to develop a cocktail of reprogramming factors that efficiently and reliably transduces somatic cells to iPS cells.

Attention has focused on p53 as a key player in the efficiency of iPS cell transduction. In 2008, it was demonstrated that adding p53 siRNA (small interfering RNA) to the Oct4/Sox2/Klf4/c-Myc reprogramming factors increased the rate of iPS cell colony formation by up to 100-fold [77]. However, many of the resulting iPS cells were only partially reprogrammed, and none yielded teratomas in vivo.

The central role of p53 in controlling iPS cell transduction has been better defined in several newly published papers. The Yamanaka group showed that in homozygous p53 knockout MEFs, 10% of the cells could be transduced to iPS cells with three reprogramming factors (Oct4/Sox2/Klf4) [78]. They further showed that terminally differentiated, p53 null T cells could be turned into iPS cells. Another group arrived at similar conclusions when they transfected cells with p53 shRNA (small hairpin RNA), and when they transduced $p53^{+/-}$ and $p53^{-/-}$ MEFs into iPS cells [79]. They further showed that reducing p21 and Bax levels, two factors downstream of p53, also increased the efficiency of iPS cell transduction. Two other groups focused on the Ink4/Arf locus, which is responsible for inhibiting Mdm2, which in turn is the main destabilizing enzyme of p53 [80,81]. One group found that downregulating tumor suppressors contained in the Ink4/Arf locus increased the efficiency of iPS cell transduction. The other group showed that cells with low endogenous Ink4a/Arf locus products are more readily reprogrammed into iPS cells, and that genetic ablation of p53 converts non-reprogrammable somatic cells into cells that could be transduced to iPS cells. Lastly, it was also shown that p53 is responsible for preventing

10013-GOLIGORSKY-9780123809285

iPS cell transduction of G3 Terc$^{-/-}$ MEFs, which are cells with short telomeres [82]. Taken together, these data show that the molecular network surrounding p53 strongly inhibits iPS cell transduction and the disruption of this network increases the efficiency of iPS cell generation many-fold.

While the precise molecular mechanisms behind p53 inhibition of iPS cell reprogramming is unknown, a newly published study suggests that the very process of reprogramming upregulates tumor suppressor expression. It was found that the Oct4/Sox2/Klf4/c-Myc reprogramming factors induced DNA damage and chromatin remodeling, thereby resulting in the development of senescence characteristics, including p16, p21 and p53 expression [83]. Vitamin C improves mouse and human iPS cell generation by reducing p53 levels and alleviating cell senescence while maintaining an intact DNA repair machinery [84].

COMPLETE PLURIPOTENCY

Previously, iPS cells had not been shown to contribute fully to all cell types in adult organisms. In a significant breakthrough, three separate groups have reported that in mice, MEF-derived iPS cells could be injected into tetraploid blastocysts and result in the live birth of mice entirely derived from iPS cells [85–87]. Different iPS cell lines were varyingly effective at producing viable offspring, with some cell lines showing early termination of fetal development. The success of this approach seems related to the age of the somatic cells from which the iPS cells were derived. With these reports, the debate over the equivalence of ESCs and iPS cells with regard to pluripotency has been resolved, at least in mice.

DISEASE MODELING WITH PATIENT-DERIVED INDUCED PLURIPOTENT STEM CELLS

Currently, there is one major question being addressed by iPS cell research. Researchers are seeking to produce iPS cell lines that capture the genotypes of disease. These cell lines would offer an unprecedented opportunity to understand pathobiology and pathophysiology, identify abnormalities in the development or function of differentiated cells affected by disease, develop therapies that render these cells resistant to disease and provide sources of material for cell replacement therapy. Ultimately, the goal is to develop new therapies where treatment is either non-existent or insufficient.

Numerous groups have reported the generation of disease-specific iPS cell lines. A Harvard group has generated iPS cells from patient fibroblasts of a familial form of amyotrophic lateral sclerosis (ALS) [88]. Another Harvard group has produced iPS cells from patients with 10 different genetic diseases, including Parkinson's disease, type 1 diabetes, Duchenne and Becker's muscular dystrophy, adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman–Bodian–Diamond syndrome, Gaucher's disease type III, Huntington's disease, Down's syndrome and the carrier state of Lesch–Nyhan syndrome [89]. Another two groups have added to the list of disease-specific iPS cells by creating human cell-based models of spinal muscular atrophy (SMA) [90] and Parkinson's disease [69].

iPS cells were also generated from skin fibroblasts of a patient with homozygous beta-thalassemia and subsequently differentiated into hemoglobin-producing hematopoietic cells [44]. In theory, these cells could be treated with gene therapy to yield autologous hematopoietic cells that function normally. This goal was advanced by another group when they derived iPS cells from dermal fibroblasts harvested from Fanconi anemia patients, corrected the genetic defect using lentiviral vectors encoding for FANCA and FANCD2, and subsequently derived hematopoietic progenitor cells that were phenotypically disease free [91].

