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MINI REVIEW

Mechanism of human somatic reprogramming to iPS cell

Rika Teshigawara, Junkwon Cho, Masahiro Kameda and Takashi Tada

Somatic reprogramming to induced pluripotent stem cells (iPSC) was realized in the year 2006 in mice, and in 2007 in humans, by transiently forced expression of a combination of exogenous transcription factors. Human and mouse iPSCs are distinctly reprogrammed into a 'primed' and a 'naïve' state, respectively. In the last decade, puzzle pieces of somatic reprogramming have been collected with difficulty. Collectively, dissecting reprogramming events and identification of the hallmark of sequentially activated/silenced genes have revealed mouse somatic reprogramming in fragments, but there is a long way to go toward understanding the molecular mechanisms of human somatic reprogramming, even with developing technologies. Recently, an established human intermediately reprogrammed stem cell (iRSC) line, which has paused reprogramming at the endogenous *OCT4*-negative/exogenous transgene-positive pre-MET (mesenchymal-to-epithelial-transition) stage can resume reprogramming into endogenous *OCT4*-positive iPSCs only by change of culture conditions. Genome-editing-mediated visualization of endogenous *OCT4* activity with GFP in living iRSCs demonstrates the timing of *OCT4* activation and entry to MET in the reprogramming toward iPSCs. Applications of genome-editing technology to pluripotent stem cells will reshape our approaches for exploring molecular mechanisms.
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REPROGRAMMING OF SOMATIC CELLS

Reprogramming somatic cells into induced pluripotent stem cells (iPSCs), which possess unique properties of self-renewal and differentiation into multiple cell lineages, is achieved by transduction using a defined set of transcription factors: Oct4 (Pou5f1), Sox2, Klf4, and c-Myc (OSKM) in mice,[1,2] and humans.[3,4] The success of iPSC generation opens a way to produce patient-specific pluripotent stem cells with less ethical issues than embryonic stem cells (ESCs) generated from fertilized pre-implantation embryos. Personalized iPSCs are expected to contribute to the exploration of cure and cause of diseases, drug screening, and tailor-made regenerative medicine. iPSC generation methodology has improved with different delivery systems, including non-integrating vectors, deletion after integration, DNA-free transduction, and chemical induction.[5–11] Furthermore, novel approaches for iPSC production have been developed, including combinations of alternative transcription factors.[12] In addition to those, reprogramming-susceptible cell types have been identified.[13–15] Even with studious effort for methodological and technical improvements, the efficiency of reprogramming remains ~0.1% in humans, and ~1.0% in mice.[16] Mechanisms of somatic reprogramming have important implications for iPSC applications. Furthermore, iPSCs could be of great use in exploring molecular mechanisms of many diseases and embryonic development as models. However, the low efficiency and stochastic nature of reprogramming hinders the understanding of reprogramming mechanisms.

MECHANISMS OF REPROGRAMMING IN MICE

In mice, the specific order of reprogramming events has been determined as (i) activation of alkaline phosphatase, (ii) silencing of somatic-specific expression, (iii) expression of SSEA1, and (iv) progressive silencing of exogenous genes with concomitant upregulation of endogenous *Oct4* and *Nanog*[17–19] (Figure 1a). *Nanog* is a key player of the stem cell regulatory network critical for acquiring a pluripotent state.[20] EpCAM, c-Kit, and PECAM1 were identified as other surface markers of early, intermediate, and late genes of SSEA1-positive cells, respectively.[21,22] Information on roadblock genes during reprogramming and stage-specific markers for enrichment of intermediately reprogrammed cells prone to forming iPSCs is being accumulated using several advanced technologies.

In addition to the marker genes, pluripotency-associated mmu (Mus Musculus) -microRNAs (miRNAs) are sequentially expressed, and implicated with induction, maturation, and stabilization of unique characteristics of iPSCs[23–25] (Figure 1a). In chromatin reprogramming, pioneer

Department of Developmental Epigenome, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan
Correspondence: Professor T Tada, PhD, Department of Developmental Epigenome, Institute for Frontier Life and Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: ttada@infront.kyoto-u.ac.jp

