detectable by forming colonies on laminin-521 in Essential 8 in the process of hMSC differentiation. These results indicate that a culture system utilizing a combination of laminin-521 and Essential 8 medium provides a direct and highly sensitive method for detecting undifferentiated hPSCs. To our knowledge, this is the first report to show a direct and highly-sensitive *in vitro* method for detecting undifferentiated hPSCs as impurities in CTPs.

In this study, highly efficient amplification of undifferentiated hPSCs has been uniquely applied to quality control of CTPs. Amplified hPSC colonies were visible using phase-contrast microscopy and also immunofluorescence staining using pluripotency antibodies, which enabled direct detection of hPSCs contaminating CTPs. Our method distinguished between undifferentiated cells and other cells *in vitro*, and overcame the disadvantage of other *in vitro* methods such as flow cytometry and qRT-PCR. The flow cytometry analysis detects known marker molecules expressed in undifferentiated hPSCs using antibodies and proteins. Signals originating from non-specific detection commonly affect sensitivity of the assay as background. Our *in vitro* method can lower the background arising from non-specific detection and is expected to specifically detect residual undifferentiated hPSCs in CTPs. The qRT-PCR method is highly sensitive and can rapidly quantify undifferentiated cell contamination in CTPs. However, in the present study, gene expression levels of pluripotency markers during the differentiation process of hiPSCs into MSCs varied markedly among those marker genes (Figure 6B). Moreover, there remains a possibility that expression signals of marker genes were not derived from totally undifferentiated hPSCs, but from partially differentiated cells. Indeed, the expression level of *LIN28* did not decrease so much during the differentiation as those of the other genes, which was not obviously associated with the differentiation status of the cells in EBs on Day 6 (Figure 6B), although we have previously reported that *LIN28* was a useful marker for monitoring the level of residual hiPSCs in RPE cells derived from hiPSCs [3]. Thus, it is difficult to determine the presence of residual hiPSCs simply by qRT-PCRs. In contrast, direct detection method using the highly efficient amplification system can clearly detect the presence of intact undifferentiated cells. Based on the result from direct detection of residual hiPSCs when tested the cells on Day 6 of differentiation (approximately 0.01%–0.1%) (Figure 6C-D), it is conceivable that the qRT-PCR signals for the pluripotency marker genes (Figure 6B) are partly derived from residual hiPSCs but mainly derived from partially differentiated cells. Similarly, in the case of the cells at Day 14 of differentiation, the majority of the qRT-PCR signals of *OCT3/4* and *LIN28* (Figure 6B) are considered to be attributable to partially differentiating cells but not to intact hiPSCs. Combination of the *in vitro* methods including our cell culture method would mutually support useful quality assessment of CTPs to detect undifferentiated hPSCs.

In addition to the detection of undifferentiated cells, this culture system using laminin-521 and Essential 8 medium allows further characterization of the undifferentiated cells if they are maintained *in vitro* or inoculated into immunodeficient animals. Analyses for the properties of the residual undifferentiated cells would be necessary not only for the quality assessment of CTPs, but also for improvement of quality specifications of hPSCs as a raw/intermediate material for production of CTPs.

Here, we showed that our culture system is able to detect 0.01% of 409B2 hiPSCs and 0.001% of 253G1 hiPSCs, both of which were spiked into hMSCs (Figure 4). The detection sensitivity for hiPSCs spiked into hMSCs was different between the two hiPSC lines, although such a difference in cell growth on laminin-521 was not found between these two cell lines (Figure 3). This difference may be attributable to the difference in the growth potential of hPSCs in the specific environment provided by CTPs. Kanemura *et al.* have recently demonstrated that hiPSCs co-cultured with iPSC-derived RPE undergo apoptosis by pigment epithelium-derived factor (PEDF) secreted from hiPSC-derived RPE [15], showing that CTPs themselves have the potential to affect cell growth of hPSCs. In the present study, the influence of the co-culture system with hMSCs to the proliferation of hiPSCs might have been different between the two cell lines.

The mechanism by which laminin-521 and Essential 8 medium enhance hiPSCs cell proliferation remains unclear. Rodin *et al.* have recently shown that addition of E-cadherin to laminin-521 permitted the efficient clonal expansion of hESCs [7]. E-cadherin is known to be the primary cell-cell adhesion molecule and essential for hESC survival [16]. We observed that anti-E-cadherin antibody decreased growth potential of hiPSCs under our experimental conditions (data not shown). Therefore, E-cadherin signaling may play some important roles in the rapid cell growth on laminin-521 in Essential 8 medium.

Tumorigenicity is one of the major safety concerns for CTPs derived from hPSCs that are transplanted into patients. However, testing strategies for the tumorigenicity of hPSC-derived CTPs have not yet been established. Here, we introduced a novel testing method for directly detecting a trace amount of undifferentiated hPSCs *in vitro*. The ability of each tumorigenicity-associated test should be taken into consideration to evaluate tumorigenicity of residual undifferentiated hPSCs as impurities in products. *In vivo* tumorigenicity tests using immunodeficient animals can detect tumorigenic cells including undifferentiated hPSCs, but this method is costly and time-consuming. The flow cytometry analysis and qRT-PCR are rapid, but these methods indirectly detect tumorigenic cells depending on marker molecules. Risk of tumorigenicity in hPSCs-derived CTPs should be assessed, based on the results from an appropriate combination of these tumorigenicity-associated tests. Our novel method will contribute to establishment of the testing strategies for tumorigenicity in products, following evaluation of the quality of CTPs derived from hPSCs for the future regenerative medicine/cell therapy.

## Supporting Information

**Figure S1** (A) Quantification of the number of dissociated 201B7 cells expanded on laminin-521 or Matrigel in Essential 8 or mTeSR1 medium. Data are presented as the mean ± standard deviation (SD) of three independent experiments (**$P<0.01$, two-way ANOVA followed by a Bonferroni post-hoc test). LN521, laminin-521. MG, Matrigel. (B) Quantification of the number of dissociated 201B7 cells expanded on laminin-521 or LM511-E8 in Essential 8 or mTeSR1 medium. Results are presented as the mean ±SD (n = 3) (***$P<0.001$, two-way ANOVA followed by a Bonferroni post-hoc test).
(TIF)

**Figure S2** (A-B) Expression levels of undifferentiated markers (*OCT3/4, NANOG, SOX2* and *LIN28*) in 201B7 cells (A) and 409B2 cells (B) subcultured on laminin-521 in Essential 8 were determined using qRT-PCR. Relative mRNA expression levels are presented as ratios to the level of that in control cells subcultured on Matrigel in mTeSR1 medium by colony passage. Results are presented as the mean ± SD (n = 3). (C-D) Expression levels of markers for the differentiation of embryoid bodies (EBs) derived from 201B7 cells (C) and 409B2 cells (D): endoderm (*GATA6, SOX17*), mesoderm (*CDH5, FOXF1*), and ectoderm (*SOX1, PAX6*). Relative mRNA expression levels are presented as

ratios to the level of that in control cells (EBs at day 10). Results are presented as the mean ± SD (n = 3).
(TIF)

**Figure S3 Quantification of the number of 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium.** Cell numbers were counted at day 6, 9, and 12 after plating at $8.0\times10^3$ cells/cm$^2$ or $8.0\times10^2$ cells/cm$^2$.
(TIF)

**Figure S4 Morphologies of forming colonies derived from 253G1 cells spiked into hMSCs are shown (images in the left).** 253G1 cells (1%, 300 cells; 0.1%, 30 cells; 0.01%, 3 cells; 0%, 0 cells) were spiked into hMSCs (30,000 cells) and co-cultured on 12-well plates coated with laminin-521 in Essential 8 medium for 9 days. Expression of the undifferentiated cell marker, TRA-1-60, in these colonies was assessed using immunofluorescence staining (images to the right). Each experiment was carried out in duplicate.
(TIF)

**Figure S5 Phase contrast images of the cells at day 18 of differentiation (at the stage of passage 0 MSCs) are shown.** Expression of MSC marker, CD105, in these cells was examined using immunofluorescence staining (images to the right). Arrowheads indicate the cells that were positive for CD105.
(TIF)

**Table S1 Sequences of the primers and probes for qRT-PCR.**
(DOCX)

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## Author Contributions

Conceived and designed the experiments: KT SY YS. Performed the experiments: KT. Analyzed the data: KT SY TK HS AU YS. Contributed reagents/materials/analysis tools: KT SY TK HS AU YS. Wrote the paper: KT SY YS. Acquired the funding: HS AU YS.

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PATHOBIOLOGY IN FOCUS

# A practical guide to induced pluripotent stem cell research using patient samples

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Approximately 3 years ago, we assessed how patient induced pluripotent stem cell (iPSC) research could potentially impact human pathobiology studies in the future. Since then, the field has grown considerably with numerous technical developments, and the idea of modeling diseases 'in a dish' is becoming increasingly popular in biomedical research. Likely, it is even acceptable to include patient iPSCs as one of the standard research tools for disease mechanism studies, just like knockout mice. However, as the field matures, we acknowledge there remain many practical limitations and obstacles for their genuine application to understand diseases, and accept that it has not been as straightforward to model disorders as initially proposed. A major practical challenge has been efficient direction of iPSC differentiation into desired lineages and preparation of the large numbers of specific cell types required for study. Another even larger obstacle is the limited value of *in vitro* outcomes, which often do not closely represent disease conditions. To overcome the latter issue, many new approaches are underway, including three-dimensional organoid cultures from iPSCs, xeno-transplantation of human cells to animal models and *in vitro* interaction of multiple cell types derived from isogenic iPSCs. Here we summarize the areas where patient iPSC studies have provided truly valuable information beyond existing skepticism, discuss the desired technologies to overcome current limitations and include practical guidance for how to utilize the resources. Undoubtedly, these human patient cells are an asset for experimental pathology studies. The future rests on how wisely we use them.