In a publication demonstrating the broad utility of disease-specific iPS cells, the generation of iPS cells was reported from patients with familial dysautonomia (FD). The iPS cells were redifferentiated into cells of all three germ layers and tissue-specific missplicing of the protein responsible for FD was demonstrated [92]. During redifferentiation, novel insights were also gained into the pathogenesis of FD: a possible mechanism for the tissue specificity of FD was demonstrated, and defects in cell differentiation and migration were uncovered. Lastly, the in vitro model was successfully used to screen candidate drugs. These approaches using disease-specific iPS cells can be also applied to genetic disorders in the nephrology field.

TOXICOLOGY SCREENING USING EMBRYONIC STEM CELLS OR INDUCED PLURIPOTENT STEM CELLS

Other clinical applications using ESC or iPS cell technology include the use of stem cell-derived cells or tissues for in vitro toxicology screening. For example, it has been shown that human ESC or iPS cell-derived cardiomyocytes can be used to examine toxic effects of drug compounds on cardiomyocytes, such as drug-induced QT interval prolongation which can lead to sudden cardiac death and is a major safety concern for the drug industry [93]. Similar approaches may include the use of ESC or iPS cell-derived hepatocytes and renal

10013-GOLIGORSKY-9780123809285

cells in testing the hepatotoxicity and nephrotoxicity of drug compounds, which are common and major problems in clinical practice.

FUTURE DIRECTIONS IN INDUCED PLURIPOTENT STEM CELL RESEARCH AND TECHNOLOGY

In the rapidly developing field of iPS cell research, improved and more efficient iPS cell derivation protocols are expected in the near future (Fig. 13.3). Key will be the development of methods that do not rely on the integration of transgenes but are still highly efficient. As shown by the recent tetraploid complementation studies, the age at which somatic cells are harvested plays a key role in the pluripotency of the derived iPS cells. As such, it will be important to identify somatic cell types that are easily harvested and harbor the fewest mutations. It may be advisable to collect cord blood from newborns as they have been shown to be candidates for reprogramming and would at that time harbor very few mutations. In addition, cord blood cells possess the immunological immaturity of newborn cells and several hundred thousand immunotyped cord blood units are readily available through a worldwide network of cord blood banks. Two separate groups have already reported the successful derivation of human iPS cells from human cord blood cells [94,95].

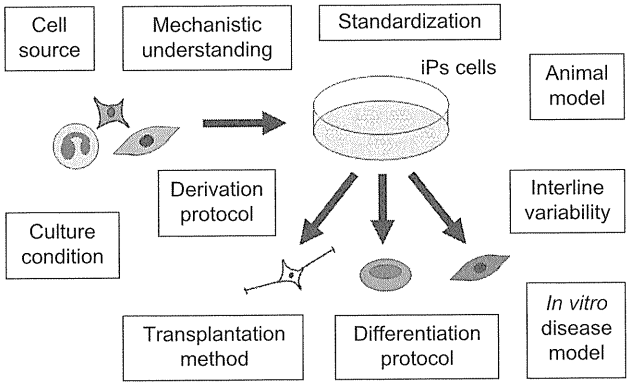

FIGURE 13.3 Questions and future directions in induced pluripotent stem (iPS) cell research and technology. Suitable somatic cell sources, safer and more efficient derivation protocols, and both derivation and maintenance culture conditions need to be established for iPS cells in the near future. Elucidation of the mechanisms of iPS cell reprogramming would help to overcome the problems of low frequency and inefficient iPS cell transduction. The criteria for iPS characterization and standardization need to be developed, which would make it possible to solve the problem of interline variability of iPS cell lines. Protocols to efficiently differentiate iPS cells into various organ lineages including kidney remain to be established. The creation of animal disease models using iPS cells from species other than mouse and disease modeling using patient-derived iPS cells would contribute to curing patients with intractable disorders. Strategies for the transplantation of iPS-derived specific cell types into diseased organs also need to be developed for the embodiment of cell replacement therapies.

We are only beginning to understand the mechanism and kinetics of iPS cell reprogramming. Elucidation of these would overcome the current problems of low-frequency and inefficient iPS cell transduction. An integrative genomic analysis of the reprogramming process demonstrated that the repression of lineage-specific transcription factors and DNA demethylation are critical and inefficient steps [74]. The question of quality standards for iPS cells has begun to be addressed [96]. A minimum set of criteria for iPS characterization includes: (i) pluripotent stem cell morphology and unlimited self-renewal; (ii) expression of pluripotency markers and downregulation of differentiation markers; (iii) reprogramming factor independence; and (iv) "proof of functional differentiation through the highest stringency test acceptable".

The promise of iPS cell technology includes applications in both patient care and advanced cellular research. Currently, incomplete silencing of viral transgenes and even continued dependence on exogenous factors to maintain pluripotency are barriers to fulfilling the promise of iPS cells.

Significant differences exist in the differentiation potential of different human ESC lines, even though the observable differences in the pluripotent state are marginal [97]. These results also suggest that pluripotent stem cells derived by other means, including iPS cells and ESCs produced by SCNT, may show a similar variability. Thus, although the demonstration of complete iPS cell pluripotency via the tetraploid complementation studies was significant, it remains necessary to develop and standardize differentiation protocols that assess the differentiation potential of iPS cell lines, which would address the problem of interline variability.

