Disease modeling of immunological disorders using induced pluripotent stem cells

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Abstract
Disease-associated induced pluripotent stem cells (iPSCs) established from patients are now widely used for disease modeling. They can provide an unlimited source of hematopoietic cells that carry the patients’ genetic background, making them advantageous for modeling immunological disorders. To obtain functional immune cells from human iPSCs, we have developed a differentiation system that generates immortalized myeloid cells including neutrophils and monocytic cells. By using this strategy, we have established in vitro models of many immunological disorders. These models have proven useful for genetic diagnosis and elucidation of the disease mechanism.

1. Introduction
Because of their pluripotency and capacity for self-renewal, human pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can be potential cellular sources for regenerative medicine, drug screening, toxicology testing and the investigation of disease mechanisms [1,2]. iPSCs are PSCs reprogrammed from somatic cells by introducing sets of transcription factors and possess ESC-like characteristics [3,4]. Disease-associated iPSCs established from patients are now widely used for disease modeling, because these cells share an almost identical genetic background with the patients.

Immunological disorders are abnormalities of the immune system, which consists mainly of hematopoietic cells in the blood, lymphoid system and tissues. To understand their cellular pathophysiology, studies using patient-derived primary hematopoietic cells or animal models are usually conducted. However, these approaches have limitations. For example, obtaining a large number of patient-derived cells from the blood can be invasive, and the in vitro functions of the cells are often affected by the patients’ conditions, such as the presence of therapeutic agents. On the other hand, although genetically modified animals provide an indispensable opportunity to study in vivo pathophysiology, differences in immunological development between humans and other animals can cause discrepancies in the resulting phenotypes. Modeling immunological disorders with patient-derived iPSCs can circumvent these issues, because they can be an unlimited source of hematopoietic cells that inherit the patients’ genetic background [5].

Autoinflammatory syndrome is defined as disorders associated with dysregulation of the innate immune systems [6]. In typical cases, patients with autoinflammatory syndrome show various inflammatory symptoms, including periodic fever, skin rash and sterile serositis [7,8]. PSC models of autoinflammatory syndrome are attractive, because (1) most typical autoinflammatory disorders are monogenic, (2) the responsible cells are usually innate immune cells, which can be robustly differentiated from PSCs, and (3) there is a high demand for better therapeutics and diagnostics. In this review, we introduce our strategy for modeling immunological disorders using human PSCs with special attention on autoinflammatory disorders and application of these models to the identification of the genetic and biological backgrounds of the patients’ pathophysiology.

2. Hematopoietic differentiation and establishment of myeloid cell lines
To obtain functional hematopoietic cells from human PSCs, we have developed an original monolayer differentiation system that generates hematopoietic lineages (Figure 1) [9]. In this system, human PSCs can be
differentiated into mesodermal lineage cells that give rise to both hematopoietic cells and vascular endothelial cells [10]. The resulting hematopoietic progenitor cells are further differentiated into various hematopoietic lineages such as erythroid, megakaryocytic and myeloid cells. By optimizing the cytokine setting, monocytic cells including macrophages and dendritic cells can also be obtained efficiently [11] and used to model autoinflammatory disorders [12].

Although these cellular progenies can be used for functional analysis, one of the obstacles facing disease-associated iPSC studies is that direct differentiation from human PSCs is often time- and labor-intensive. Furthermore, the differentiation efficiency and the results of the functional analysis can often show high variance. To overcome these issues and stably obtain a large number of mature hematopoietic cells from human PSCs, researchers have tried to immortalize PSC-derived hematopoietic progenitors by introducing several genetic factors and differentiating the cells towards terminally differentiated hematopoietic progenies (Figure 2) [13–17]. We have used this approach to obtain proliferating myeloid cell lines (iPS-MLs). The Satoru Senju Laboratory was the first to establish iPS-MLs from iPSC-derived monocytes by introducing three transgenes: cMYC, BMI1 and MDM2 [16,17]. iPS-MLs can be expanded in the presence of M-CSF and show an immature monocytic appearance with the expression of CD33 and CD14. Importantly, iPS-MLs can be differentiated into macrophages or dendritic cells and used for various functional analyses. By combining our robust differentiation protocol [9,11] and myeloid immortalization, we can obtain an unlimited number of functional myeloid cells established from the iPSCs of patients with immunological disorders.