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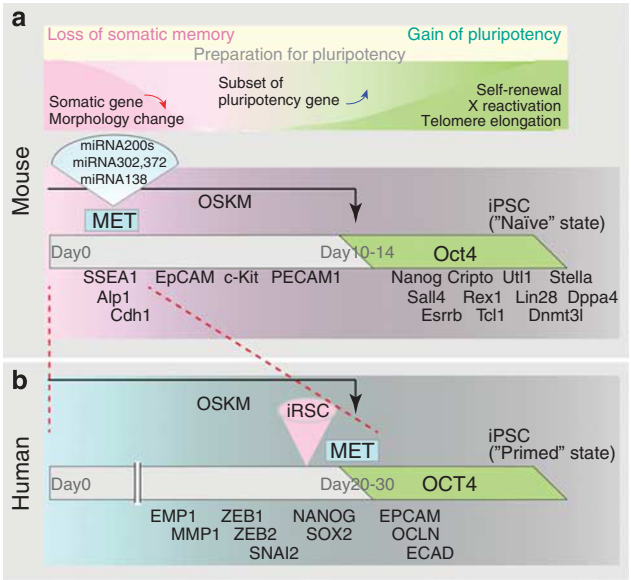

Figure 1 Sequential events, changes of gene and microRNA expression in reprogramming mouse (**a**) and human (**b**) somatic cells to induced pluripotent cells (iPSCs). Mesenchymal-to-epithelial transition (MET) occurs at differential timing in the road of reprogramming. Duration of expression of exogenous reprogramming factors, Oct4, Sox2, Klf4, and c-Myc (OSKM) is indicated by black arrow. Expression of endogenous Oct4/OCT4 is indicated by the green rectangle. Derivation of timing of intermediately reprogrammed stem cells (iRSCs) in the reprogramming process is shown by the pink triangle in humans (**b**).

transcription factors bind directly to condensed chromatin and elicit a series of chromatin remodeling events, including histone modification of H3 lysine 4 and 27 (ref. 26) that lead to opening of chromatin during cell division, which creates opened chromatin situation that other transcription factors are readily accessible.[27] In cell cycle reprogramming, rapid proliferation with a characteristic cell cycle structure of short G1- and G2-gap phases is signature to iPSCs accompanying self-renewal and pluripotency.[28]

MET (mesenchymal-to-epithelial transition), which is the hallmark critical event toward the derivation of iPSCs from mouse embryonic fibroblasts (MEFs), occurs at an early stage of reprogramming.[23,29,30] MET is characterized by upregulation of epithelial genes, *E-Cadherin*, *Cdh1*, *Epcam*, and epithelial-associated mmu-miRNA-200 family, and down-regulation of mesenchymal genes, *Snail1/2*, *Zeb1/2*, and *N-Cadherin*.[31–33] Exogenous Oct4 and Sox2 bind to the promoter regions of mmu-miRNA-141/200c and the mmu-miRNA-200a/b/429 cluster, respectively, and induce transcription activation of the mmu-miRNA-200 family (miRNA-200s).[29]

MET is driven by a strong bone morphogenetic protein (BMP) response through induction of mmu-miRNA-200s and 205 according to the BMP-miRNA-MET pathway.[23] Repression of mmu-miRNA-200s with specific inhibitors results in repression of MET and iPSC generation. Furthermore, the effects of mmu-miRNA-200s and 205 were blocked by *Zeb2* overexpression. Collectively, the mmu-miRNA-200/Zeb2 pathway critically functions in promoting MET at the early stage of somatic reprogramming.[29,34] Moreover, MET is controlled under the orchestrated regulation of epigenetic modification modulated, in part, by H3K36 demethylases Jhdm1a/1b[35] and H3K79 methylase Dot1L.[36] Consequently, MET accompanied by changes in morphology from the somatic to pluripotent type cell induces expression pattern changes in several thousands of genes.[23,24]

MECHANISMS OF REPROGRAMMING IN HUMANS

Human iPSC colonies exhibit characteristic flat-shaped morphology, which is clearly distinct from mouse iPSC colonies that exhibit bowl-shaped morphology. Mouse iPSCs are reprogrammed into a 'naïve' state similar to the state of mouse embryonic stem cells (ESCs), whereas human iPSCs are in a 'primed' state similar to the state of human ESCs, and mouse Epistem cells (EpiSCs)[37,38] (Figure 1b). Mouse ESCs, but not mouse EpiSCs, are germline-competent in blastocyst-injection-mediated chimeras. Notably, 'primed'-state human and 'naïve'-state mouse iPSCs make differential responses in mouse ground state culture conditions with N2B27+2i+LIF medium.[39,40] Distinct pluripotent states between human and mouse iPSCs are also demonstrated by X-chromosome reactivation of female somatic cell reprogramming.[41] Collectively, the final cell fate by somatic reprogramming through forced expression of the same exogenous OSKM transcription factors is distinctive between humans and mice (Figure 1a and b). Thus, it is predicted that parts of the reprogramming process are shared with humans and mice, whereas others are unique to humans or mice.