Although the transcriptional and genomic characterization of iPS cells is fairly established, no proteomic characterization has been performed. The recent discovery of microRNAs presents another area of potential research and characterization; a Stanford group performed the first "miRNA-ome" analysis of human iPS cells compared with human ESCs and fibroblasts [98].

CUES TO REGENERATE KIDNEY LINEAGE CELLS FROM INDUCED PLURIPOTENT STEM CELLS AND EMBRYONIC STEM CELLS

To date, few reports have described the generation of renal lineage cells from iPS cells. However, the phenotype of iPS cells is indistinguishable from ESCs and accumulated experiences and knowledge using ESCs to

10013-GOLIGORSKY-9780123809285

generate mesodermal and renal lineage cells are potentially applicable to iPS cell differentiation experiments.

By mimicking signals used in embryonic development, to the extent that they are known, a stepwise protocol was explored to differentiate ESCs or iPS cells into some specific cell types in adult organs, such as pancreas and liver [12–14,99]. For the generation of insulin-producing cells in pancreas, human ESCs were first differentiated into definitive endoderm, then foregut endoderm, followed by pancreatic progenitors, then endocrine precursors, and eventually insulin-expressing endocrine cells [99]. These stepwise approaches may be used in generating renal lineage cells from ESCs or iPS cells, and should involve directing the pluripotent stem cells first to form intermediate mesoderm, then the renal progenitors, followed by the eventual formation of functional renal cells found in adult kidney.

There are significant differences in the differentiation potential among different human ESC lines, and some cell lines have a propensity to differentiate into certain lineages or cell types [97]. These findings suggest that iPS cell lines may show similar differences in differentiation potential, and underscore the importance of using suitable stem cell lines for renal lineage differentiation.

Directed differentiation of pluripotent stem cells into specific organ lineages is usually carried out through the combination of cell–cell interactions (coculture with primary cells or immortalized cell lines), exposure to diffusible factors (growth factor, cytokine, chemical) and other positional cues (extracellular matrix) that ultimately alter gene expression, conferring a specific cellular identity and function. Direct genetic manipulations on pluripotent stem cells using the overexpression of cDNA, siRNA or shRNA have also been performed to generate specific cell types. It has recently been demonstrated that high-throughput screening of chemical compounds can be used to identify a small molecule that has a potential to induce the differentiation of pluripotent stem cells into specific tissue lineages [100]. In the report, a chemical compound, (−)-indolactam V, known to activate protein kinase C (PKC) signaling, was shown to induce the differentiation of a substantial number of pancreatic progenitor cells from human ESCs. It was also shown that the activated PKC signal is involved in pancreatic specification in human ESC differentiation culture [100]. Similarly, chemical screens in an unbiased high-throughput method can be applied for the identification of chemical compounds to induce mesodermal and renal lineage differentiation from pluripotent stem cells and the elucidation of mechanisms or pathways involved in renal lineage commitment. By taking the cues described into account, continued investigations are needed to establish methods to efficiently differentiate pluripotent stem cells into renal lineage.

CONCLUSION

In a short period, iPS cells have proven to be a major new frontier in biological research. iPS cells have been created in human and several animal models including mice, rats, pigs and primates. They have been generated from numerous somatic cell types, and disease-specific iPS cells have been created from dozens of diseases. iPS cells have also been shown to develop autonomously into full-term mice via tetraploid complementation. Over the coming months and years, iPS cell generation efficiency will be improved.

Research towards the kidney regeneration strategy using iPS cells is in its infancy. Continued efforts to elucidate the developmental mechanisms of mesoderm formation, specification into kidney lineages and nephron specification should be made. These investigations are necessary for the establishment of differentiation protocols from iPS cells into renal lineages. Further investigations using pluripotent stem cells as a model for kidney development will facilitate our understanding of kidney precursor specification, which eventually will lead to the development of new cell-based therapies for the treatment of renal diseases, in addition to other clinical applications such as drug discovery, disease modeling and toxicology (Fig. 13.4). iPS cell-based disease modeling and drug discovery will yield new discoveries, culminating in the development of low-cost, patient-specific cell-based therapies. Because of the annual increase worldwide in the number of patients with end-stage renal failure who have to undergo dialysis therapy and need a renal transplantation, regenerative medicine strategies using iPS cells need to be developed.

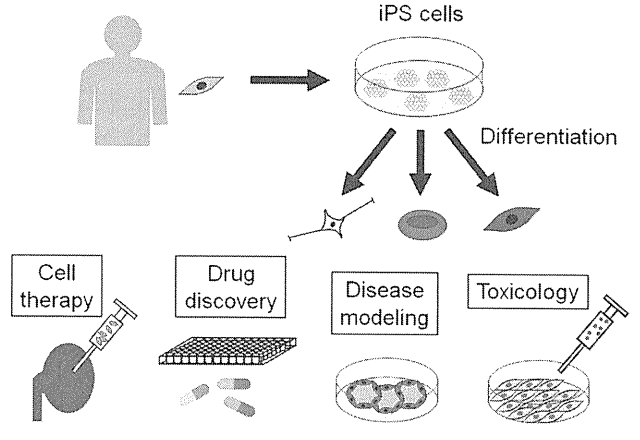

FIGURE 13.4 Possible applications of induced pluripotent stem (iPS) cell technology. iPS cells generated from somatic cells of patients with intractable diseases can be differentiated into the affected cell types and used to develop cell therapy and to study disease mechanisms (disease modeling). The cells can also be used as tools in drug discovery and toxicology.