3. Identification of somatic NLRC4 mutation as a cause of autoinflammation

Neonatal-onset multisystem inflammatory disease [NOMID; OMIM #607715, also known as chronic infantile neurological cutaneous and articular syndrome (CINCA)] is an autoinflammatory disease with severe inflammatory symptoms persisting from the neonatal period [18,19]. Approximately 90% of NOMID patients carry heterozygous gain-of-function mutations in the NLR family pyrin domain containing 3 (NLRP3) gene either as a constitutive mutation [20,21] or somatic mosaicism [22,23]. Mutant NLRP3 causes excessive amounts of the proinflammatory cytokine IL-1β, resulting in IL-1β-dependent autoinflammation. However, in the remaining 10% of NOMID patients, the responsible genetic factor(s) remains unidentified. To understand the underlying pathophysiological and genetic mechanism for these mutation-negative patients, we recruited one NOMID patient without known genetic etiology. The patient is a Japanese male with typical NOMID symptoms [24,25] but negative for any NLRP3 mutation even as low-frequency somatic mosaicism [23,26]. A whole-exome sequencing analysis using DNA samples from the patient and his parents identified 26 genes as candidates, but the candidate list could not be narrowed further. Interestingly, despite the absence of any NLRP3 mutation, the clinical phenotype of the patient was driven by IL-1 based on the response to anti-IL-1 treatment [24]. We established iPSC clones from the fibroblasts of the patient and differentiated them to monocytes. IL-1β secretion under LPS stimulation was then evaluated, because monocytes derived from NOMID patient-specific iPSCs are known to show excessive IL-1β production without any secondary activating signals, such as ATP or silica [12]. When
multiple iPSC clones were differentiated and evaluated, their phenotype was clearly separated into two groups based on the IL-1β secretion (high and low). Since this inter-clonal phenotypic heterogeneity seemed associated with the genetic heterogeneity of the iPSC clones, we performed whole exome sequencing of representative iPSC clones to identify mutations exclusively shared among clones with the disease-associated phenotype. As a result, we identified a heterozygous NLRC4 mutation, p.T177A (NC_000002.11:g.32476404T>C, NM_021209.4:c.529A>G), as the cause. Because each iPSC clone is derived from a single somatic cell [27], this finding indicated that the patient has the somatic NLRC4 mutation. Indeed, the identical mutation was identified from the patient’s primary blood cells. The frequency of the mutant allele was calculated as approximately 30–34%. Since the mutation is heterozygous, the estimated mosaic frequency is therefore calculated as about 60–68%.

Our data support the possibility that the patient has a disease-specific somatic NLRC4 mutation. To confirm the mutant NLRC4 is indeed disease causing, we knocked out the NLRC4 locus of a mutant iPSC clone using CRISPR/Cas9-based mutagenesis and established iPS-MLs both from the knocked-out clones and the original mutant clones. When the iPS-MLs from knockout clones were differentiated into macrophages, the excessive IL-1β secretion was completely abolished. Therefore, we concluded the somatic NLRC4 mutation found in the patient was indeed disease causing.

NLRC4 mutations were recently reported as causes of other autoinflammatory disorders [28–30]. Interestingly, their phenotypes can be distinguished into two distinct entities: a very severe neonatal-onset phenotype and a much milder phenotype of cold-induced urticaria [29]. The two extreme phenotypes of NLRC4-associated autoinflammatory disorders are in contrast to the phenotype of NLRP3-associated proinflammatory conditions, which show a clinical continuum with regard to the genotype-phenotype correlation [18]. However, one recent clinical case showed intermediate clinical symptoms of NLRC4-associated autoinflammatory disorders, suggesting they too have a clinical continuum.

This above case demonstrates that iPSC-based forward genetic approaches can be used to identify the genetic etiology of rare sporadic cases of autoinflammatory syndrome (Figure 3). Further, iPSC technology enables researchers to dissect the patient’s phenotype at the single cell level, which can be used to reveal genetic heterogeneity of the patient. To the best of our knowledge, this was the first report in which iPSC technology played an essential role in the identification of the precise genetic background of a patient. By connecting iPSC-based phenotype analysis with other technologies, such as next-generation sequencing and genome editing technology, iPSCs models can be used to investigate the detailed pathophysiology of rare immunological diseases, even when only a single patient is known.

4. Pluripotent stem cell model of Blau syndrome

Blau syndrome (OMIM: #186580) is a congenital monogenic granulomatosis caused by mutations in Nucleotide-binding oligomerization domain-containing
Since the disease onset is usually before 4 years old, Blau syndrome was alternatively named as early-onset sarcoidosis. Patients with Blau syndrome develop intractable granulomas in the eye, skin and joints. Although inflammatory symptoms such as fever and rush are relatively mild, these granulomas cause severe complications such as joint contraction and blindness. Recently, anti-TNF therapy was reported effective for controlling the disease [33,34], but the long-term effectiveness is still unestablished. Therefore, specific curative therapeutic options are being considered.