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The potential influence of induced pluripotent stem cell (iPSC) technology for pathobiology studies is revolutionary.[1] Once established from any given patient, iPSCs serve as enduring resources to provide various functional cell types, essentially forever, which retain genomic information from the original patient. For this reason, as well as based upon expectations of their applications for cellular transplantation therapy, iPSC research has been growing exponentially within the short number of years since the original method was published by Takahashi and Yamanaka in 2006.[2] Technical feasibility and high reproducibility are two additional reasons why the method has prevailed worldwide so quickly. Fundamentally, iPSC generation does not require sophisticated equipment or technical expertise, and all the materials required for generation are commercially available. Owing to more recent technological advances, one can now routinely generate iPSCs from patient peripheral blood cells without concern of exogenous gene integration. Accordingly, we can say iPSC technology has become a standard research tool in experimental medicine, like polymerase chain reaction, small interfering RNA, knockout mice and others.

Basic approaches to utilize patient iPSCs for disease mechanism studies are well demonstrated in the literature. Essentially, when patient iPSCs are differentiated into disease-relevant cell types, they can recapitulate, at least in part, molecular and phenotypic changes seen in patients. Using this system, we can further investigate how disease-related phenotypes develop 'in a dish', or even test whether novel therapeutic approaches can reverse these changes. Pioneering

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studies proved that these concepts are indeed valid for certain clinical disorders of both monogenic and polygenic origins. Thus, the future looks quite promising in general. However, when the concept is applied to model a wide range of diseases, we often encounter practical limitations and obstacles for their genuine application to understand diseases, and realize their application has not been as straightforward as initially proposed. First, despite numerous published protocols, *in vitro* differentiation of iPSCs is challenging, often requiring tremendous effort for optimization until the system becomes useful in other laboratories. Second, even after differentiation is successfully achieved, a major obstacle frequently resides in limited value of the *in vitro* outcomes, which may not closely represent disease conditions.

As we have witnessed triumphal examples and experienced many practical obstacles at the same time, we are gradually recognizing ways to utilize patient iPSCs more wisely. Three years have passed since we wrote the previous review in *Laboratory Investigation*,[1] and during this time, we have had the opportunity to manage a core facility for patient iPSC research at the University of Florida. Thus, we feel this is a good time to revisit the issue of 'modeling diseases in a dish' using patient iPSCs, and try to elucidate where we are now with the technology. We target general experimental pathologists as primary readers of the present review, particularly those who are interested in starting patient iPSC research to study a disease of their interest, but not yet sure whether the direction will justify the effort. As there are many outstanding review articles available for recent technological advances in iPSCs,[3–5] here we will focus more on introducing practical issues and solutions for pathobiology applications, leaving extensive details to the references.

## EXEMPLARY CASES

To understand how patient iPSC research is generally conducted, it is useful to introduce a few exemplary cases briefly, in which patient iPSCs have been wisely and beneficially utilized. As iPSCs retain genomic information from the original patient, theoretically we can analyze phenotypic and functional characteristics manifested from changes in the individual genome. Initially, early-onset monogenic disorders, where a single genetic aberration is considered to cause severe deleterious effect on cellular function, have been studied preferentially using iPSCs.

### Early-Onset Monogenic Disease

An exemplary work proving the concept, 'modeling diseases in a dish' was first published in January 2009 by Ebert *et al.*[6] The authors successfully established iPSCs from patients with spinal muscular atrophy, differentiated them into motor neurons, and demonstrated the premature death of neurons *in vitro*, a phenotype reflecting the disorder. Importantly, the study further proposed that disease iPSCs could be utilized to screen novel drugs that could de-repress the *SMN2* gene, a close homolog of the mutated *SMN1* gene. SMN2 is normally not expressed in neurons but could mitigate the disease phenotype when induced. It should be noted that the *SMN2* gene only exists in humans but not in rodents, thus this type of drug screening would only be possible using human neurons.

### Late-Onset Monogenic Disease

Modeling late-onset disease in a dish is a more difficult task because some environmental factors, for example, oxidative stressors, may be involved in disease progression. Nevertheless, Nguyen *et al*[7] demonstrated, for instance, that a phenotype of a familial Parkinson's disease (PD) can be evaluated *in vitro*. The authors generated iPSCs from a patient with a mutation in the leucine-rich repeat kinase 2 (*LRRK2*) gene and differentiated the iPSCs into dopaminergic neurons. The resultant dopaminergic neurons were more susceptible to oxidative stressors (hydrogen peroxide, MG-132 and 6-hydroxydopamine), compared with those from control iPSCs. The study also demonstrated that the patient iPSC-derived dopaminergic neurons had an increase in α-synuclein, which is one of the major components of Lewy bodies, a hallmark of PD pathology.

### Proving the Causal Mutation and Elucidating a Novel Mechanism

*LRRK2-G2019S* is the most commonly identified mutation, but it is only found in a few percent of the sporadic PD patients. Genome-wide association studies suggested that many other polymorphisms in other genomic loci are linked to the disease phenotypes and clinical courses. To that end, the exact pathological mechanism caused by the *LRRK2-G2019S* mutation needed to be elucidated using isogenic controls. Reinhardt *et al*[8] applied genomic engineering technology to correct the *G2019S* mutation in patient iPSCs. They confirmed *LRRK2-G2019S* indeed induced pathological changes of dopaminergic neurons such as deficit in neurite outgrowth, defect in autophagy, increase in α-synuclein, and higher susceptibility to oxidative stress. Furthermore, the study demonstrated the *LRRK2-G2019S* mutation is associated with activation of extracellular signal-regulated kinases (ERKs), which leads to transcriptional dysregulation of CPNE8, MAP7, UHRF2, ANXA1 and CADPS2, resulting in neural degeneration. By demonstrating an ERK inhibitor-mediated amelioration of the neurodegeneration, the study indeed indicated a novel therapeutic approach for patients with PD.

### Polygenic Disorder or Disease of Unknown Causes

In the case of polygenic disorders or sporadic diseases with unknown causes, it is more challenging to obtain useful outcomes using patient-derived iPSCs. Israel *et al*[9] successfully investigated neural phenotypes derived from both familial and sporadic Alzheimer's disease. One of the

| | Helpful | Harmful |
|---|---|---|
| Internal Origin | **Strengths**<br>❑ Clear merits to use patient iPSCs<br>❑ Strong research history for the disease<br>❑ Accessibility to number of patients (or iPSC clones)<br>❑ Preexisting collaborative strengths to develop the study | **Weaknesses**<br>❑ May take time to establish differentiation protocols in your lab<br>❑ Differentiated cells may not be pure or mature enough for your study |
| External Origin | **Opportunities**<br>❑ High expectations for developing novel model systems<br>❑ High expectations from societies of particular diseases<br>❑ External and internal grant opportunities | **Threats**<br>❑ Competitors working on similar directions<br>❑ Competing animal models |

**Figure 1** SWOT analysis before start patient iPSC research. It is critical to analyze all the strengths and potential problems you have before you initiate patient iPSC research. A local iPSC core facility may also assist you to analyze individual projects and create a research design.

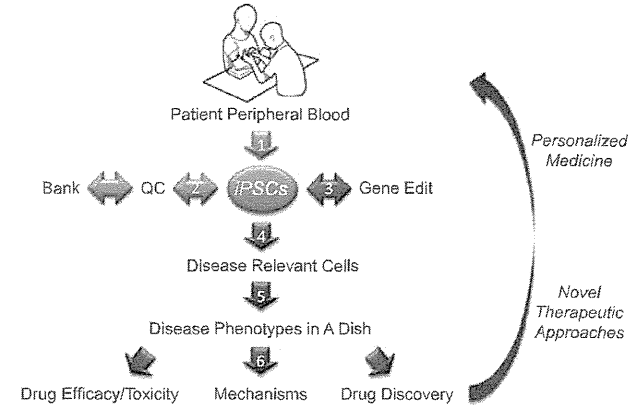


**Figure 2** A typical work flow of patient iPSC research and tips for individual steps. (**1**) iPSC generation (~3 weeks)—multiple clones from multiple patients using non-integrating reprogramming vectors; (**2**) Quality control (QC) and storage (1–4 weeks)—first by morphology and pluripotency markers, then ideally by gene expression profiling, teratoma formation, karyotyping, exome analysis, and mycoplasma testing; (**3**) Isogenic controls made by gene editing serve as ideal controls; (**4**) Differentiation (2–10 weeks)—consult a local iPSC core or colleagues to identify the best available protocols; (**5**) Disease recapitulation—set realistic goals to demonstrate unique pathological changes *in vitro*; (**6**) Study further disease mechanisms—molecular 'omic' analyses are often used here. 'Green' highlighted parts are usually taken care by a local iPSC core facility (if desired), whereas 'blue' highlighted parts will typically be performed by individual investigators.

two sporadic patient's iPSCs showed higher levels of the pathological markers amyloid-$\beta$ (1-40), phosphor-tau(Thr231) and active glycogen synthase kinase-3$\beta$ (aGSK-3$\beta$), as those derived from familial Alzheimer's disease, while the other case did not. These observations offered new opportunities to investigate the mechanisms underlying heterogeneity among sporadic cases. For such studies, however, a larger number of patients and controls would ideally be required.

