Human AK2 links intracellular bioenergetic redistribution to the fate of hematopoietic progenitors

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Abstract
AK2 is an adenylate phosphotransferase that localizes at the intermembrane spaces of the mitochondria, and its mutations cause a severe combined immunodeficiency with neutrophil maturation arrest named reticular dysgenesis (RD). Although the dysfunction of hematopoietic stem cells (HSCs) has been implicated, earlier developmental events that affect the fate of HSCs and/or hematopoietic progenitors have not been reported. Here, we used RD-patient-derived induced pluripotent stem cells (iPSCs) as a model of AK2-deficient human cells. Hematopoietic differentiation from RD-iPSCs was profoundly impaired. RD-iPSC-derived hemoangiogenic progenitor cells (HAPCs) showed decreased ATP distribution in the nucleus and altered global transcriptional profiles. Thus, AK2 has a stage-specific role in maintaining the appropriate energy level of each organelle by the intracellular redistribution of ATP is important for controlling the fate of progenitor cells.

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1. Introduction

Reticular dysgenesis (RD, OMIM: 267500) is an extremely rare autosomal recessive disorder caused by hypofunctional mutations in adenylate kinase 2 (AK2) [1, 2]. RD patients suffer from severe combined immunodeficiency (SCID) with profound T lymphopenia and neutrophil maturation arrest [3]. Although the recapitulation of hematopoietic manifestations has been reported in vitro in cultured AK2 knockout cells [1, 4] and patient-derived induced pluripotent cells (iPSCs) [5] and in vivo in a zebrafish model [2, 5], the underlying pathophysiology linking the function of AK2 to the fate of hematopoietic progenitors has not been fully clarified.
Additionally, in those previous models, although the dysfunction of hematopoietic stem cells (HSCs) has been implicated, earlier developmental events that affect the fate of the HSCs and/or hematopoietic progenitors have not been reported.

AK2 is an isoenzyme of adenylate kinases (AKs, EC 2.7.4.3). The phosphotransfer network of AKs is an important intracellular system that contributes to energy redistribution among distinct subcellular compartments [6]. AKs are evolutionally conserved enzymes that catalyze the reversible adenine nucleotide phosphor transfer of adenine nucleotides through the reaction \( \text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP} \) [6]. AK2 localizes to the intermembrane spaces of mitochondria and is involved in various cellular biological functions [4,5,7–9]. The unique subcellular localization suggests a distinct role of AK2 on energy communication between intra- and extra-mitochondrial compartments. However, the impact of AK2 on the intracellular ATP concentration in each subcellular compartment has not been directly evaluated.

Adenosine triphosphate (ATP) is an essential energy currency in cells. ATP is mainly produced in the mitochondria and cytosol, and is consumed by various organelles including the nucleus. The main sites of ATP production transition dynamically during differentiation [10]. Additionally, alterations in the intracellular energy metabolism in stem/progenitor cells affect the fate of progenies. For example, cellular bioenergetic homeostasis has been linked to the fate of HSCs [11,12]. Cytosolic energy sensors and glycolytic enzymes play critical roles in maintaining and regulating the stemness of HSCs [13,14]. The fate control of hematopoietic progenitor cells is also affected by the altered metabolism [15]. However, since current methods for evaluating the transition of the metabolic status during differentiation are mainly whole-cell based, whether metabolic miscommunication among subcellular compartments affects the fate of progenitor cells remains to be elucidated. To address this possibility, tracing the distribution of intracellular ATP during hematopoietic differentiation is necessary.

In the present study, we used RD-patient-derived iPSCs [16] as a model for understanding the underlying mechanism linking AK2 function and hematopoietic differentiation. We focused on human AK2, because the embryonic lethality observed in animal models of homozygous AK2 deletion [17,18] (including our unpublished observation in mice) indicates the existence of unique redundant AK2 pathways in humans [8]. By using these models, we found that the differentiation propensity was impaired in early-stage hematopoietic progenitors derived from AK2 mutant iPSCs.

2. Materials and methods

2.1. Primers and antibodies

The primers and antibodies used in this study are listed in Supplementary Tables 3 and 4, respectively.