Indeed, partially diverged interactions of pluripotency-associated miRNAs and the target mRNAs between humans and mice have been summarized.[25] This is consistent with the divergence of sequential reprogramming events between humans and mice. In mice, MET occurs early in reprogramming of MEFs to iPSCs, which precedes the activation of endogenous *Oct4*;[21,23] however, in humans, MET occurs at a later stage of reprogramming with the same timing of endogenous *OCT4* activation (Figures 1a and b). It is likely that MET is a checkpoint for entry into a 'primed' state of pluripotency, whereas activation of an *OCT4/Oct4* is a key step for commitment to further cellular reprogramming through composing OCT4/Oct4-induced pluripotency molecular network. In this context, human iPSCs acquired OCT4-induced pluripotency under a 'primed' state prior to conversion to a 'naïve' state. This implies that, in mice, a 'primed ' state is generated at a much earlier stage, with additional steps required prior to Oct4-induced pluripotency. MET is an event separable from activation of endogenous OCT4/Oct4, as shown by differential timing of entry to MET between human and mouse reprogramming.

The generation of human 'naïve' iPSCs, which demonstrated molecular characteristics and functional properties similar to mouse ESCs/iPSCs, was reported with the

chemically defined culture conditions, NHSM (naïve human stem cell medium).[42] Conversion from a 'primed' state to a 'naïve' state is facilitated by forced expression of exogenous *Klf4* under ground state culture conditions in mice.[43] Furthermore, it was revealed that the pluripotency-associated hsa (Homo sapiens) -miRNA-290/302 family of microRNAs regulates the transition of ESCs from a 'naïve' to 'primed' state of pluripotency.[44] However, in humans, mechanisms involved in 'primed-to-naïve' conversion are largely unknown.

In human ESCs, OCT4, SOX2, and NANOG, which play essential roles in somatic reprogramming to iPSCs, co-occupy a substantial portion of more than 300 target genes with collaboration to form regulatory circuitry consisting of autoregulatory and feedforward loops,[45] suggesting that appropriate transcription of OCT4, SOX2, and NANOG is required to stabilize a pluripotency molecular network for facilitating the maturation of the somatic reprogramming process toward iPSC generation. Prior to stabilization of the pluripotency network through MET and endogenous *OCT4* activation in human reprogrammed cells, a 'primed' pluripotency competent state may be induced by forced expression of *c-Myc* and *Klf4* as demonstrated by mouse reprogramming cells.[30] Recently, several 'naïve'-specific, but not 'primed'-specific, cell surface marker proteins were demonstrated by comprehensive profiling of cell surface proteins by flow cytometry in 'naïve' and 'primed' human pluripotent stem cells (PSCs).[46] It is unclear whether stabilization of human iPSCs under a 'primed' state but not a 'naïve' state resulted from passing through a transient 'naïve' state during reprogramming. Newly identified 'naïve'-specific marker proteins could facilitate to define the human pre-iPSC and iPSC state in the progress of somatic reprogramming.

EXPLORING REPROGRAMMING MECHANISMS WITH AN INTERMEDIATELY REPROGRAMMED STEM CELL LINE

In mice, intermediately reprogrammed cells characterized by silencing somatic genes, activated SSEA1, and the potential of conversion to iPSCs were predicted as a transient cell population, whereas partially reprogrammed cells