### Imprinting Disorders

In addition to genetic diseases, the iPSC models facilitate investigation of epigenetic-related diseases such as Beckwith–Wiedemann syndrome, Silver–Russell syndrome, Angelman syndrome and Prader–Willi syndrome. Unlike genetics based on the DNA sequence, epigenetic processes involve DNA methylation and histone modulation. One of the most important epigenetic phenomena is genomic imprinting by which genes are expressed in a parent-of-origin-specific manner. Abnormality of the imprinting mechanism during development causes epigenetic diseases. The methylation status of imprinting genes is maintained during iPSC generation and subsequent cultivation, implying that imprinting disease iPSCs are worth investigating to elucidate mechanism of imprinting abnormality.[10] Patient iPSCs from Angelman and Prader–Willi syndrome have been established and utilized for examination of epigenetic and transcriptomic abnormalities, and for testing compounds aimed at correcting the epigenetic aberrations.[11,12] One must use caution when analyzing epigenetic aberrations in imprinting disease iPSCs because the process of iPSC generation is associated with epigenetic dynamics that may bias interpretation.[13] However, iPSCs with *in vitro* multipotency have been an invaluable tool to clarify molecular mechanisms as a simulator of developmental defects.[14,15]

## PRACTICAL ADVICE BEFORE YOU BEGIN

These exemplary cases certainly make us feel hopeful that we can apply patient iPSCs to various diseases. Taking all the progress and current issues into consideration, which we discuss more in detail in the following section, we have compiled practical tips you may find useful when starting patient iPSC research. First, it is essential to analyze whether the project is worth pursuing, as with any other new research projects. A SWOT analysis, for an example as shown in Figure 1, will guide you to identify the potential internal and external strengths and weaknesses of your direction. Unfortunately, the field is highly competitive, and the funding is scarce; thus it is critical to fully analyze the status of your project before beginning. In the end, the most important factor in the analysis is whether you have unique and significant question(s) that are likely answered using patient iPSCs.

When the analysis is positive, Figure 2 illustrates an actual workflow of the study with estimated time lines. Unless you have extensive experience in human pluripotent stem cell culture, it is easiest to consult with an iPSC core facility or colleagues to generate patient iPSCs. In a typical study of a monogenic disorder, generation of three iPSC clones from three individual patients is minimally required, along with an equivalent number of controls; however, such number can vary considerably depending on your questions. The quality of iPSC clones should also be controlled by the core facility to

**Table 1 List of iPSC banks and registries by disease**

| Diseases | Institute | Website |
|---|---|---|
| General | Coriell Institute/NIGMS | http://ccr.coriell.org/Sections/Collections/NIGMS/ipsc_list.aspx?PgId=696 |
| | American Type Cell Collection | http://www.atcc.org/Products/Cells_and_Microorganisms/Stem_Cells/Human_IPS_Pluripotent.aspx |
| | RIKEN Bioresource Center Cell Bank | http://www.brc.riken.jp/lab/cell/english/index_hps.shtml |
| | Wi-Cell | http://www.wicell.org/home/stem-cell-lines/order-stem-cell-lines/obtain-stem-cell-lines.cmsx |
| | Boston University, Center for Regenerative Medicine | http://www.bu.edu/dbin/stemcells/ips_cell_bank.php |
| | U MASS International Stem Cell Registry | http://www.umassmed.edu/iscr/Genetic-Disorders-Lines/ |
| | U Connecticut Stem Cell Core | http://stemcellcore.uchc.edu/services/distribution.html |
| | Harvard Stem Cell Institute | http://stemcelldistribution.harvard.edu/shoppingCart/index |
| Neural | Coriell Institute/NINDS | http://ccr.coriell.org/Sections/Collections/NINDS/ipsc_list.aspx?PgId=711&coll=ND |
| Mental | NIMH Stem Cell Center | http://nimhstemcells.org/catalog.html |

Currently available information of iPSC bank and registry (June 2014). Please note that the list here may not cover all the available sites.

meet the current standard, as discussed later. If patient iPSC clones or fibroblasts already exist in publicly available libraries, you can save substantial amount of time and cost. Table 1 shows a list of disease iPSC bank and registry, in which you may be able to find the lines of your interest. Additional information is available in a recent review article specifically discussing this topic.[16]

As we discuss later, iPSC differentiation should ideally be performed in collaboration between your lab and the iPSC core or a person who has iPSC expertise. In the steps of disease recapitulation and further mechanism studies, it is particularly important to set practical goals for patient iPSC research. First, you should accurately estimate purity, quantity and maturation status of the resultant iPSC-derived differentiated cells. Depending on those factors, you can identify what types of assays can be performed with the prepared cells. In general, patient iPSCs will hold the most value in identifying molecular changes caused by pathogenic mutations in certain human cell types. 'Omic' level screening will be particularly useful there; and isogenic iPSC clones with the mutation corrected through gene editing would serve as ideal controls in such tests, as discussed later.

## TECHNICAL IMPROVEMENT AND REMAINING CHALLENGES

### iPSC Generation

*Viral methods*

Methods for achieving reprogramming have progressed significantly from the groundbreaking work completed by Yamanaka and colleagues. The variety of reprogramming approaches stems from an interest to develop methods that do not integrate DNA into the host genome, making them feasible for eventual use in clinical applications. Virus-mediated reprogramming is commonly used for its capacity to efficiently transduce cells of interest. Original methods using retrovirus[2,17] and lentivirus[18] remain widely used. The disadvantage is that these viruses integrate transgenes randomly into the host genome upon infection. This has the potential to cause unpredictable changes in the genome and result in aberrant transgene expression, which can potentially impact data interpretation and differentiation potential. Although scientists have devised ways to remove the transgenes after reprogramming is complete (using loxP sites and Cre recombinase),[19] it is still necessary to thoroughly screen clones for confirmation of excision and loxP site retention that may alter endogenous gene expression. For these reasons, methods to reprogram cells have since focused on naturally non-integrating approaches.

Improvements using viruses that do not integrate into the genome, including adenovirus and Sendai virus, are becoming increasingly popular. The use of adenovirus was first applied to iPSC reprogramming shortly after the initial reprogramming reports.[20,21] Adenovirus was chosen for its inability to integrate into the genome and ability to provide high transgene expression for a limited amount of time as the virus is reduced with each cell division. Although successful, the incidence of tetraploid cells following reprogramming has limited its usefulness.[20]

Sendai virus has recently been developed for reprogramming because it is non-cytopathic and remains in the cytoplasm of host cells.[22] In addition, it has the ability to reprogram peripheral blood mononuclear cells (PBMCs) in addition to other somatic cells (including fibroblasts). In addition to the non-integrating nature of this virus, it is cleared by culturing cells at an elevated temperature, or treatment with siRNA against the large protein (L-gene) of

the virus. Recently, a modification has also been introduced that enables clearance by microRNA 302L, naturally produced by pluripotent cells, which recognizes an inserted microRNA targeting sequence that was incorporated into the vector (Nakanishi, personal communication).

### *Non-viral methods*

Non-viral methods include minicircle and episomal plasmids, piggyBac transposon, RNA transfection, protein transduction, and microRNA transfection. Traditional transfection was successfully used to reprogram mouse cells using polycistronic plasmids.[23,24] However, extensive screening was necessary to find clones without integration. In addition, repeated transfections were necessary to maintain high transgene expression. Minicircle DNA was first applied to reprogram adipose stem cells.[25] Polycistronic minicircle is advantageous because transfection efficiency is improved and it is diluted out more slowly during cell division, thus reducing the number of transfections required. Unfortunately, both conventional and minicircle DNA reprogram at much lower efficiency and also require more hands on time due to multiple transfections. Episomal plasmids can be stably introduced into cells using drug selection, and can be removed after drug selection is discontinued. Yu *et al*[26] first showed feasibility of this method in 2009 by reprogramming human foreskin fibroblasts, although unfortunately this method also yielded low efficiency. The piggyBac transposon system enables the removal of all exogenous elements, cleaner than the Cre-loxP system. In 2009, multiple groups demonstrated high efficiency reprogramming using tetracycline-inducible or polycistronic expression of the reprogramming factors.[27–29] Although removal of the transgenes was demonstrated by sequencing, transposase-mediated excision of transgenes was shown to also induce microdeletion of genomic DNA, which could pose a problem for future use.

Methods described thus far carry the risk of unexpected persistence or genetic modification. To circumvent this, scientists have been devising methods, which do not introduce DNA into the host cell. mRNA synthesized *in vitro* from cDNA of the reprogramming factors was demonstrated to be successful in 2010.[30] This method utilized host cells translation machinery, although it requires five consecutive transfections to be successful. Protein delivery is an alternative to nucleic acid introduction. Harnessing the ability of reprogramming factors tagged with C-terminus poly-arginine domains to transduce through the cell membrane, two groups showed feasibility.[31,32] Protein delivery method eliminates the need to screen for integration of transgenes. However, efficiency was lower, and multiple rounds of transduction were necessary. In 2011, mature double-stranded microRNA including mir-200c, mir-302s and mir369s family of microRNAs were shown to reprogram somatic cells by direct transfection.[33] Although this method resulted in lower efficiency, it provides a viable method compatible with clinical use.

Ultimately, these methods and modifications have laid the groundwork for improving methodology. Combination of these methods with small molecules has been shown to improve reprogramming. In 2013, Deng's group used a cocktail of seven compounds to reprogram mouse somatic cells into iPSCs at efficiency comparable to standard reprogramming techniques.[34] The ability to apply this technique to human cells would be an exciting advance in the field. Although many methods focus on efficiency, it is important to note that efficiency alone is not the most important aspect of the reprogramming process. In the end, it is more important to obtain a number of high quality iPSCs clones. Generally, fewer than 10 clones per individual are needed, especially if using a non-integrating method where exhaustive transgene screening is not necessary.

### *Practical considerations*

Starting cell type before reprogramming is an important consideration. Dermal fibroblasts and PBMCs are the most common starting cells, and while most methods nearly always reprogram dermal fibroblasts successfully, using a method that also works for PBMCs increases flexibility. Benefits include reduced processing time (biopsy outgrowth can require up to 1 month *vs* isolation of PBMCs from a blood draw can be completed within an hour). In addition, a blood draw is less invasive and particularly useful for obtaining cells from pediatric patients. Ultimately, starting cell type may vary depending on the questions to be asked. If initial assays using fibroblasts can be useful for disease understanding, it may be advantageous to reprogram those cells. Regardless of delivery method (virus, plasmid and so on), utilizing polycistronic plasmids to introduce all reprogramming factors at once is easier and increases the likelihood of successfully reprogramming.