2.2. Ethical matters

This study was approved by the Ethics Committee of Kyoto University, and written informed consent was obtained from the patients’ guardians in accordance with the Declaration of Helsinki. Pretransplant bone marrow stromal cells were obtained from two independent RD patients (RD1, CIRA00068; and RD2, CIRA00067).

2.3. Establishment of RD-iPSCs

Bone marrow stromal cells were reprogrammed towards iPSCs using a pseudotyped retroviral transduction protocol with six factors (OCT4, SOX2, KLF4, LMYC, LIN28 and NANOG) or a combination of episomal plasmid vectors encoding seven factors (OCT4, SOX2, KLF4, LMYC, LIN28, EBNA1 and p53 carboxy-terminal dominant-negative fragment). pMXs retrovirus vectors encoding each factor together with pMG2.G (Addgene, 12259) were introduced into the Platinum-GP packaging cell line (Cell Biolabs, Inc.). The viral supernatant was concentrated with PEG-it Virus Precipitation Solution (System Biosciences). Transduction of the retroviral vectors and the subsequent harvesting of PSC-like colonies were performed as previously described [16]. Episomal plasmid vectors encoding each factor were transduced into bone marrow stromal cells by electroporation [19].

2.4. Hematopoietic cell differentiation from PSCs

The hematopoietic cell differentiation protocol was modified from a previously described serum and feeder-free monolayer system [20,21].

2.5. T lymphocyte differentiation from PSCs

T-lineage cells were obtained from RD-iPSCs or control T cell-derived iPSCs after coculturing with C3H10T1/2 and OP9-DL1 cells, as previously described [22].

2.6. FRET imaging

ATeam1.03s with and without a mitochondrial or nuclear targeting signal were cloned into a piggyBac vector and transfected into each PSC. PSCs were cultured on a iMatrix 511 (nippi)-coated 9.5 mm multi-well glass-bottom dish (Matsunami), and adherent CD34 + KDR + HAPCs were sorted using FACS, suspended in hematopoietic differentiation medium and transferred onto a multi-well glass bottom dish at a concentration of 1–2 × 10^5 cells. After overnight incubation at 37 °C, PSCs and HAPCs were imaged under identical gain and exposure settings using a FX1000 confocal microscope with high sensitivity GaAsP detector (Olympus). An image analysis was performed using Fiji software. Following noise reduction and background subtraction, the YFP and CFP emission ratio was calculated from the YFP and CFP intensity obtained from the same region of the cells.

2.7. Gene expression data

RNA probes were hybridized to SurePrint G3 Human GE 8 × 60K Microarrays (Agilent Technologies) according to the manufacturer’s protocols. The microarrays were scanned, and the data were analyzed using the Bioconductor package limma. The complete dataset for this analysis is available at the NCBI Gene Expression Omnibus using accession no. GSE76761.

2.8. Statistical analysis

All statistical analyses were performed using Microsoft Excel, Python 2.7 and R software programs. A description of each analysis is available in the Methods, Supplementary Figures and associated legends.

3. Results

3.1. Generation and evaluation of RD-iPSCs

We obtained bone marrow stromal cells from two RD patients carrying compound heterozygous mutations in AK2 (patients RD1 and RD2, Fig. 1A) and introduced reprogramming factors. We have obtained multiple clones to exclude any clonal artifacts (Supplementary Fig. 1A, Supplementary Table 1A). AK2-supplemented (AK2(+)) iPSC
clones from patient-derived cells were used as controls for AK2-deficient (AK2(-)) iPSC clones. We finally obtained multiple iPSC clones from both patients and used 3 AK2(-) and 3 AK2(+) iPSC clones from RD1 and 3 AK2(-) and 2 AK2(+) iPSC clones from RD2 to evaluate hematopoietic differentiation capacity (Supplementary Table 1A).