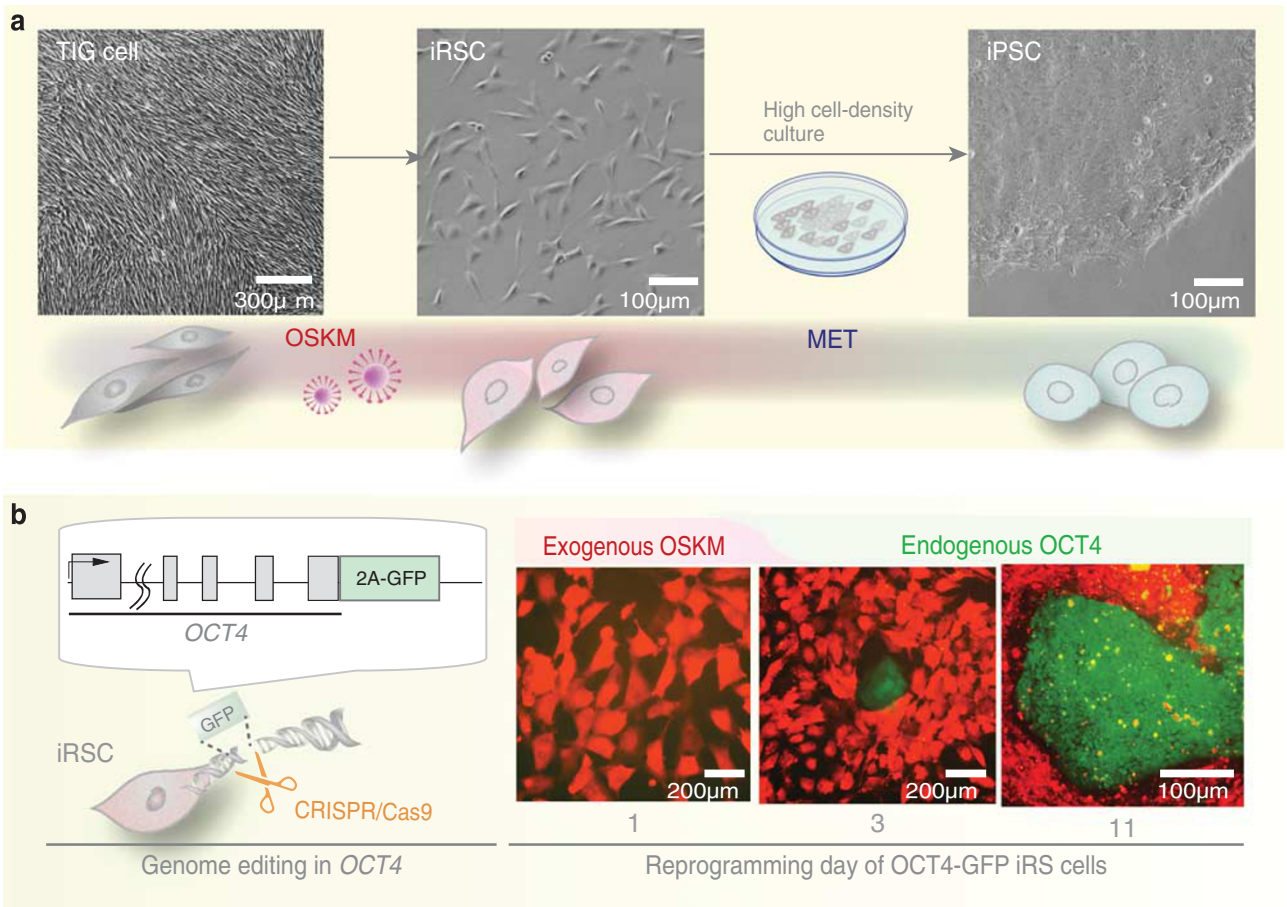

Figure 2 (**a**) Sequential changes of cell morphology from human TIG fibroblast cell, intermediately reprogrammed stem cell (iRSC), to induced pluripotent stem cell (iPSC) in somatic reprogramming. OSKM; reprogramming factor, Oct4, Sox2, Klf4, and c-Myc. (**b**) CRISPR/Cas9-mediated genome editing of the endogenous *OCT4* gene in human iRSCs to visualize the activity of OCT4 by fluorescence marker, GFP (Green Fluorescence Protein) in the reprogramming process of iRSCs to iPSCs. MET; mesenchymal-to-epithelial transition.

characterized by expression of reprogramming transgenes, activated proliferation genes, silencing of pluripotency genes, and aberrant expressing lineage genes were established as several endogenous *Oct4*-negative pre-iPSC lines without potential of conversion to iPSCs.[2,47,48] In humans, partially reprogrammed iPSCs resumed reprogramming by upregulation of *KLF4*,[49] and pre-iPSC-like cell lines were established as cancer stem cell lines.[50] Metabolome profiling demonstrated that human partially reprogrammed iPSCs shared only 74% similarly expressed metabolites with human ESCs,[51] indicating that transcriptome of partially reprogrammed stem cells is considerably different from that of ESCs. Therefore, establishment of iPSC lines makes no promise to establish stable lines of human partially reprogrammed iPSCs. Under such a state, derivation of human intermediately reprogrammed stem cells (iRSCs) as stable lines was unexpected.