Commercial availability of multiple reprogramming methods is also increasing. Although cost may be an issue, it is possible to send samples to be reprogrammed using various non-integrating methods or to purchase ready to use reagents to complete the procedure in the lab. In addition, it is important to realize the reprogramming process itself is not the only barrier to overcome. It is imperative to learn proper culture techniques. To this end, many commercially available cell culture media are available (Life Technologies, ReproCell, Stem Cell Technologies and so on) that can ease the transition for researchers who are new to the culture techniques required to propagate these cells. Even for seasoned scientists, commercial protocols and products enable quick improvements and it is advantageous to keep up to date to reduce labor and improve quality of iPSC culture.

In addition to various culture media, there are also a number of different substrate iPSCs can be cultured on (mouse embryonic fibroblasts, Matrigel, Vitronectin, Geltrex and so on). In addition, iPSCs themselves are generally an

intermediate resource before differentiation to various lineages. As such, the vast variety of differentiation protocols generally has different starting cell culture conditions. Usually, these are referred to as feeder dependent or feeder free. For this reason, it may be advantageous to generate frozen stocks cultured by feeder-dependent and feeder-free techniques to reduce the labor involved if testing out a number of protocols.

## Quality Control

Variability within iPSC clones (either genetic, epigenetic or phenotypic) has been a concern in patient iPSC research. Unless each iPSC clone is carefully evaluated, researchers could potentially run into issues with data misinterpretation when using this approach.

### *Quest for genome stability*

To investigate characteristics of iPSCs derived from monogenic disorders, one of the important issues is to validate retention of the gene mutation in iPSCs and to identify additional mutations introduced during iPSC generation. By comparing genomes of parental cells and iPSCs, exome analysis may be a prerequisite for subsequent medical research of pathogenesis and drug discovery. Whole-exome analysis covering protein-coding sequences is sufficient to investigate pre-existing and additional mutations, although the recent platform of exome analysis has expanded to include not only coding but also untranslated, non-coding RNA and their adjacent regions. The number of single-nucleotide mutations per cell genome was estimated from 22 human iPSCs by extensive exome analysis on protein-coding sequences.[35] Generally, iPSCs are considered to have a comparable nucleotide substitution rate independent of donor cells, except for cells from patients with a genome instability syndrome, a DNA repair disorder or a DNA damage response syndrome. However, acquisition of novel mutations during passages is indeed unavoidable, and banking of early passage iPSC clones is therefore essential once suitable disease iPSCs are established and characterized.

### *Quest for quality control*

In addition to genomic analysis, general characteristics of disease iPSCs such as morphological analysis, *in vitro* differentiation by embryoid body formation, teratoma formation by injection of iPSCs into immunodeficient animals, karyotypic analysis, short tandem repeat analysis, pluripotency markers such as Oct4/3, Sox2, Nanog, SSEA4, Tra-1-60 and Tra-1-81, and gene expression of exogenous and endogenous pluripotency-associated genes are usually performed. Before banking, contamination of mycoplasma, bacteria, virus and endotoxins should ideally be tested. Generally, morphology of iPSCs provides us enormous information including purity, quality, transformation, undifferentiated state and other cell contamination. In addition to these standard quality controls, profiling of RNA expression, DNA methylation and glycans can be added for monitoring when necessary.[10,13,36,37] These comprehensive analyses would also elucidate pathogenic states such as aberrant genomic methylation and gene expression of patient iPSCs.

### *Quest for suitable controls of disease iPSCs*

In addition to disease-derived iPSCs, preparation of suitable control iPSCs are required for elucidation of disease mechanisms and drug discovery. One of the ideal controls is genetically corrected iPSCs. To correct gene mutation in disease iPSCs, ZFN, TALEN and CRISPR/Cas-based methods for genome editing can be used. Alternatively, introduction of exogenous genes that are mutated in disease iPSCs may be used, but the expression level of the exogenous gene may bias phenotypes. Another control is iPSCs obtained from the same age, gender and ethnic group. Usually, iPSCs from more than three independent patients and from more than three independent healthy donors need to be analyzed to conclude that observed pathogenic phenotypes are due to endogenous genotypes of the disease iPSCs. However, genetic correction and preparation of age-, gender- and ethnic-matched controls is labor intensive. To circumvent this, commonly available iPSCs from healthy donors may be used for comparison. MRC5-derived (fetal lung fibroblast) iPSCs have been utilized as a control in several previous reports,[13,37–41] and can be obtained from the public bank. If MRC5-iPSCs do not demonstrate pathogenic phenotypes that disease iPSCs do under the same experimental condition, MRC5-iPSCs would serve as a practical control.

## Differentiation

### *Lack of practical differentiation protocols*

Depending on the desired disease or field of study, there may be ample protocols for investigators to turn to (as in the case of neurodegenerative disease modeling).[3,5] However, unless the particular lab is well versed in the biology of both pluripotent stem cells and differentiated cell types, the likelihood of reproducing a protocol in a reasonable time is uncertain. In general, differentiation protocols take advantage of particular cytokines, culture media and extracellular matrices, thus making these protocols quite expensive. Often, after differentiation, cell populations of interest need to be separated using specific surface markers to achieve sufficient purity. In addition to the expense, most protocols are time consuming and slow in data collection. In general, common obstacles in published differentiation protocols include low reproducibility, low yield, high cost and multiple steps, which often utilize complicated procedures. Thus, except for a few relatively straightforward lineages such as neural progenitors, we are still lacking very practical protocols to prepare a large number of disease-relevant cell types. Developing simple, easy and affordable methods, where the process can be applied to robust

large-scale cell differentiation from patient iPSCs, is truly desired in the field.

*Uncertain quality of differentiated cells*

Depending on the cell type, iPSC differentiated cells may not proliferate well in the long term. As with human primary cells, doubling times while maintaining proper phenotype will most likely be limited, making it more difficult to carry out desired experiments. Furthermore, the possibility of freezing a batch of cells for later use may be unrealistic, giving investigators a 'one shot' per differentiation scenario to obtain meaningful data. This can become taxing if a differentiation protocol takes months from start to finish as in the case of vascular cell differentiation with a 2-month long protocol.[42] Also, unless the differentiation protocol is well established in an investigator's own hands, a portion of the obtained cells will need to be used to assess the proper phenotype. Despite a successful differentiation protocol, investigators may run into issues if these cells are to be used in functional assays. iPSC-derived cells may have the proper phenotype but may be too immature to also possess the normal function of the cells. In that case, investigators will have to optimize such conditions for their specific interests keeping in mind the physiological relevance of their *in vitro* assays.

*Practical considerations*

Although there remain many issues to be improved, some iPSC differentiation protocols are relatively straightforward, and have been successfully used by multiple groups to obtain mesoderm,[42–44] endoderm[45] and ectodermal[46–48] lineages. These protocols utilize available materials, the procedures are uncomplicated, the methods include simple cell purification steps such as sorting, and their reproducibility and usefulness have been demonstrated by other investigators. There are many additional protocols available in the literatures (many that share commonalities, while others are distinct). As the field is constantly changing, updated information is best obtained through an iPSC core facility or colleague scientists. We emphasize here again that one should try to reproduce the protocol(s) in a side-by-side collaboration with a scientist who has expertise in iPSCs and another scientist with experience of the targeted differentiating lineage. Knowing the biology of both ends, the cells you start with and those you end up with, is critical to reproducing protocols in a reasonable time.

## Disease Modeling

*How to fill the discrepancies from real disease*

Although generation of disease-relevant cell types from patient-derived iPSC is a standard strategy for studying a 'disease in a dish' as described above, many human diseases arise from multicellular interactions in the context of tissue architecture, organ or whole-body homeostasis. Therefore, it is essential to further advance model systems to represent a more complex physiological environment similar to the body. When your hypothesis requires the interactions of different cell types for pathogenesis, multiple cell types in a co-culture setting will certainly provide further functional insights for the disease. As an exemplary work, the co-culture of glial cells from ALS iPSCs with neurons from normal iPSCs demonstrated the non-cell autonomous effect of diseased glial cells for aberrant survival of neurons.[49] Similarly, aberrant controls in vasculature tone would be better understood when co-culturing endothelial and vascular smooth muscle cells together rather than using a single cell type.

Admirably, iPSCs possess pluripotency comparable to embryonic stem cells (ESCs), which are originated from the embryonic blastocyst stage embryo. Both iPSCs and ESCs are competent to early developmental cues. Once proper cues are given, initial specification occurs to induce differentiation. The multiple types of differentiated cells are autonomously organized and interact with each other leading to subsequent fate specification like the cascade of embryonic development. To take maximum advantage of this self-organizing ability of pluripotent stem cells, several groups have developed sophisticated 3D culture protocols for making organoid structures *in vitro*. One example is the so-called 'mini-brain' consisting of tissue layers that mimic the brain cortex. Using this culture technique, Knoblich's group demonstrated that iPSCs derived from a microcephalic patient indeed formed a smaller brain than iPSCs from a healthy control.[50] Similarly, several organoid culture techniques for iPSCs have evolved to generate other tissue types and organs (optic cup, pituitary gland).[51,52] Lack of vascular supply is the major limiting factor to grow more functional units in organoid culture. Remarkably, Taniguchi's group was able to generate a transplantable small liver unit from human iPSCs. They co-cultured hepatic endoderm cells differentiated from iPSC with human mesenchymal stem cells and human umbilical vein endothelial cells in a loosely solidified extracellular matrix. These cells autonomously formed the functional units of the liver *in vitro* with the support of microvasculature. Upon transplantation of the unit into immunodeficient mice, the liver bud quickly connected to the host vascular networks and further functional maturation occurred.[53]

Advances in differentiation protocols heavily rely on our knowledge of the molecular mechanisms of embryonic development. Our knowledge is not sufficient to provide the optimal environment for desired morphogenesis from iPSC *in vitro* culture. Nevertheless, simple inoculation of iPSCs into immunodeficient animals is able to form teratomas, which comprise cells from all three germ layers (endoderm, mesoderm and ectoderm). As mature tissue organization (gut epithelial, cartridge and so on) can be observed in the tumor, it will be feasible to assess the histopathological phenotype of patient-derived iPSC using this methodology. For instance, iPSCs from dominant genetic disorders with oncogenesis may develop cancer in teratomas over time. Patients with familial adenomatous polyposis develop

adenoma and adenocarcinoma in colon. Similarly, iPSCs from familial adenomatous polyposis may generate adenoma and adenocarcinoma in colon-like mucosa in teratomas. iPSCs from degenerative disorders may exhibit degeneration or apoptosis of cells in corresponding tissues of teratomas. It is also noteworthy that histopathological analysis of implanted cells into immunodeficient animals may support *in vitro* phenotypes of iPSCs during the differentiation process.