10013-GOLIGORSKY-9780123809285

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III. STEM CELLS IN REGENERATIVE PROCESSES

10013-GOLIGORSKY-9780123809285

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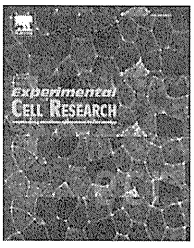

Review

In vitro regeneration of kidney from pluripotent stem cells

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ABSTRACT

Although renal transplantation has proved a successful treatment for the patients with end-stage renal failure, the therapy is hampered by the problem of serious shortage of donor organs. Regenerative medicine using stem cells, including cell transplantation therapy, needs to be developed to solve the problem. We previously identified the multipotent progenitor cells in the embryonic mouse kidney that can give rise to several kinds of epithelial cells found in adult kidney, such as glomerular podocytes and renal tubular epithelia. Establishing the method to generate the progenitors from human pluripotent stem cells that have the capacity to indefinitely proliferate *in vitro* is required for the development of kidney regeneration strategy. We review the current status of the research on the differentiation of pluripotent stem cells into renal lineages and describe cues to promote this research field.

Contents

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Introduction

An increasing number of patients with end-stage renal failure undergo dialysis therapy around the world. It causes both medical and medico economical problems. Renal transplantation has proved a successful therapy for most patients with end-stage renal failure, as the therapy results in a significant improvement in the patient's quality of life, and prolongs survival. And it is also considered cost-effective [1]. However, the annual increase in the number of new patients with end-stage renal disease who need a renal transplant and the widening gap between the demand for and the supply of donor kidney, have led to a progressive shortage of donor organs for transplant around the world. This has become a serious issue and is even worsened by limited graft survival due to immune rejection [1].

Among strategies to overcome these problems is kidney regeneration using stem cells. Stem cells contain two large categories; organ-specific or somatic stem cells and pluripotent stem cells. In contrast to organ-specific stem cells that generally have the limited potential for growth and differentiation, pluripotent stem cells, such as embryonic stem (ES) cells [2–4] and induced pluripotent stem (iPS) cells [5–7], have a virtually unlimited replicative capacity on culture dishes and are theoretically able to give rise to any cell types in the body. The cell types have increasingly been used as a model system for understanding developmental mechanisms. In addition, *in vitro* culture and the differentiation of the stem cells offer unique opportunities for disease modeling, drug discovery, toxicology and cell replacement therapy [8]. The generation of specific functional cell types from ES cells has been demonstrated, including neuron, vascular endothelia, cardiomyocytes, hematopoietic cells, pancreatic insulin-producing cells, and hepatocyte-like cells [9]. However, the protocol for *in vitro* differentiation of pluripotent stem cells into renal lineage cells has not been fully established.

Other approaches to regenerate kidney have also been investigated using organ-specific local stem cells within the kidney and bone marrow-derived hematopoietic stem cells [10]. Kidney regeneration using mesenchymal stem cells localized in bone marrow has also been investigated [11].

This review first summarizes the mechanisms of kidney development, then focuses on the directed differentiation of pluripotent stem cells into renal lineages based on the knowledge of kidney development, and finally describes potential applications of this strategy for various research fields. *In vitro* generation of kidney using the undifferentiated cell mass in amphibian eggs, similar to mammalian pluripotent stem cells in that the cell mass can differentiate into various organs *in vitro*, is also described as references to kidney regeneration in mammals.

Mechanisms of kidney development

Vertebrates develop successively three kidneys; pronephros, mesonephros and metanephros. The three kidneys consist of a basic functional unit, the nephron, although the number of nephron differs among the kidneys [12]. The kidneys are derived from a portion of the early embryonic germ layer, intermediate mesoderm that is located between lateral and paraxial mesoderms [13]. Lineage tracing experiments have recently demonstrated that intermediate mesodermal cells expressing *Odd-skipped related* (*Osr*) *-1*, an essential transcriptional factor for intermediate mesoderm and kidney development, give rise to all components consisting of mammalian adult kidney metanephros [13]. Metanephros is formed by reciprocal interaction between two tissues derived from intermediate mesoderm — metanephric mesenchyme and ureteric bud [12]. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules. By creating a novel culture system which uses the co-culture with the cell line expressing a renal-epithelializing factor *Wnt4*, we previously demonstrated that mouse metanephric mesenchyme contains multipotent progenitor cells that can give rise to several kinds of epithelial cells found in adult kidney, such as glomerular podocytes and epithelia of proximal and distal renal tubules and Henle's loop [14] (Figs. 1A, B). The progenitor cells are contained only in the cell population strongly expressing *Sall1*, a zinc finger transcriptional factor that is essential for kidney development. It was also shown that these progenitor cells can reconstitute a three dimensional kidney structure *in vitro*, which contains glomeruli- and tubule-like components in an organ culture setting (Figs. 1C, D). The presence of the progenitor cells was also demonstrated *in vivo* by using a lineage tracing experiment for *Six2*, which is also an essential transcriptional factor for kidney development [15].