Both AK2(-) and AK2(+) clones were morphologically comparable to human embryonic stem cells (ESCs), expressed pluripotency-associated surface markers (Fig. 1B, Supplementary Table 1A) and exhibited a normal karyotype (Supplementary Fig. 1B, Supplementary Table 1A). Each established iPSC clone maintained parental AK2 mutations (Supplementary Fig. 1C, Supplementary Table 1A). The exogenously introduced reprogramming factors were efficiently silenced (Supplementary Fig. 1D), while PSC-specific transcription factors were well expressed in all patient-derived iPSCs (OCT3/4, SOX2, NANOG, LIN28 and REX1, Supplementary Fig. 1D, Supplementary Table 1A). All AK2(-) and AK2(+) iPSCs successfully differentiated into three germ layers in a teratoma formation assay (Fig. 1C, summarized in Supplementary Table 1A). AK2(-) and AK2(+) iPSCs were efficiently expressed in AK2(+) iPSC clones (Fig. 1D, Supplementary Figs. 1E and 1F). The promoter regions of OCT3/4 and NANOG were effectively demethylated in iPSCs (Supplementary Table 1A). We confirmed that exogenously expressed Flag-tagged AK2 was located in the mitochondria (Supplementary Fig. 1G). The AK2 enzymatic activity was profoundly impaired in AK2(-) iPSCs and efficiently recovered by complementation of AK2 in AK2(+) iPSCs (Fig. 1E and Supplementary Fig. 1H). Overall, we successfully established disease-specific iPSCs from RD patients and AK2-supplemented isogenic counterparts, and the basic characteristics of iPSCs such as pluripotency was comparable between AK2(-) and AK2(+) clones.

3.2. Defective hematopoietic differentiation in RD-iPSCs

In order to evaluate whether the in vivo phenotype of RD is recapitulated, we next differentiated RD-iPSCs into hematopoietic lineages using a step-wise protocol that produces functional erythroid and myeloid lineages in vitro [20,21]. When differentiated into myeloid lineages, the AK2(-) clones exhibited a significant defect in the maturation of neutrophils, which was completely rescued by the supplementation of wild-type AK2 (Fig. 2A and B). This finding of neutrophil maturation arrest is consistent with the pathology of the patient’s bone marrow (Fig. 2A). The maturation defect of neutrophils was observed over longer time courses (Supplementary Fig. 2A), indicating that the defect does not reflect a delay of maturation, but rather an inability to produce mature neutrophils. This in vitro phenotype was reproducibly recapitulated in multiple iPSC clones from both RD1 and RD2 patients (Supplementary Fig. 2B).

Since it remains unknown at which stage T-lymphocyte differentiation is impaired in RD patients, we next differentiated RD-iPSCs to T-lymphocyte lineage using a protocol that can derive
functional T cells in vitro [22]. While CD7⁺CD5⁻ pro T1 cells were maintained, CD7⁺CD5⁺ pro T2 cells [23] were deficient in AK2(-) clones (Fig. 2C, Supplementary Fig. 2C), indicating that the initial commitment to the CD7⁺CD5⁺ pro T2 stage is impaired in the T-lymphopoiesis of RD patients. Overall, the differentiation of mature neutrophils and T-lymphocytes was severely impaired, which is consistent with the in vivo phenotype of RD patients.

3.3. Defective hematopoietic output from RD hemoangiogenic progenitors

To examine which progenitors were responsible for the hematopoietic defect, we next evaluated the quantity and quality of earlier hematopoietic progenitors. We therefore compared the frequency of CD34⁺KDR⁺HAPCs at day 6 and CD34⁺CD43⁺ early MPCs at day 9. As shown in Fig. 2D and E, while the fraction of CD34⁺KDR⁺HAPCs was increased in AK2(-) clones, the CD34⁺CD43⁺ early MPCs fraction was decreased in AK2(-) clones. Apoptotic cells were not increased in either CD34⁺KDR⁺HAPCs or CD34⁺CD43⁺ early MPCs (Supplementary Fig. 2D). Therefore, the output of CD34⁺KDR⁺HAPCs seemed impaired in RD patients. In accordance with this hypothesis, the clonogenicity of purified CD34⁺KDR⁺ cells in the AK2(-) clones was almost completely defective (Fig. 2F and Supplementary Fig. 2E). Taken together, the earliest impairment of the differentiation propensity of AK2(-) iPSCs to hematopoietic lineage was found at the stage of CD34⁺KDR⁺HAPCs.