To model systemic disease, it is compelling to reconstitute the human pathological process in experimental animals. For example, type I diabetes is recognized as a type of autoimmune disease, in which three major cell lineages (hematopoietic cells, pancreatic $\beta$ cells and thymus epithelial cells) have important roles. Melton's group has reconstituted the human version of these three lineages into animals by transplantation into immunodeficient mice.[54] A more rigorous approach is led by Nakauchi's group, where they successfully generated a whole kidney or pancreas derived from iPSCs in the pig by blastocyst complementation. They transferred donor pig iPSC into pancreatogenesis- or nephrogenesis-disabled blastocyst stage pig embryos, and demonstrated the embryos were born as chimeras having pancreas or kidney exclusively derived from the donor pig iPSCs.[55] Any blastocyst complementation using human iPSC into animals has not been performed yet because of ethical issues, but theoretically it is feasible to generate whole functional human organs in animals using the same strategy. This humanized animal or hybrid animal approach using patient-derived iPSC would be a next-generation disease model for studying human pathology.

## Gene Editing

Rapidly evolving gene-editing technology has been shown valuable in patient iPSC research as well, as described above with an exemplary case.

### *TALEN*

Transcription activator-like effector nucleases (TALENs) are composed of a DNA-binding domain that is capable of directing the *Fok*I nuclease to a specific target site. Two TALENs, recognizing left and right arms of the target site, respectively, can bring two *Fok*I monomers close together for the formation of a functional dimer, which generates a DNA double-strand break (DSB) on the target site.[56,57] The TALEN-induced DSBs activate the DNA repair system within cells, which stimulates non-homologous end joining (NHEJ) in the absence of a homologous DNA template. The error-prone nature of this repair mechanism results in the introduction of nucleotide mismatches, insertions or deletions. However, in the presence of a homologous template DNA, the DSB triggers homologous recombination, introducing desired DNA sequence alterations. The TALENs have rapidly gained prominence as a novel genome-editing tool, which were successfully applied to create site-specific gene modifications in model organisms such as yeast, plants, zebra fish, mouse, rat and human cells, including human pluripotent cells.[58–62] TALEN has also been used to generate single base-pair mutations, linking single-nucleotide polymorphisms to specific human disease.[63] Furthermore, TALENs have even been utilized to eliminate the mutant form of mitochondrial DNA from patient-derived cells.[64] Currently, TALEN plasmids targeting 18 740 protein-coding human genes have been assembled using a high-throughput Golden-Gate cloning system.[65] Delivery of these TALENs can be achieved by injection of DNA or mRNA encoding TALENs or even the TALEN proteins directly.[62,66,67]

### *CRISPR*

The CRISPR system is another effective genome-editing tool, which utilizes Cas9 nuclease to cleave DNA and chimeric guide RNA (gRNA) to target Cas9 to a specific region in the genome.[68,69] The Cas9-gRNA-mediated genome editing has been shown to have improved efficiencies over TALENs and it is also easier to implement.[68–72] Moreover, it allows simultaneous editing of more than one site through expression of multiple gRNAs.[68,69] This approach was used to create mice carrying five different mutant genes in a single step,[73] and also was shown to generate large deletions of genomic regions by directing Cas9 cleavages at the two sites flanking the desired deletion.[68] Wu *et al*[74] have even shown in mice that a dominant mutation in *Crygc* gene that causes cataracts could be rescued by a Cas9-mediated DSB on the mutant allele, which triggered homology-directed repair based on the endogenous WT allele. More recently, a clone library encoding short gRNAs targeting all open reading frames in the human genome has been generated. Combined use of this library with Cas9 enabled the generation of random gene knockouts in the human genome, which can be screened for desired phenotypes to link genes to their functions.[75,76] The CRISPR technology has been used to cure a mouse model of a human fatal liver disorder (type I tyrosinemia) caused by a single genetic mutation in the fumarylacetoacetate hydrolase gene.[77] This defect in tyrosine catabolism causes toxic accumulation of the amino acid, leading to liver failure. CRISPR-mediated genome editing could one day help treat many diseases caused by single mutations, such as hemophilia and Huntington's disease.

A mutant version of the Cas9 was further reported which cleaves only one strand of the target DNA, generating single-strand nicking, thus favors HR DNA repair over NHEJ (error prone), increasing desired DNA changes over random mutations.[70] Recently, a nuclease-defective Cas9 enzyme has been utilized to label genomic loci, allowing for visualization of *in vivo* of their partitioning in live cells.[78] Most interestingly, the catalytically inactive Cas9 nuclease, in complex with a gRNA, can bind to a specific site, which physically blocks the RNA polymerase, thus silencing the target gene.[79] Similarly, the catalytically inactive Cas9 was fused to known transcriptional activator domains and targeted to specific promoter regions by corresponding gRNAs, upregulating the target gene expression.[80] The

ability to artificially control the expression of specific target genes not only enables us to better understand gene functions but also to manipulate cell fate through controlled expression of desired sets of pathway genes.

## CONCLUDING REMARKS

Undoubtedly, patient iPSCs are an enduring asset for experimental pathology studies, with some exemplary applications introduced above and many more in published literature. Additional technical improvement, particularly in iPSC differentiation methods and three dimensional cultures, as well as expansion of patient iPSC banking, will further accelerate the field. From a pathologist perspective, patient iPSC banking will serve as a powerful addendum to existing tissue banks. Their value is unlimited, as once established, they serve as an enduring and expandable resource for live patient cells. For instance, it is almost impossible to obtain hepatocytes from a rare metabolic disease through liver biopsy of a large number of patients at one given time and place. However, through iPSC banking, such resources will be available to any researcher, any place in the world, and at any time. Banking iPSCs of large patient cohorts with a clinical and GWAS database would be particularly useful in order to identify molecular mechanisms underlying certain genetic links to the disease or individual patients' drug efficacy and toxicity. The future rests on how properly we prepare the resource and how wisely we use it.

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### DISCLOSURE/CONFLICT OF INTEREST

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EDITORIAL

# Stem Cells Bond Our Organs/Tissues and Engineering Products

Masashi Toyoda, PhD; Akihiro Umezawa, MD, PhD

The human body is made up of trillions of cells that work together. Cells make up tissues and organs, and a group of organs performs a certain function for homeostatic maintenance. Many diseases result from a homeostatic imbalance. Stem cells in organs and/or tissues play important roles in the regulation of homeostasis. Stem cell-based therapy has become a promising strategy for the treatment of many diseases. Therefore, regenerative medicine may one day restore the function of damaged organs or tissues. On the other hand, tissue engineering, such as cell-sheet and artificial organs that might supplement or completely replace the functions of impaired or damaged tissues, has developed remarkably.[1] An integrated strategy of tissue engineering including artificial organs and stem cell-based therapy medicine should give rise to a new regenerative medicine for organ failure.

Article p1762

Stents have been used for the treatment of coronary artery disease (CAD) for more than a decade. A coronary stent is placed in an artery to keep the vessel patent to maintain blood flow. Stent implantation is a major treatment option for CAD (eg, in bypass surgery) and has saved many patients' lives.

But no therapy is without risk. In fact, in clinical practice, persistent inflammation occurs around the stent, and can result in coronary restenosis and thrombosis. Until now, however, there has not been an appropriate experimental in vivo model to analyze the mechanisms of these side effects.

In this issue of the Journal, Sato et al[2] report on the established animal models of coronary stenting. In the past, many researchers used the endothelial injury model of CAD, in which there is transient injury to the endothelium, and then the mechanisms of restenosis of the artery are analyzed. But the stent implantation model has had difficulties because of inflammation around the stent. Sato et al used the stenting model of the rat abdominal aorta and showed that transplantation of adipose tissue-derived stem cells (ASCs) prevents this persistent inflammation.

Stem cells can self-renew and differentiate into multiple types of cells and have varying degrees of differentiation potential:

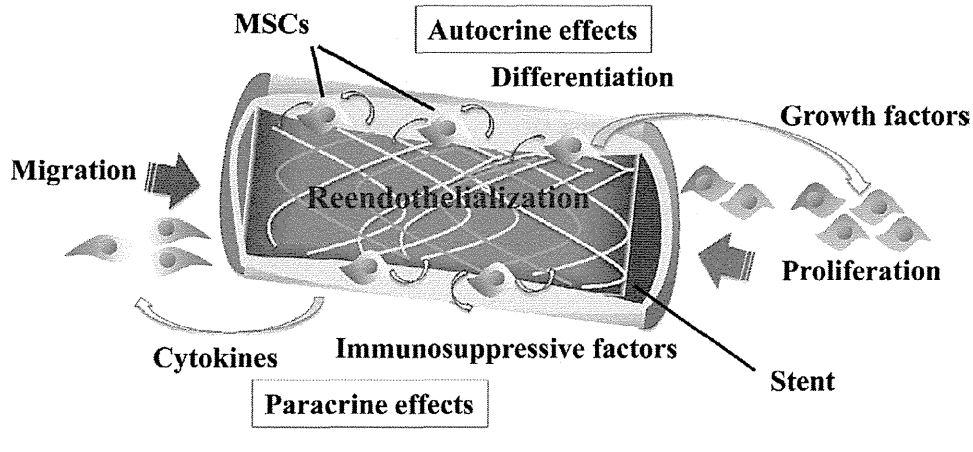


**Figure.** In stent implantation, administration of mesenchymal stem cells significantly stimulates reendothelialization through paracrine and autocrine effects on cell migration, proliferation, differentiation and cell fusion, leading to tissue/organ regeneration.