The progenitors in metanephric mesenchyme differentiate into epithelia after Wnt stimulation, but a cell fate decision is required for further differentiation toward glomerular podocytes, proximal or distal renal tubules, or Henle's loop. Molecular mechanisms of the cell fate decision are largely unknown, although it was demonstrated that *Notch2* is required for the differentiation of proximal nephron structures, such as podocytes and proximal renal tubules, as *Notch2* deletion leads to the impaired formation of these proximal structures [16]. There may be many other molecules involved in the lineage commitment, and they remain to be elucidated. Metanephric mesenchyme may well contain at least two other progenitor populations in addition to the epithelial progenitors; 1) vascular progenitor cells that can give rise to vascular and glomerular endothelial cells, vascular smooth muscle cells (pericytes) and mesangial cells, and 2) stromal progenitors eventually developing into interstitial cells within adult kidney. The ureteric bud is known to elaborate the lower urinary tract system — from collecting ducts through renal pelvis and ureters to a part of the urinary bladder [12]. Most of the mechanisms of the lineage commitment, by which the intermediate mesoderm gives rise to ureteric bud, stromal and vascular cells, are unknown. Further examinations will be required to elucidate the mechanisms, which will eventually help us completely understand the commitment of multiple cell lineages within a kidney and then reproduce it *in vitro* from pluripotent stem cells.

Animal cap in fertilized eggs of amphibians

Amphibians have been used as experimental animals for the research of developmental biology mainly because of its easiness to handle and observe. In amphibian eggs, the ectodermal cell mass of mid-blastula embryos, called animal cap, is similar to ES and iPS cells in mammals in that it has a potential of multipotent differentiation. The animal cap can be easily excised from fertilized eggs and cultured as explants *in vitro* in simple saline solution.

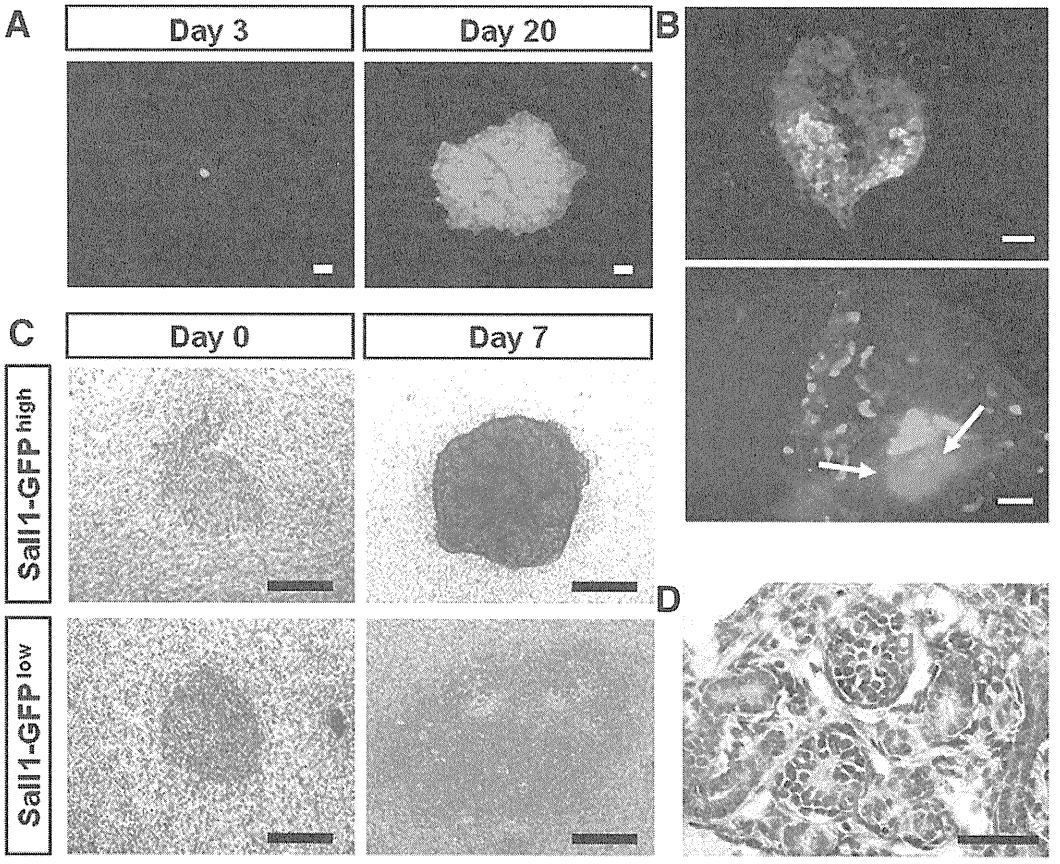

Fig. 1 – Multipotent progenitor cells in metanephric kidney. (A) *In vitro* colony formation from a single progenitor cell within metanephric mesenchyme on feeder cells expressing Wnt4. (B) A single progenitor cell divides and differentiates into two renal lineages; (upper panel) podocytes (red: peanut agglutinin; PNA) and proximal tubular epithelia (green: *Lotus tetragonobulus* lectin; LTL), (lower) proximal (green: LTL) and distal tubular epithelia (red: E-cadherin, arrows). (C) Only cell population strongly expressing *Sall1* (Sall1-GFP^{high}) that contains multipotent progenitors (upper panels) differentiates into kidney structure in organ culture, whereas that weakly expressing *Sall1* (Sall1-GFP^{low}, lower) disappears. (D) Hematoxylin–eosin staining of sections of Sall1-GFP^{high} aggregates at day 10. Tubule- (t) and glomerulus-like structures (g) are seen. Bars: (A, B) 50 μm, (C) 500 μm, and (D) 25 μm.