3.4. Intracellular ATP redistribution is impaired in AK2(-) hematopoietic progenitors

The most important putative role of AK2 is to regulate cellular adenine nucleotides [17], suggesting AK2 is critical for maintaining ATP levels in specific subcellular components. Since the above data indicate that AK2 plays a critical role in mitochondrial homeostasis in a stage-specific manner, we next evaluated whether the intracellular redistribution of ATP is affected in RD patients. We first evaluated the total concentration of intracellular metabolites using capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) metabolome analysis. At day 6, the loss of AK2 did not reduce the intracellular ATP concentration of differentiated cells (Supplementary Fig. 3A). The intracellular balance of adenine nucleotide, as indicated by the energy change, was not different at the whole cell level (Supplementary Fig. 3B). We next introduced ATime, a fluorescence resonance energy transfer (FRET)-based genetically encoded indicator of ATP, into iPSCs [24]. The fluorescence of ATime is invariant at the physiological level of pH fluctuation [24]. In iPSCs, the intracellular distribution of ATP was not dependent on the status of AK2 (Fig. 3A). However, when differentiated into HAPCs, the level of ATP was consistently elevated in
the mitochondria and decreased in the nuclei of AK2(-) clones (Fig. 3B–D). The cytosolic ATP level remained comparable between AK2(-) and AK2(+) clones (Fig. 3B–D). Overall, these data provide direct evidence that AK2 affects the intracellular redistribution of ATP at a specific stage of hematopoietic development, which leads to the retention of ATP in the mitochondria and a relative shortage of ATP in the nuclei of HAPCs.

3.5. Altered transcriptional profiles of RD-HAPCs

Because the control of cell fate in RD-HAPCs was profoundly impaired and the nuclear ATP concentration decreased during hematopoiesis in RD-iPSCs, we hypothesized that the relative shortage of ATP in the nucleus during hematopoietic differentiation alters the transcriptional profiles during hematopoietic development, thereby determining the fate of hematopoietic progenitor cells (HPCs). To address this issue, we evaluated whether the transition of the global expression pattern during hematopoiesis is perturbed in RD-iPSCs. We first compared the expression profiles of ESCs, AK2(-) and AK2(+) clones during four sequential developmental stages: undifferentiated PSCs, day 6 CD34(+)KDR(+)HAPCs, day 13 CD34(+)CD45(+)MPCs and day 22 CD13(+)CD14(-)/neutrophils (Supplementary Fig. 4). A subsequent statistical analysis extracted 27,413 probes and showed significant changes among the four stages in at least one clone. We next selected 11,611 probes that showed a similar transition in the expression pattern between ESC and AK2(+) clones through hematopoietic development (Fig. 4A). Among them 6173 probes (53.2%) displayed a different expression pattern in AK2(-) clones (Fig. 4B). Interestingly, when common probes that were continuously expressed or continuously suppressed during the differentiation of ESC and AK2(+) clones (Fig. 4B, consistent pattern) were selected from the 6173 differentially expressed probes, distinctly expressed probes in the AK2(-) clones were predominantly observed at the stage of CD34(+)KDR(+)HAPCs (77.2% of the 6173 probes). Next, to identify which stage was most affected by the loss of AK2 activity, we investigated the 6173 probes at each differentiation stage (Fig. 4C). Among these probes, 4800 probes (77.8%) were discriminated in only one developmental stage (Fig. 4C). Further, among these 4800 probes, 54.8% were concentrated at the stage of day 6 CD34(+)KDR(+)HAPCs (77.2% of the 6173 probes). On the other hand, despite the presence of distinct maturation arrest in AK2(-) clones, the number of differentially expressed probes in myeloid-committed cells (day 13 and day 22 cells) was relatively small, emphasizing the significance of the global alteration of the transcriptional profiles in undifferentiated multipotential progenitors. Gene ontology (GO) analysis for the stage of CD34(+)KDR(+)HAPCs revealed that genes promoting hematopoietic differentiation were downregulated among the differentially expressed genes (Fig. 4D and Supplementary Table 2).

Collectively, these data support the hypothesis that defective hematopoietic development in RD patients is associated with an alteration of the transcriptional network in hematopoietic progenitors. Importantly, AK2 has an impact on the transcription...
profiles of the genes associated with hematopoiesis in a stage-specific manner.