Human iRSCs, established from the reprogrammed fetal lung fibroblast cell lines, TIG1 and TIG3, by retroviral transduction of OSKM reprogramming factors were characterized by silencing of somatic genes, activated reprogramming transgenes, self-renewal ability, activated SSEA4, and potential of conversion to iPSCs[52] (Figures 1b and 2a). Notably, iRSCs efficiently resumed the reprogramming process toward iPSC generation under specified culture conditions at a high cell density. Molecular stimuli involved in resuming reprogramming from iRSC to iPSC are elusive. It has been reported that high cell density is a potent negative regulator of cell cycle and expression of genes including retrotransposon,[53] implying that exogenous OSKM genes were silenced as a consequence of high cell densitiy of iRSCs. iRSCs were marked by endogenous expression of core pluripotency factors, *SOX2* and *NANOG* but not *OCT4*, in addition to exogenous OSKM reprogramming factors. Endogenous *OCT4* was activated along with entry to MET and silencing of exogenous transgenes. GFP (green fluorescence protein) knockin to the endogenous *OCT4* locus by CRISPR/Cas9-mediated genome editing-enabled visualization of the *OCT4* activation kinetics in living reprogramming cells transit from iRSCs to iPSCs (Figure 2b). It was revealed that activation of endogenous *OCT4* simultaneously occurring with silencing of exogenous OSKM reprogramming factors is induced prior to entry into MET.

Interestingly, time-lapse analyses of endogenous *OCT4* activity demonstrated that *OCT4*-positive reprogramming cells created *OCT4*-positive and negative daughter cells through asymmetric cell division soon after *OCT4* activation, while *OCT4*-positive cells enabled symmetric cell division to form two daughter cells with the same pluripotent identity in larger growing colonies.[52] It is likely that instability of endogenous *OCT4* is linked to the cell characteristics of symmetric or asymmetric division. It has been debated whether reprogramming entails a hierarchic or stochastic process.[54] Once OSKM factors are silenced and endogenous *OCT4* is activated in a stochastic manner, further reprogramming is proposed to progress in a hierarchical manner.[12] Contrary to this, it was proposed that endogenous *Oct4* activation is insufficient for progression of subsequent events in mouse somatic reprogramming.[55,56] In the maturation process of iRSC-to-iPSC conversion, endogenous *OCT4* activation is essential for iPSC generation, but not sufficient for determining cell fate to be iPSCs.

It is controversial whether the reprogramming pathway from somatic cell to iPSC is a single pathway. This is linked to hypotheses as to whether the reprogramming process occurs in a hierarchic or stochastic manner. It has been proposed, in mice, that the pluripotency spectrum can encompass multiple, unique cell states, including an alternative somatic reprogramming path to iPSCs through a *Nanog*-positive transient state, in addition to the preconceived *Nanog*-negative transient state.[57,58] Collectively, reprogramming mechanisms of cellular reprogramming from somatic cells to iPSCs are more complicated rather than those we expected when OSKM-mediated somatic reprogramming was discovered.

iRSC APPLICATIONS

Understanding of molecular mechanisms involved in human somatic reprogramming will not directly contribute to curing specific diseases of patients, but will be useful for investigating medical biology, including human embryonic development, anti-aging, cell physiology, and epigenetics. To do so, application of genetic manipulation to human iPSCs, which is desirable for repair of genetic mutations and deficiencies, is one of the crucial approaches. Single-cell sub-cloning is an inevitable process for genetic manipulation. However, dissociation-induced pro-apoptosis takes place in subcultures of iPSCs,[59] even using the anti-apoptosis molecule, Rho-associated kinase (ROCK) inhibitor Y-27632.[60] An advantageous property of iRSC use is that they are readily expandable from a single cell after conventional gene modifications. Afterwards, reprogramming from gene-manipulated iRSCs to iPSCs can be feasibly resumed by the change of culture conditions. iRSCs will be a powerful cell source for applying recently developed genome-editing technologies.[61,62] Furthermore, iRSC-based identification of marker genes modulating different reprogramming stages would greatly facilitate the understanding of epigenetic events that occur at each stage by enabling enrichment of subpopulations of reprogramming cells. Integration of a inducible gene expression/repression control system enabled by genetic modifications with iRSCs could help for exploring genes responsible for conversion of iRSCs to 'naïve' iPSCs in the reprogramming. Only a part of the reprogramming mechanism is understood in humans. Further investigation of mechanisms of somatic reprogramming by developing new technologies, and integration with new scientific fields may shed light on the fundamental question of 'what is life'.

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DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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