After the treatment with differentiation-inducing factors, animal cap can differentiate into various tissues and organs *in vitro* [17]. The pronephros is a simple excretory organ of *Xenopus* larvae with only one nephron, consisting of glomus, a filtering unit equivalent to glomerulus in metanephros, tubule and duct. When animal caps are treated with the combination of activin A, a protein inducer belonging to transforming growth factor (TGF)-β superfamily, and retinoic acid, the differentiation into pronephric tubules was observed [18]. In the same differentiation system, the marker gene expression for pronephric glomus was detected [19]. We demonstrated that pronephric duct, a third component of pronephric kidney, can be generated in the explants, in addition to pronephric tubule and glomus, and that the pronephros formed *in vitro* is similar to that in *Xenopus* embryos both histologically and in gene expression (Fig. 2) [20]. The pronephric tubule and duct also have the similar ultrastructure to those of *Xenopus* embryos by the examination with electron microscopy.

The pronephros formed from animal cap *in vitro* shows temporarily a similar gene expression pattern to that in *Xenopus* embryos. Using the *in vitro* system to generate pronephric tissue, the molecular mechanisms underlying pronephros development have been investigated and some molecules involved in the formation of pronephros were identified [17]. Although the pronephros produced *in vitro* from animal cap cannot be directly translated to clinical application, this system should facilitate the study of kidney regeneration and may promote a shift from tissue engineering to clinical applications. Indeed, this induction method established for animal cap using activin A and retinoic acid has been applied for a differentiation experiment from mouse ES cells into renal lineages [21].

ES cell

ES cells are pluripotent stem cells derived from the inner cell mass of fertilized eggs in mammals. The first derivation of mouse ES cells by Evans and Martin was described in 1981 [2,3] and human ES cells were established in about 20 years later [4]. Both mouse and human ES cells have a virtually unlimited replicative capacity and are theoretically able to give rise to any cell types in the body. Since its derivation, mouse ES cells have mainly been used as a research tool to generate experimental mouse models by the combination with gene targeting techniques. However, after the success in human ES cell derivation, regenerative medicine strategy has been