4. Discussion

We herein provided the first direct evidence that the subcellular local distribution of ATP is regulated via AK2-dependent phototransfer. Notably, this AK2-dependent redistribution of ATP is specific to HAPCs. The loss of AK2 may hamper the transport of ATP outside the mitochondria, which subsequently leads to elevated ATP levels in the mitochondrial matrix. Importantly, the AK2-dependent shortage of nuclear ATP occurs in a developmental stage-specific manner, which indicates that AK2-dependent direct bioenergetic communication between the mitochondria and the nucleus may exist in HAPCs.

The causative relationship between nuclear ATP levels and altered transcriptional profiles remains to be demonstrated. Cells alter the epigenome and transcriptome sequentially during directed differentiation, alterations that demand the continuous supply of intra-nuclear energy. Nuclear ATP shortage may affect ATP-dependent chromatin remodeling, the epigenetic landscape or RNA polymerase II-mediated transcription. Indeed, a role of ATP-dependent nucleosome remodeling enzymes on hematopoietic differentiation was previously implicated [25]. RNA metabolism may also be affected by the level of ATP [26].

In contrast to most SCID cases caused by defective signaling pathways necessary for direct differentiation [27], the underlying pathophysiology of RD seems more complicated. Our study elucidated that AK2 defects provide a significant global and local impact on the metabolic and transcriptional networks in HAPCs. AK2(-) HAPCs exhibit defective neutrophil and T cell differentiation, a phenotype also seen in RD patients. Intriguingly, HAPCs and MPCs were not spontaneously apoptotic in our experiments, consistent with the finding that AK2 mediates FADD-dependent apoptosis in human cells [8] and in contrast to the enhanced apoptosis observed in patients’ primary fibroblasts and T-lymphocytes [2,28]. Additionally,
activated unfolded protein response [7,29,30] may also account for the defective hematopoiesis.

With the use of iPSC technology, we were able to track in vitro hematopoiesis in a stepwise manner and to establish isogenic AK2-supplemented counterparts and genetically-modified FRET-based ATP reporter lines. Since conducting a comprehensive analysis of patient-derived progenitors is not feasible otherwise, iPSC technology provides an unprecedented opportunity to dissect human congenital developmental abnormalities.

Acknowledgements

We thank Y. Jindai, S. Nakamura, Y. Sasaki, K. Kobayashi, Y. Kawasaki, H. Matsubara, M. Terashima and M. Yamane for technical assistance, H. Watanabe for administrative assistance, J. Kuwabara and N. Amato for the bisulfite sequencing analysis, O. Ohara for the AK2 sequencing, P. Karagiannis for reading the manuscript, and M. Yanagimachi, T. Tanaka, R. Ohta and N. Morone for critical discussions.

Funding was provided by grants from the Ministry of Health, Labour and Welfare to S.N. and T.N., a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to T.N. and K.Oshima (23791169), grants from the Leading Project of MEXT to T.N., a grant from the Funding Program for World-Leading Innovative Research and Development on Science and Technology (FIRST Program) of the Japan Society for the Promotion of Science (JSPS) to T.N., a grant from JSPS to JSPS Fellows to N.S. (15J06514), grants from the JSPS to T.N. and M.K.S. and a grant from Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics to K.Oshima, the Core Center for iPSC Cell Research of Research Center Network for Realization of Regenerative Medicine from the Japan Agency for Medical Research and Development (AMED) to T.N. and M.K.S., the Program for Intractable Diseases Research utilizing Disease-specific iPSC cells of AMED (15652070 to I.A., T.N. and M.K.S. and 17935423 to M.K.S.), Practical Research Project for Allergic Diseases and Immunology (Research on Allergic Diseases and Immunology) of AMED (14525046) to M.K.S., Practical Research Project for Rare/Intractable Diseases of AMED (17930095 and 16686375) to M.K.S., and Research Project for Practical Applications of Regenerative Medicine from AMED to M.K.S.

Transparency document

 Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.02.139.

Appendix A. Supplementary data

Supplementary Material includes Supplementary Methods, four Supplementary Figures, and four Supplementary Tables and can be found with this article online at https://doi.org/10.1016/j.bbrc.2018.02.139.

References