**NOD2** is an intracellular pathogen recognition receptor and recognizes muramyl dipeptide (MDP), a substance common in bacteria [35,36]. NOD2 activates the nuclear factor kappa B (NF-κB) pathway in a ligand-dependent manner, which up-regulates proinflammatory cytokines and chemokines [35–39]. On the other hand, the function of disease-associated mutant NOD2 is controversial. In several reports using HEK293 cells [40] or in vitro protein reconstitution systems, ligand-independent enhanced NF-κB activity is reported, while a knock-in mouse model [41] demonstrates a decreased proinflammatory response, indicating the mutations are loss-of-function. However, since the mouse model did not show any phenotype associated to Blau syndrome, a human model that is able to evaluate the unique function of mutant NOD2 in a strictly controlled condition is needed. Importantly, specific perturbations that evoke the proinflammatory response to NOD2 mutant cells remain unidentified.

To address these issues, we established iPSC clones from a Blau syndrome patient harboring heterozygous R334W (1000C>T) mutation and corrected the mutation by genome-editing to obtain an isogenic counterpart. By using the same targeting strategy, a knocked-in clone with heterozygous R334 mutation was generated from a wild-type iPSC clone. By using these two isogenic pairs for disease modeling, we confirmed consistency in the in vitro phenotype associated with the disease-associated NOD2 mutation using a strictly controlled genome background. Since NOD2 is mainly expressed in hematopoietic lineage cells, especially monocytic cells [37], and the granulomas contain many macrophages, we next differentiated the iPSCs towards myelomonocytic lineage cells and sought a specific trigger that evokes the proinflammatory response to these cells. Interestingly, IFN-γ upregulated the expression of NOD2 in iPSCs-derived macrophages, as previously described [42–44], indicating a priming role of IFN-γ in the NOD2-NF-κB signaling pathway. We established iPS-MLs from myeloid progenitors obtained from iPSCs and used iPS-ML-derived macrophages (iPS-MPs) for further analysis. Both wild-type and mutant iPS-MPs were able to react to MDP and activate the NF-κB pathway. IFN-γ increased the NF-κB reporter activity and facilitated the nuclear translocation of NF-κB p65 subunit in mutant iPS-MPs even in the absence of MDP. In line with this finding, IFN-γ treatment significantly increased the production of cytokines such as IL-6, IL-8 and TNF-α in mutant iPS-MPs without

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**Figure 3.** Identification of somatic NLRC4 mutation from a patient with autoinflammation. The patient’s genetic background was identified through iPSC-based phenotype analysis.
secondary MDP stimulation. Therefore, IFN-γ primes mutant iPSC-MPs to activate the NF-κB pathway in a ligand-independent manner, resulting in the enhanced production of proinflammatory cytokines. This finding was supported by RNA-seq analysis using iPSC-MPs, as IFN-γ treatment specifically enriched several inflammatory pathways in mutant iPSC-MPs compared to their wild-type counterparts.

To confirm that these findings are indeed associated with patient cells, but not an artifact only recognized in iPSC-MPs, we investigated the in vitro phenotypes of primary monocyte-derived macrophages obtained from another Blau patient. As expected, IFN-γ treated monocyte-derived macrophages from the patient exhibited an enhanced activation of the NF-κB pathway and production of proinflammatory cytokines, confirming the validity and usefulness of our iPSC-based model.

Using iPSCs, we identified IFN-γ as a specific trigger that induces the pro-inflammatory response seen in Blau syndrome (Figure 4). Substantial evidence has already indicated a pathophysiological role of IFN-γ in the pathophysiology of the disease. For example, IFN-γ-expressing cells were reported positive in and around the granulomas of Blau syndrome patients [45]. Also in some patients, BCG vaccination, which evokes an INF-γ response, was sometimes associated with the onset of the disease [46,47]. Furthermore, humans lacking IFN-γ receptors such as IFN-γR1 or IFN-γR2 do not display any mature granulomas [48], indicating an indispensable role of IFN-γ in the formation of granulomas. However, it was through an iPSC-based disease model that we could reveal a previously undefined IFN-γ-dependent pathway that activates NOD2-mutated macrophages in Blau syndrome.

5. Conclusions

Here, we provide some examples of using iPSC technology for studying immunological disorders. When combined with other novel technologies such as next generation sequencing and genome editing, these models provide a phenotypic analysis that can identify extremely rare genetic conditions, showing the future possibility of iPSC-based personalized diagnosis in rare diseases. The development of therapeutic approaches for immunological disorders using iPSC technology are also anticipated.

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No potential conflict of interest was reported by the author.

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