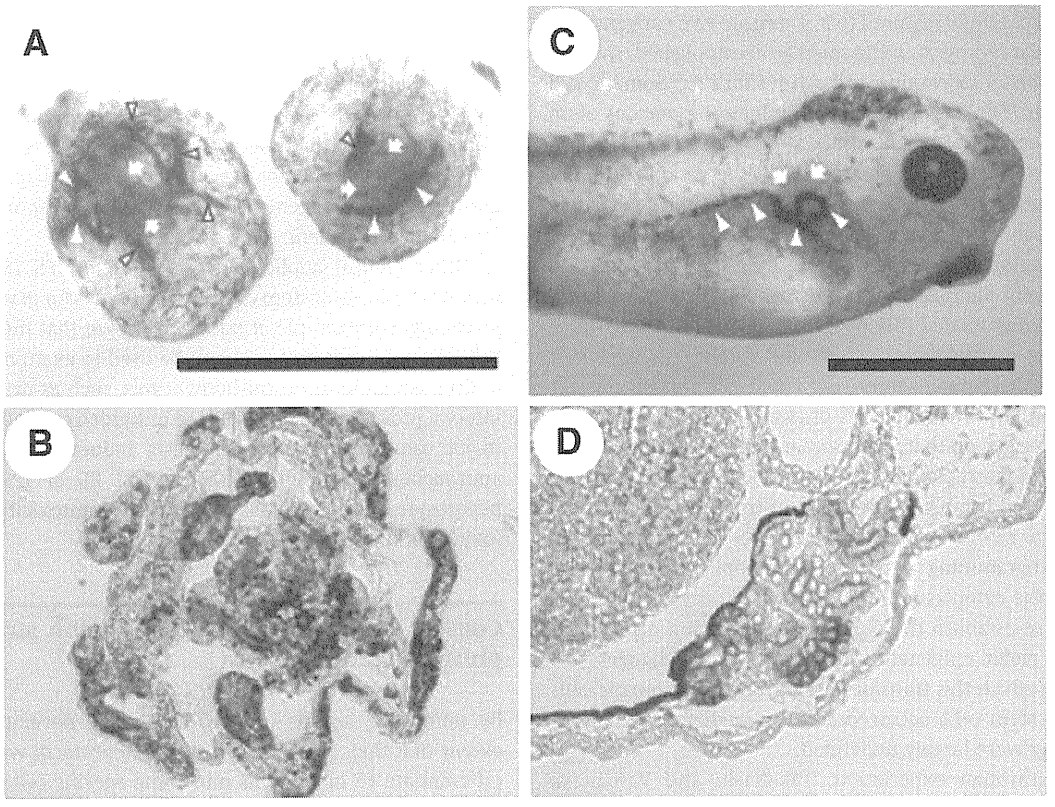

Fig. 2 – Pronephric kidney formed *in vitro* from undifferentiated cells in an amphibian. Double immunostaining of stage 42 equivalent explants (A, B) and a stage 40 *Xenopus* larvae (C, D) using pronephric tubule-specific antibody 3G8 (red) and pronephric duct-specific antibody 4A6 (blue). Whole-mount immunostaining patterns (A, C) and photomicrographs of sections (B, D) are shown. (A, B) Explants treated with activin A and retinoic acid contain both 3G8-positive (pronephric tubules, arrows) and 4A6-positive tubular structures (pronephric ducts, arrowheads). Bars: 1 mm.

considered closer to clinical applications, which aim at accomplishing the functional recovery of dysfunctional organs by transplanting organ-specific cell types generated from ES cells *in vitro*. The research on the *in vitro* differentiation of ES cells into specific cell types of various organs has since been more vigorously investigated to develop cell transplantation therapies.

In vivo injection of ES cells into immunocompromised mice can produce teratoma, which are tumors containing cells of all three embryonic germ layers (ectoderm, mesoderm and endoderm). It was shown that glomerulus- and tubule-like structures can be formed in teratomas derived from mouse [22] and human ES cells [4], which demonstrate that ES cells do have a potential to differentiate into kidney lineages. Furthermore, fundamental research aiming to establish the methods to efficiently induce mouse ES cells into renal progenitors and fully differentiated renal cells has already been carried out [21,23–26]. Mouse ES cells treated with the combination of hepatocyte growth factor (HGF) and activin A in addition to the transfection of renal-epithelializing factor Wnt4 [23], the combination of activin A, bone morphogenetic protein (BMP)-7 and retinoic acid [21], activin A alone [24], BMP-4 alone [25], or the combination of four chemical compounds [26], produced cells expressing markers for intermediate mesoderm, developing kidney and fully differentiated renal cells. In some reports, the induced cells formed tubule-like structures in or were incorporated into developing mouse kidneys [21,23,24]. Few reports have described the directed differentiation of human ES cells into renal lineage. It was shown that human ES cells differentiated *in vitro* into cells expressing WT1 and rennin, marker genes for glomeruli, following the treatment with growth factors [27]. Recently it has been reported that the combinational treatment with activin A, retinoic acid, and BMP-4 or BMP-7 also induces marker gene expression for intermediate mesoderm and developing kidney from human ES cells [28].

In summary, these data suggest that ES cells are a potential source for kidney regeneration, although the efficiency or the induction rate of generation of renal cells from ES cells is unknown in many cases. It remains to be determined whether kidney lineage cells produced from ES cells have a function as kidney. It is also unknown whether tubule-like structures or cells integrated into kidney are metanephric or of earlier kidneys (pronephros and mesonephros). The research on kidney regeneration using ES cells is at its beginning, and further examinations are needed to develop this research field.

iPS cell

One of the more remarkable observations in the past century is that the differentiation is not a unidirectional process. Instead, we can turn back the clock on differentiated cells. This rewinding of the developmental clock is termed nuclear reprogramming and is often defined as the process whereby an adult somatic nucleus has

developmental potential restored to it. There are two conventional ways that nuclear reprogramming could be accomplished: somatic cell nuclear transfer or cloning and cell fusion [29]. Somatic cell nuclear transfer (SCNT), a procedure in which the nucleus of an adult cell is injected into an unfertilized egg whose chromosomes have been removed, has demonstrated that the genome of adult cells can be reset to an embryonic state. Using this strategy researchers have generated cloned embryos that possess the potential to develop into an adult animal or become an ES cell line that is genetically identical to that of the donor nucleus. These experiments demonstrated that no irreversible changes are made to the genome during development and further showed that animal oocytes harbored factors that could accomplish nuclear reprogramming. In a related series of experiments, a number of researchers have shown that when somatic cells are fused with ES cells, the resulting tetraploid hybrid cells silence the expression of somatic genes and establish a program of transcription indistinguishable from ES cells, indicating that ES cells contain the necessary reprogramming activities to accomplish this transformation. Thus, the cytoplasm of the enucleated oocyte and the ES cell is able to re-establish the pluripotent state via a mechanism dependent on global epigenetic and transcriptional changes. The mechanism by which this transformation from adult somatic cells to pluripotent stem cells occurred and the mediators of nuclear reprogramming were largely undefined.

In a breakthrough experiment, Takahashi and Yamanaka identified four factors normally found in ES cells – Oct3/4, Sox2, c-Myc, and Klf4 – and reported that they were sufficient to reprogram both mouse and human somatic cells to closely resemble mouse and human ES cells [5–7]. They called the cell type induced pluripotent stem (iPS) cells. Since the reports, the technique has been rapidly confirmed, improved and subsequently applied to successfully reprogram somatic cells.