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pluripotency, ES cells and iPS cells; multipotency, somatic stem cells; and unipotency, or precursor cells. Mesenchymal stem cells (MSCs) are multipotent cells that can be derived from a variety of fetal and adult tissues such as bone marrow and adipose, and possess an immunosuppressive effect.[3] Therefore, MSCs are being used in clinical studies of a variety of diseases, and can be used in allogeneic settings without immunosuppressive therapy, and as cellular immunosuppressants that have the potential to control steroid-refractory acute graft vs. host disease.[4] In addition, the results of Sato et al suggest that the immunosuppressive effect of MSCs enables construction of a model to analyze the in vivo risk of stent therapy. The authors also show that transplantation of ASCs stimulates reendothelialization and inhibits neointimal formation after stent implantation in the animal model (**Figure**). They have previously reported that ASCs stimulate reendothelialization and inhibit neointimal formation in a wire injury model.[5] In the current experiment, they reproduced the effect of reendothelialization by ASC stimulation in the stent model and used 2 types of stents: a Driver coronary stent (bare metal) and a Cipher stent (sirolimus-eluting). Treatment with the Driver implant resulted in more effective reendothelialization by ASC stimulation than with the Cipher stent.

MSCs produce and secrete a broad variety of cytokines, chemokines, and growth factors, which influence the microenvironment through paracrine and autocrine effects on cell migration, proliferation, differentiation and cell fusion (**Figure**).[6,7] In fact, these factors are potentially involved in cardiac repair. The implantation of a cell-sheet over the damaged area of a failing heart has been shown to improve cardiac function through a paracrine effect.[8] Cell-sheets have recently been developed as a tissue engineering technology, and then put to practical use in several clinical studies. Paracrine effects of the cell-sheets by myoblasts, MSCs and cardiac progenitor cells improve cardiac function.[9] Major components of the regenerative mechanism in cell-sheet implantation would be both angiogenesis and the recovery of diastolic function in the heart. Most likely, MSC-released factors lead to tissue/organ remodeling, repair and regeneration in vivo. With stent implantation, MSC administration significantly stimulates reendothelialization and inhibits neointimal formation through paracrine factors.

Sato et al caution that the effect of reendothelialization might depend on the material type of the stent. In the future, factors affecting coronary restenosis after stent deployment in the coronary artery need to be identified. Peripheral technologies have indeed been developing along with the progress of regenerative medicine research, and development of stent design and efficiency and the safety of stent therapy should be expected in the form of combination products with cell-based products.

Cardiovascular disease is a growing problem in our aging society. It will be more important than ever to use stents as treatment for CAD. There are several animal disease models for hypertension, obesity and diabetes, and researchers will use these animals to study the mechanisms of heart disease and the development of stent therapy. The report by Sato et al therefore contributes to the growing number of clinical and preclinical studies of effective stent therapies for heart disease.

## Disclosures

None declared.

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TOPIC HIGHLIGHT

WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

# Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells

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## Abstract

Hormone replacement therapy is necessary for patients with adrenal and gonadal failure. Steroid hormone treatment is also employed in aging people for sex hormone deficiency. These patients undergo such therapies, which have associated risks, for their entire life. Stem cells represent an innovative tool for tissue regeneration and the possibility of solving these problems. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. In particular, they can effectively be differentiated into steroidogenic cells by expressing nuclear receptor 5A subfamily proteins (steroidogenic factor-1 and liver receptor homolog-1) with the aid of cAMP. This approach will provide a source of cells for future regenerative medicine for the treatment of diseases caused by steroidogenesis deficiencies. It can also represent a useful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.



**Key words:** Steroid hormone; Adrenal; Gonad; Steroidogenic factor-1; Liver receptor homolog-1; Mesenchymal stem cells; Differentiation

**Core tip:** Stem cells can be a potential source of cells for regenerative medicine for diseases caused by steroidogenesis deficiency. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. This system can also provide a powerful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

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## INTRODUCTION

In mammals, steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. Steroid hormones are essential for glucose metabolism, the stress response, fluid and electrolyte balance, sex differentiation and reproduction via binding to cognate receptors in target tissues. Therefore, a steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands[1,2]. Impaired cortisol and aldosterone pro-

duction increases adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism. In males, testosterone concentrations decline with age, causing various clinical symptoms such as obesity and hypertension[3-6]. Postmenopausal women often suffer from osteoporosis caused by estrogen deficiency[7,8]. Hormone replacement therapy has been well established for the treatment of such patients, although they require hormone replacement for their entire lifetime. In addition, these patients suffer from various side effects (liver and kidney damage, immune system dysfunction) and risks associated with long-term replacement therapy (cancer). Therefore, another therapy is needed to resolve these problems. Stem cells represent an innovative tool for tissue regeneration and gene therapy, which could possibly solve these problems. In this review, we provide an overview of differentiation and regeneration of steroidogenic cells using mesenchymal stem cells (MSCs), preceded by a description of the development of steroidogenic organs. We also describe molecular events, such as coactivator function and epigenetic modifications, which occur during differentiation.

## DEVELOPMENT OF STEROIDOGENIC ORGANS AND NUCLEAR RECEPTOR 5A SUBFAMILY

Steroidogenesis begins with conversion of cholesterol into pregnenolone in mitochondria by the P450 side chain cleavage enzyme (P450scc/CYP11A1/Cyp11a1), a rate-limiting enzyme in the synthesis of all steroid hormones. Thereafter, various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases[9,10]. Although adrenal glands and gonads produce various steroid hormones in adult life, they have a common developmental origin, a so-called adrenogonadal primordium (AGP) that mainly originates from the intermediate mesoderm and is localized on the coelomic epithelia of the developing urogenital ridge[11-13]. As development proceeds, AGP separates into two distinct populations, adrenocortical and gonadal primordia, characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively, which originate and migrate from other germ layers. During differentiation, adrenal glands and gonads synthesize tissue-specific steroid hormones by specific expression patterns of steroidogenic enzymes.

Steroidogenic factor-1 (SF-1, also known as Ad4BP) is one of the earliest markers of the appearance of AGP[11,14]. Because SF-1 knockout mice fail to develop adrenal glands and gonads, SF-1 represents a master regulator of the development of these organs[15-17]. SF-1/Ad4BP is also important for steroidogenesis by regulating the transcription of steroidogenic genes. SF-1/Ad4BP was originally discovered by Keith Parker and Ken Morohashi as a transcription factor that binds to the Ad4 sequence in promoter regions of all cytochrome P450 steroid hydroxylase genes for transactivation[18,19]. They concluded from the expression of SF-1 in steroidogenic cells and its regulation of all steroid hydroxylase genes that SF-1 is a determinant factor in cell-specific expression of steroidogenic enzymes. In addition to steroidogenic enzymes, diverse groups of SF-1 target genes, such as other steroidogenic genes, pituitary hormones and cognate receptors, and sex differentiation-related genes have been identified thus far[17,20,21]. SF-1 belongs to the nuclear receptor (NR) superfamily. NRs are lipophilic ligand-dependent and independent transcription factors and essential for various physiological phenomena[22,23]. A large number of family members have been identified from invertebrate to mammals. There are a total of 48 family members on the human genome. They share a common structural organization: zinc finger DNA-binding domain and a carboxyl-terminal ligand-binding domain. The NR superfamily can be broadly divided into four classes based on their characteristics (steroid hormone receptors, RXR heterodimers, dimeric orphan receptors and monomeric orphan receptors). SF-1 is categorized into monomeric orphan receptors, although Ingraham and colleague argued the possibility that phosphatidylinositols are ligands for SF-1[24]. SF-1 is very similar to liver receptor homolog-1 (LRH-1). LRH-1 was originally identified in the liver[25] and is known to function in metabolism, cholesterol and bile acid homeostasis by regulating the transcription of a number of genes[26-29]. In addition to the liver, LRH-1 is highly expressed in tissues of endodermal origin. It is also expressed in gonads and involved in steroidogenesis; in particular, its ovarian expression levels are the most abundant among tissues[30]. These factors constitute one of the NR subfamilies and are designated as NR5A proteins (Table 1, SF-1 is NR5A1 and LRH-1 is NR5A2). SF-1 and LRH-1 have various common characteristics, such as binding sequences, target genes and cofactors[24,31-38].

Consistent with its role in steroidogenesis, SF-1 expression is detected in adults in three layers of the adrenal cortex (zona reticularis, zona fasciculata and zona glomerulosa), testicular Leydig and Sertoli cells, ovarian theca, granulosa cells and, to a lesser extent, in the corpus lutea[39,40]. In the corpus lutea, LRH-1 rather than SF-1 is highly expressed and is important for progesterone production[36,41,42]. LRH-1 is also expressed in testicular Leydig cells[12,43,44].

SF-1 knockout mice die shortly after birth because of adrenal insufficiency and exhibit male-to-female sex reversal in external genitalia[15]. These phenotypes are caused by the complete loss of adrenal glands and gonads. Although the initial stages of adrenal and gonadal development occur in the absence of SF-1, they regress and disappear during the following developmental stage.

