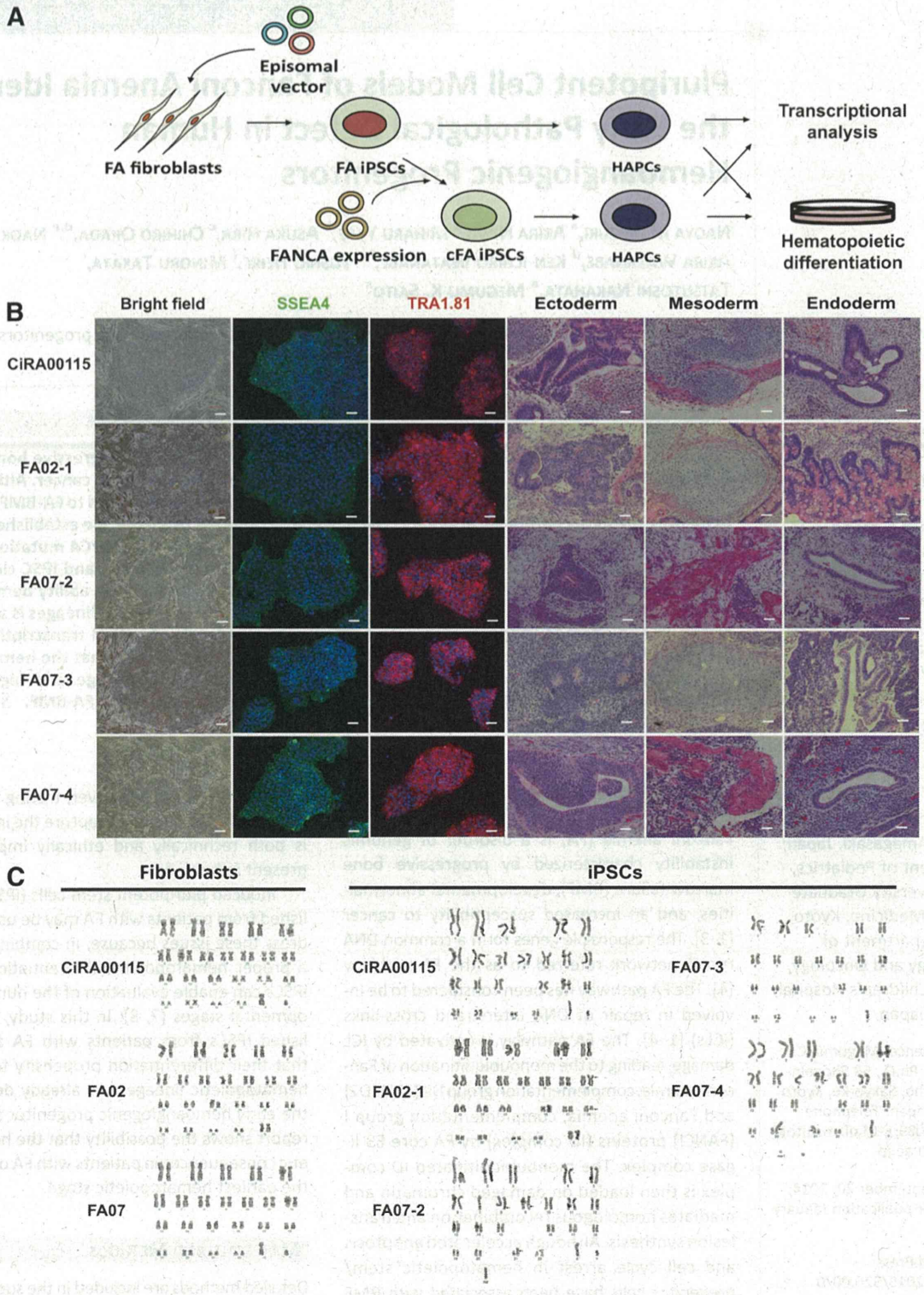

Figure 1. Establishment of FA-iPSC lines from patients with FA and mutations in *FANCA*. CiRA00115 is a control iPSC line. **(A):** The experimental scheme. **(B):** The morphology and immunostaining of pluripotent markers of FA-iPSCs and teratoma formation by the FA-iPSC lines (ectoderm: neural rosette; mesoderm: cartilage; endoderm: gut-like structure). Scale bars indicate 100 μm. **(C):** The results of karyotype analysis of fibroblasts and iPSCs from FA patients. The karyotypes of the fibroblasts were 46XX and 46XY for FA02 and FA07, respectively. The karyotype of FA-iPSCs from FA02 was summarized as 46, XX, t(12;18)(p11.2;p11.2) [20 cells]. The karyotypes of FA-iPSCs from FA07 were as follows: for FA07-2, 46, XY, add(1)(q32) [14 cells] and 46, XY [6 cells]; for FA07-3, 42-46, XY, add(8)(q24.1),add(11)(q23),del(11)(q23),add(18)(q21) [17 cells] and 42-46, XY, add(8)(q24.1),add(9)(q34),add(21)(p11.2) [3 cells]; for FA07-4, 45-46, XY, add(8)(q24.1),add(9)(q34),add(21)(p11.2) [20 cells]. Twenty metaphases were analyzed for each sample. Abbreviations: cFA, FANCA-complemented FA-iPSC clone; FA, Fanconi anemia; HAPC, hemoangiogenic progenitor cell; iPSC, induced pluripotent stem cell.