Therapeutic approaches using human ES cells face the two major problems. One is the ethical issue derived from the usage of human fertilized eggs, and the other is immune rejection in any cell or tissue transplantation due to histocompatibility antigenic differences between ES cells and patients. These problems have become resolvable by using iPS cells, which can be generated from somatic cells of patients. In the next step after iPS cell derivation, significant progress has been made in re-differentiating iPS cells into somatic cells including active motor neurons [30], insulin-secreting islet-like clusters [31], and a number of cardiovascular cells (arterial endothelium, venous endothelium, lymphatic endothelium, and cardiomyocytes) [32,33]. To date, few reports have described the generation of renal lineage cells from iPS cells. However, the phenotype of iPS cells is indistinguishable from ES cells and accumulated experiences and knowledge using ES cells to generate mesodermal and renal lineage cells are potentially applicable to iPS cell differentiation culture.

The iPS cell technology enables the creation of patient-specific pluripotent cell lines that carry disease genotypes. The generation of disease-specific iPS lines has been first reported from a familial form of a neurodegenerative disorder, amyotrophic lateral sclerosis (ALS) [34]. Disease-specific iPS cells have also been produced by other groups from more than 10 different genetic diseases, including Parkinson's disease, type-1 diabetes, Duchenne's and Becker's muscular dystrophy, adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman–Bodian–Diamond syndrome, Gaucher disease type III, Huntington's disease, Down's syndrome, and the carrier state of Lesch–Nyhan syndrome [35]. These cell lines could be used both as *in vitro* models for the study of disease and as potential sources of material for cell replacement therapy. Ultimately, a greater understanding of the process by which cellular identity is shaped and altered may allow for the generation of particular cell types for the treatment of degenerative diseases. These approaches can be also applied to genetic renal diseases.

Other clinical applications using ES or iPS cell technology include the usage of stem cell-derived tissues for *in vitro* toxicology screening. For example, it has been shown that human ES or iPS cell-derived cardiomyocytes can be used to examine toxic effects of drug compounds on cardiomyocytes, such as drug-induced QT interval prolongation that can lead to sudden cardiac death and is a major safety concern for the drug industry [36]. The similar approaches may well be the use of ES or iPS cell-derived hepatocytes and renal cells in testing hepatotoxicity and nephrotoxicity of drug compounds.

Cues to develop kidney regeneration strategy from pluripotent stem cells

By mimicking signals used in embryonic development, to the extent that they are known, a stepwise protocol was explored to differentiate ES or iPS cells into some specific cell types in adult organs, such as pancreas and liver [37,38]. For the generation of insulin-producing cells in pancreas, ES cells has been first differentiated into definitive endoderm, then foregut endoderm, followed by pancreatic progenitors, and eventually insulin-expressing endocrine cells [37]. These stepwise approaches may be used in generating renal lineage cells from ES or iPS cells, and it should involve directing the pluripotent stem cells first to form intermediate mesoderm, then the renal progenitors, followed by the eventual formation of functional renal cells found in adult kidney.

It has recently been shown that significant differences exist in the differentiation potential of different human ES cell lines, even though the observable differences in the pluripotent state are marginal [39]. Moreover, some human ES cell lines have a propensity to differentiate into certain lineages or cell types. These results also suggested that pluripotent stem cells derived by other means including iPS cells may well show a similar variability. The finding also underscores the importance of using suitable stem cell lines for the renal lineage differentiation.

Directed differentiation of pluripotent stem cells into specific organ lineages is usually carried out through the combination of cell–cell interactions (co-culture with some cell lines), exposure to diffusible factors (growth factor) and other positional cues (extracellular matrix) that ultimately alter gene expression conferring a specific cellular identity and function. Direct genetic manipulations on stem cells using the overexpression of cDNA, small interfering RNA (siRNA) or short hairpin RNA (shRNA) have been also performed to generate specific cell types. It has been recently demonstrated that high-throughput screening of chemical compounds can be used to identify a small molecule that has a potential to induce the differentiation of pluripotent stem cells into specific lineages [40]. In the report, a chemical compound, (−)-indolactam V, known to activate protein kinase C (PKC) signaling, was shown to induce the differentiation of a substantial

number of pancreatic progenitor cells from human ES cells. It was also shown that the activated PKC signal is involved in the pancreatic specification in human ES cell culture [40]. Similarly, chemical screens in an unbiased high-throughput method can be applied for the identification of chemical compounds to induce renal lineage differentiation from pluripotent stem cells and the elucidation of mechanisms of kidney differentiation.

Conclusion

Research for the kidney regeneration strategy using pluripotent stem cells is just at its beginning. Of course, continued efforts to elucidate the developmental mechanisms of mesoderm formation, the specification into kidney lineages, and nephron specification should be made. These investigations are necessary for the establishment of differentiation protocols from pluripotent stem cells into renal lineages. Further investigations using pluripotent stem cells as a model for kidney development will facilitate our understanding of kidney precursor specification and the development of new cell-based therapies for the treatment of renal diseases. Because of the annual increase in the number of patients with end-stage renal failure who have to undergo dialysis therapy around the world, regenerative medicine strategies using pluripotent stem cells need to be developed.

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