**Table 1 Summary of the characteristics of steroidogenic factor-1 and liver receptor homolog-1**

| Nuclear receptor | Expressing tissues | Function | Phenotypes of knockout mice |
|---|---|---|---|
| SF-1/ Ad4BP/ NR5A1 | Testis, ovary, adrenal, | Steroidogenesis<br>Sex differentiation<br>Energy homeostasis | Adrenal and gonadal agenesis<br>Sex reversal in external genitalia<br>Impaired expression of pituitary gonadotropins<br>Abnormality of ventromedial hypothalamic nucleus |
| LRH-1/ NR5A2 | Ovary, testis, liver, pancreas, intestine, early embryo | Steroidogenesis<br>Ovulation<br>Bile acid synthesis<br>Glucose metabolism | Embryonic lethal around E6.5-7.5 d |

Because gonads disappear prior to male sexual differentiation, the internal and external urogenital tracts of SF-1 knockout mice are of the female type, irrespective of genetic sex. Heterozygous SF-1 knockout mice show decreased adrenal volume associated with impaired corticosterone production in response to stress[45-47], whereas transgenic overexpression of SF-1/Ad4BP increases adrenal size and ectopic adrenal tissue in the thorax[48,49]. Total SF-1 disruption in mice demonstrated that SF-1 is crucial for the determination of steroidogenic cell fate *in vivo*. It has also been shown in Leydig cell and granulosa cell-specific knockout (LCKO and GCKO, respectively) models that SF-1 plays important roles in steroidogenesis following the development of steroidogenic organs. In LCKO mice, testicular steroidogenic acute regulatory protein (StAR) and Cyp11a1 expression is impaired, indicating a defect in androgen production[50]. Consistent with this hypothesis, the testes fail to descend (an androgen-dependent developmental process) and are hypoplastic. In GCKO mice, the ovaries are hypoplastic, adults are sterile and ovaries show reduced numbers of oocytes and lack corpora lutea[51]. Gonadotropin-induced steroid hormone production are also markedly reduced in this model.

LRH-1 knockout mouse embryos die around E6.5-7.5 d[52,53]. Moreover, heterozygous and GCKO models revealed the importance of LRH-1 in steroidogenesis[41,54,55]. In heterozygous Lrh-1-deficient male mice, testicular testosterone production is decreased along with the expression of steroidogenic enzymes and the development of sexual characteristics[54]. In addition, GCKO mice are infertile because of anovulation with impaired progesterone production[41]. It has also been demonstrated that LRH-1 has a broader role beyond steroidogenesis in these cells as they fail to luteinize.

Although SF-1 and LRH-1-deficient models revealed a common function in gonadal steroidogenesis, both factors cannot compensate for the deficiency of the other factor, even in cells expressing both factors. These facts indicate that even although SF-1 and LRH-1 control transcription by binding to the same response sequences, each has selective actions on the pattern of gene expression in the development of steroidogenic cells and steroidogenesis.

## DIFFERENTIATION OF MSCS INTO STEROIDOGENIC CELLS

In an early study, forced expression of SF-1 has been shown to direct differentiation of murine embryonic stem cells (ESCs) toward the steroidogenic lineage and then Cyp11a1 mRNA was expressed after the addition of cAMP and retinoic acid[56]. However, the steroidogenic capacity of these cells is very limited and they do not undergo *de novo* synthesis because progesterone is the only steroid hormone produced in the presence of the exogenous substrate, 20α-hydroxycholesterol. In addition, major differences between these differentiated cells and natural steroidogenic cells have been shown in cholesterol delivery and the steroidogenic pathway, including deficiencies of StAR (cholesterol delivery protein from the outer to inner mitochondrial membrane in steroidogenic cells) and steroidogenic enzymes, except for Cyp11a1 and Hsd3b1[56-58]. It is also very difficult to isolate clones expressing SF-1 from ESCs and induced pluripotent stem cells[37,57,59] because SF-1 (and LRH-1) overexpression is cytotoxic to these cells. These studies clearly indicate that SF-1 initiates the fate-determination program of the steroidogenic lineage in stem cells, although it is not completed in pluripotent stem cells.

Based on these results, we focused on MSCs[57], multipotent adult stem cells that have been shown to differentiate into mesodermal lineages, such as adipocytes, chondrocytes, osteoblasts and hematopoietic-supporting stroma, both *in vivo* and *ex vivo*[60-63]. Furthermore, MSCs are able to generate cells of all three germ layers, at least *in vitro*. Although MSCs were originally discovered in bone marrow (BM-MSCs)[60,64-66], they have also been isolated from various origins, such as fat, placenta, umbilical cord blood and other tissues[62,63,67-69]. In addition to their multipotency, MSCs have attracted considerable interest for use in cell and gene therapies because they can be obtained from adult tissues and suppress immune responses[70,71]. Indeed, their therapeutic applicability has been assessed in some cases and particularly in bone tissue engineering[72,73].

### *Induction of MSC differentiation into steroidogenic cells in vivo and in vitro*

To investigate the potential of MSCs to differentiate into steroidogenic cells, BM-MSCs from GFP-transgenic rats were transplanted into prepubertal testes (Figure 1A)[57]. In testes, there are two different steroidogenic populations, fetal and adult Leydig cells[74-76]. Even although the cells in these two populations share a common characteristic of producing androgen, they are different in their origin, ultrastructure, lifespan, steroidogenic pathway and its regulation. Fetal Leydig cells have multiple origins and

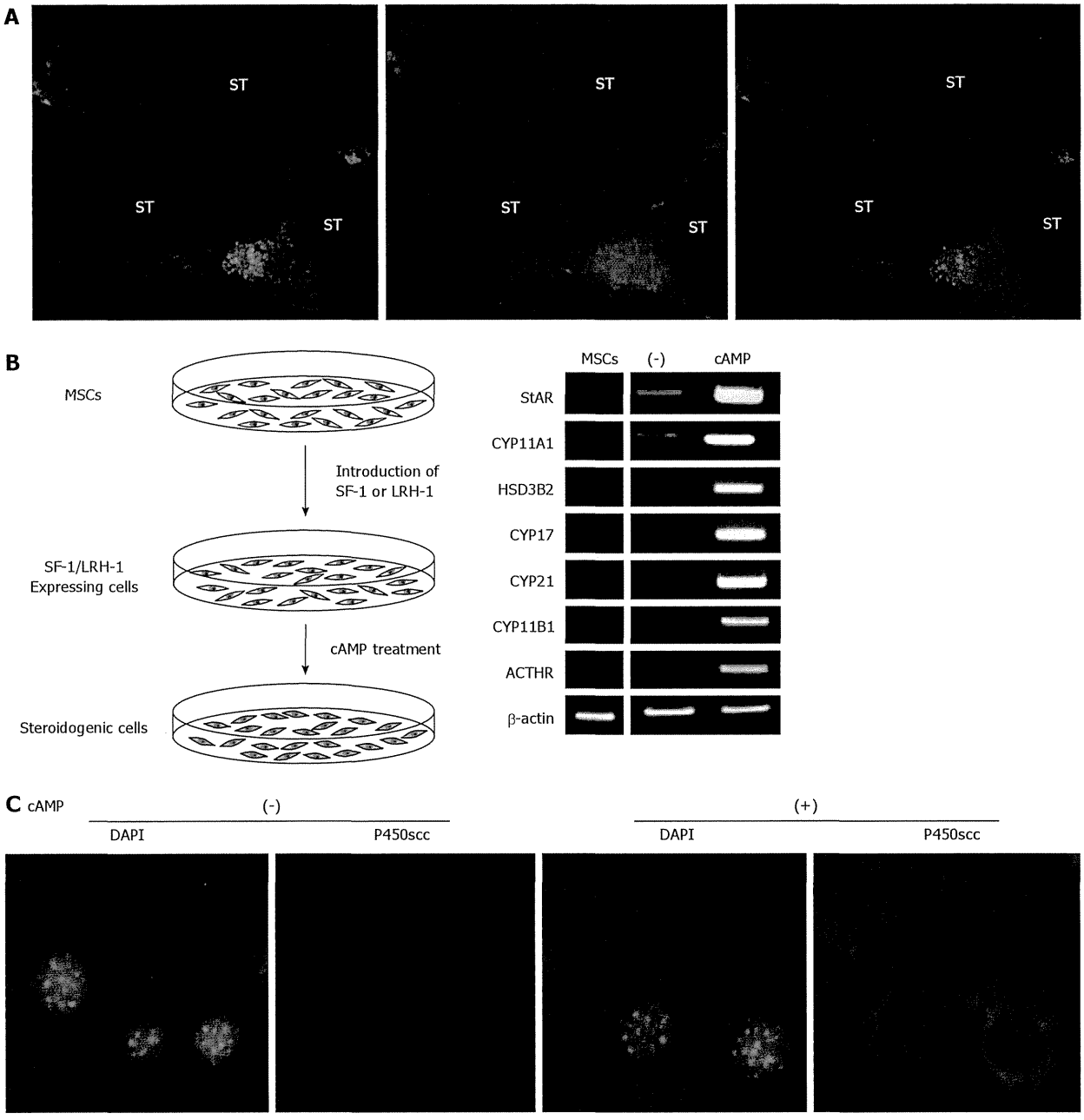


**Figure 1 Differentiation of mesenchymal stem cells into steroidogenic cells.** A: Transplantation of GFP-positive MSCs into prepubertal testis. Double staining of frozen sections from the testis 5 wk after MSC transplantation with anti-GFP and anti-P450scc antibodies; B: Protocol for generating steroidogenic cells from MSCs, and gene expression pattern of steroidogenic cells derived from hBM-MSCs; C: Fluorescence images of DAPI staining and P450scc immunostaining of SF-1 introduced BM-MSCs cultured with or without cAMP. ST: Seminiferous tubule. MSC: Mesenchymal stem cell.

appear in the interstitial space to induce sex differentiation just after the formation of the testis cord. Adult Leydig cells, which originate from mesenchymal precursor cells present in the testicular interstitium, appear to induce puberty. During the postnatal period, fetal Leydig cells are replaced by adult Leydig cells in prepubertal testis. Therefore, it should be possible to use transplanted BM-MSCs in such conditions *in vivo*. Indeed, after 3 wk, transplanted GFP-positive cells were located in the interstitium and expressed various steroidogenic enzymes for androgen production (P450scc/Cyp11a1, 3β-HSD I and Cyp17). These results indicate that MSCs have the capacity to differentiate into steroidogenic Leydig cells *in vivo*.

Although these data suggest that the injected stem cells differentiated into Leydig cells, the apparent stem cell plasticity may also be explained by possible cell-nuclear fusion between donor and recipient cells. However, purified murine BM-MSC lines spontaneously differentiate into steroidogenic cells *in vitro*[57]. A human *CYP11A1* promoter-driven GFP reporter, which consisted of a 2.3-kb fragment that drives reporter gene expression selectively in adrenal and gonadal steroidogenic cells[77],

**Table 2 Properties of steroidogenic cells derived from mesenchymal stem cells induced by steroidogenic factor-1/liver receptor homolog-1 and cAMP**

| Cells | Origin | SF-1/LRH-1 | Produced | Properties of differentiated cells |
|---|---|---|---|---|
| KUM9 | Mouse Bone marrow | Plasmid | Testosterone | Testicular leydig cells |
| hMSC- | Human | Plasmid | Cortisol | Adrenal fasciculata cells |
| TERT-E6/7 | Bone marrow | Retrovirus | Cortisol | |
| UE7T-13 | Human Bone marrow | Retrovirus | Testosterone, cortisol | Fetal adrenal-like cells |
| UE6E7T-12 | | Retrovirus | Testosterone, cortisol | |
| UE6E7T-11 | | Retrovirus | Testosterone, cortisol | |
| UCB408E6E7T-33 | Human Umbilical cord blood | Retrovirus | Progesterone cells | Ovarian granulosa-luteal cells |

has been transfected into BM-MSCs to detect cell populations committed to the steroidogenic lineage. In some transfected cell lines, GFP fluorescence was detected in very small populations that were also positive for Cyp11a1. Further analysis showed that these cells expressed several Leydig cell markers, including 3β-HSD type I and VI and luteinizing hormone (LH) receptor. These observations further support the *in vivo* findings that MSCs have the capacity to differentiate into steroidogenic cells, even under the isolated condition. Therefore, part of population of MSCs can spontaneously differentiate into steroidogenic cells *in vitro*. Interestingly, SF-1 expression was also detected in the GFP-positive cells.

### Differentiation of MSCs into steroidogenic cells induced by SF-1 and LRH-1

The above mentioned results strongly suggest that SF-1 can effectively direct the differentiation of MSCs into the steroidogenic lineage. Indeed, MSCs completely differentiate into steroidogenic cells and show their phenotype after stable expression of SF-1 (using plasmids or retroviruses) and cAMP treatment (Figure 1B)[36,37,44,57,78,79]. SF-1 by itself induces morphological changes in BM-MSCs, such as the accumulation of numerous lipid droplets, although these cells hardly express steroidogenic enzyme genes or produce steroid hormones at detectable levels. However, SF-1 expressing cells strongly become positive for CYP11A1/Cyp11a1 after cAMP treatment (Figure 1C). These cells express many other steroidogenesis-related genes (*SR-BI*, *StAR*, *3β-HSD* and other P450 steroid hydroxylases) and autonomously produce steroid hormones, including androgen, estrogen, progestin, glucocorticoid and aldosterone. Notably, this approach differentiates human BM-MSCs into high cortisol-producing cells in response to ACTH, which are very similar to fasciculata cells in the adrenal cortex (Figure 1B). Adenovirus-mediated transient expression of SF-1 also differentiates BM-MSCs into steroidogenic cells with the capacity of *de novo* synthesis of various steroid hormones[80-84]. After transplantation into animal models, these MSC-derived steroidogenic cells can improve symptoms of steroid hormone deficiencies caused by adrenalectomy. However, as mentioned above, these methods are not applicable to ESCs, embryonal carcinoma cells and terminally differentiated cells, such as fibroblasts and adipocytes[37,57,81]. These results indicate that MSCs are suitable stem cells for differentiation of steroidogenic cells. This hypothesis is supported by the fact that after predifferentiation into MSCs, ESCs can also be subsequently differentiated into steroidogenic cells using SF-1[37].

As in the case of SF-1, introduction of LRH-1 (using retroviruses) into BM-MSCs with the aid of cAMP induced the expression of steroidogenic enzymes and differentiation into steroid hormone-producing cells[44]. Expression of SF-1 was never induced in LRH-1-transduced cells and vice versa. Therefore, LRH-1 could act as another master regulator for determining the MSC fate to the steroidogenic lineage. This phenomenon is likely to represent a situation of active progesterone production in human corpus luteum; LRH-1 is highly expressed, whereas SF-1 is expressed at very low levels[36,42].

## MOLECULAR MECHANISMS OF DIFFERENTIATION

### Steroidogenic cells derived from various MSCs and their properties

In addition to BM-MSCs, various MSC types have been differentiated into steroidogenic cells by the above mentioned methods. However, their steroidogenic properties markedly vary and depend on the derivation tissues and species (Table 2)[36,42,57,83,84]. For example, hBM-MSCs differentiated into cortisol-producing adrenocortical-like cells and umbilical cord blood (UCB)-derived MSCs differentiated into granulosa luteal-like cells, which produced high levels of progesterone[36,57]. Gondo *et al*[83] also reported that steroidogenic profiles of adipose tissue-derived MSCs were markedly different from those of BM-MSCs prepared from the same mouse. However, the cell differentiation fate was consistent in each MSC. These findings suggest that the steroidogenic properties of the differentiated cells depend on the characteristics of the originating MSCs.

To determine the difference between BM-MSCs and UBC-MSCs, the fluctuations in gene expression were investigated by a DNA microarray[36,85]. Among the identified genes, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) was expressed only in UBC-MSCs at relatively high levels. Consistent with these re-

sults, the expression of PGC-1α was observed in ovarian granulosa cells. Overexpression of PGC-1α in granulosa cells induced the genes essential for progesterone synthesis, whereas knockdown of PGC-1α in granulosa cells attenuated the expression of these genes. These results demonstrate that PGC-1α represents one of the important factors for progesterone production in luteinized granulosa cells.

### *Epigenetic regulation during differentiation*

Differentiation of stem cells into specialized cells can be viewed as a process in which epigenetic changes result in alterations in genes expressed by the cell as it becomes more specialized[86,87]. Thus, stem cell differentiation is a process that involves a series of epigenetic changes in the genome: histone and DNA modifications cause chromatin structural changes and affect the profiles of gene expression. In fact, such epigenetic modifications contribute to the induction of steroidogenesis-related genes when MSCs differentiate into steroidogenic cells[44,88-90].

The histone code hypothesis predicts that post-translational modifications of histone tails, alone or in combination, function to direct specific and distinct DNA-templated programs[91]. Histone acetylation is a positive marker of transcription, while histone methylation correlates with transcriptional activation (H3K4, H3K36) and repression (H3K9, H3K27) that are dependent on their amino acid residues[92]. In hMSCs-derived steroidogenic cells, H3K27 acetylation and H3K4 dimethylation (active enhancer markers) increased in the regulatory regions of some steroidogenesis-related genes (glutathione S-transferase A and ferrodoxin reductase) after the introduction of SF-1[89,90]. Conversely, histone eviction, which has been reported in actively transcribed genes[93], took place on the promoter and the enhancer regions of the *StAR* gene[88]. Because these modifications occurred around the SF-1 binding sites, recruitment of SF-1 to the regulatory regions is likely to induce recruitment of various transcriptional regulators and histone modifiers, which in turn alter chromatin structure and lead to the expression of steroidogenesis-related genes.

In addition to histone modifications, DNA methylation at cytosine residues of the dinucleotide sequence CpG, which induces gene silencing, is essential for differentiation and development[94,95]. In MSC-derived steroidogenic cells, the DNA methylation status changes in the promoter regions of some steroidogenic genes during differentiation[44]. In undifferentiated hBM-MSCs, the *CYP11A1* promoter region is hypomethylated, whereas the *CYP17A1* promoter region is highly methylated. In SF-1/LRH-1-introduced MSCs during cAMP treatment, this condition was almost completely unchanged in the *CYP11A1* promoter region, whereas the *CYP17A1* promoter region was progressively demethylated. These methylation patterns of the *CYP11A1* and *CYP17A1* promoters closely paralleled the induction patterns of both genes by cAMP. There is a time lag associated with the induction of steroidogenic enzymes by cAMP treatment in SF-1/LRH-1-introduced MSCs[44,57]. The order of induction of the enzymes is similar to the sequential order of the steroid hormone synthesis pathway; upstream enzymes (CYP11A1 and 3β-HSD) were rapidly induced at earlier time points (6-12 h), whereas downstream enzymes (CYP17A1 and CYP11B1) were induced at later time points (24-48 h). Because this time lag disappeared by treatment with a demethylating agent, the status of DNA methylation in the promoter regions could be important for regulating the expression of steroidogenic enzymes in MSCs.

## CONCLUSION

It is clear that SF-1 represents a master regulator, not only for the development of steroidogenic organs, but also for steroidogenesis following organogenesis. LRH-1 is also important for steroidogenesis in gonads. In addition, SF-1 and LRH-1 direct differentiation of non-steroidogenic stem cells into steroidogenic cells. Among the various stem cell types, MSCs are suitable stem cells for the differentiation of steroidogenic cells. After pre-differentiation into MSCs, pluripotent stem cells can also be subsequently differentiated into steroidogenic cells using SF-1. These cells may provide a source for regenerative and gene therapies, although various problems should be resolved in future studies. It is essential to delineate the conditions that allow the directed differentiation into specific steroidogenic lineages with the characteristics of testicular Leydig cells, ovarian granulosa and theca cells, as well as various types of adrenocortical cells (reticularis, fasciculata and glomerulosa). In addition, it is necessary to establish methods for inducing SF-1 and LRH-1 expression in stem cells without gene transfer. Further studies are required for the realization of regeneration of steroidogenic tissues.

MSC-derived steroidogenic cells also provide opportunities for investigating various phenomena involved in differentiation of steroidogenic cells and steroidogenesis. In addition to the molecular mechanisms of differentiation described herein, the conservation and evolution of the androgen metabolic pathway (11-ketotestosterone production) between teleost fish and mammals has been revealed[78,96]. Genome-wide analyses of differentiated cells identified novel target genes regulated by SF-1 and LRH-1[89,90,97,98]. In addition, they contributed to the elucidation of one of the causes of steroidogenesis disorders[99-101]. Thus, progression of these studies is also important for the understanding of steroidogenesis and its related disorders.

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