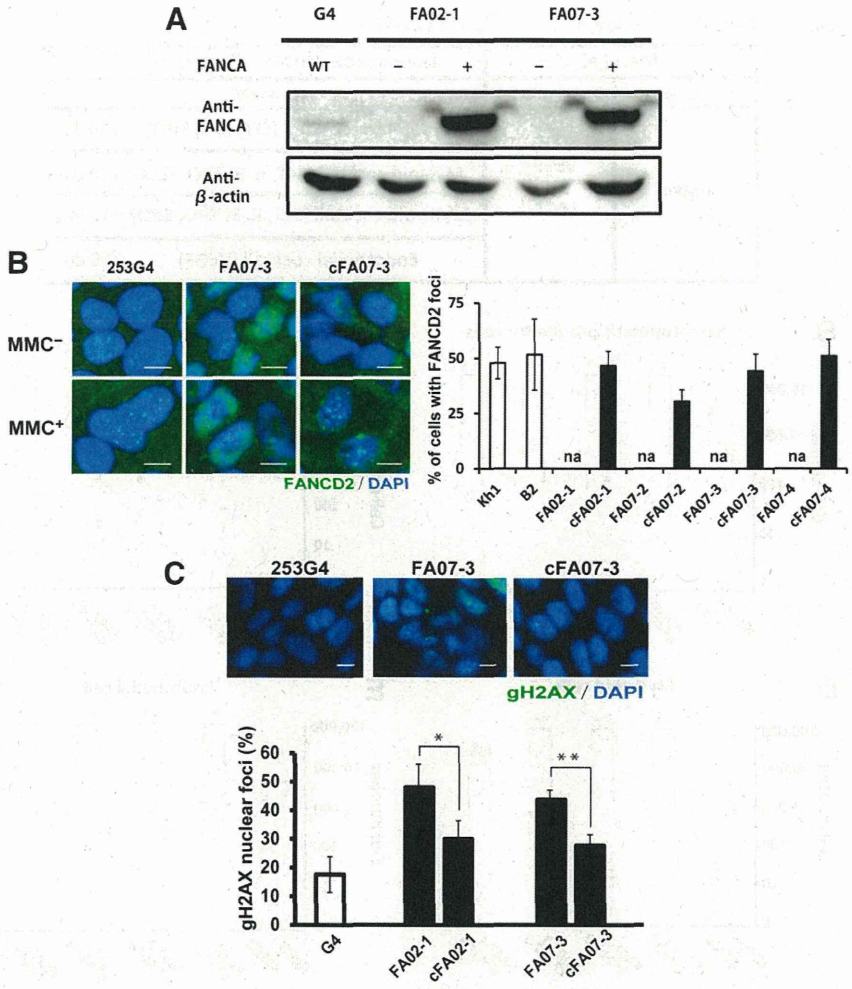

Figure 2. Validation of FA pathway in FA-iPSC lines. KhES1 is a control embryonic stem cell line, and 409B2 and 253G4 are control iPSC lines. **(A):** Immunoblotting for FANCA. **(B):** The formation of FANCD2 foci (green) in the nuclei (stained with DAPI; blue) of mitomycin C-treated FA-iPSCs (FA07) and cFA-iPSCs (cFA07) (left). The percentage of nuclei positive for FANCD2 foci (right). **(C):** Representative immunofluorescent staining (upper) and quantification (lower) of the nuclear foci of phosphorylated H2AX. The *y*-axis in the graph indicates the percentage of cells with three or more nuclear phosphorylated H2AX foci. All data are presented as mean ± SD and are representative of three independent experiments. All *p* values were determined by Student's *t* test. *, $p < .05$; **, $p < .01$ ($n = 3$). Scale bars indicate 10 μm. Abbreviations: cFA, FANCA-complemented FA-iPSC clone; DAPI, 4′, 6-diamidino-2-phenylindole; FA, Faconi anemia; iPSC, induced pluripotent stem cell; MMC, mitomycin C; WT, wild type.

patients' guardians in accordance with the Declaration of Helsinki.

Establishment and Differentiation of iPSCs From Patients With FA

Fibroblasts obtained from the six patients with FA (detail shown in supplemental online Table 1) were reprogrammed with episomal vectors encoding *OCT3/4, SOX2, KLF4, LIN28*, and *L-MYC* and a short hairpin RNA (shRNA) encoding a p53 knockdown sequence, as described previously [9]. Plasmids were kindly provided by Dr. Keisuke Okita (Kyoto University). For hematopoietic differentiation, a two-dimensional hematopoietic differentiation system was used, as described previously [10, 11].

Results and Discussion

We tried to reprogram fibroblasts obtained from six patients with FA and mutations in complementation group A (FA-A; patients with mutations in the Fanconi anemia, complementation group A [*FANCA*] gene) (supplemental online Table 1) by introducing the previously described reprogramming factors (*OCT3/4, SOX2, KLF4, c-MYC*) with retroviral [7] or Sendai viral vectors [12] under hypoxic conditions (5% O_2). Consistent with the previous reports [13], no iPSC-like colonies emerged. Recently, several groups reported that using a combination of highly efficient methods, including improved vectors such as a polycistronic OSKM cassette or additional reprograming factors under hypoxic culture conditions, could overcome the reprogramming resistance of FA cells [14–16]. Hence, we tried to establish FA patient-specific iPSCs by using episomal vectors encoding *OCT3/4, SOX2, KLF4, LMYC, LIN28*, and an shRNA-mediated p53 knockdown construct [9] under hypoxic conditions (5% O_2). As a result, iPSC-like colonies arose from all FA patient-derived fibroblasts (supplemental online Table 1); however, we could pick up and maintain only the TKFA02 and TKFA07 patient-derived iPSC (FA-iPSC) lines. The colonies arising from the TKFA03, FA44, FA45, and FA46 patients could not be picked up and propagated.

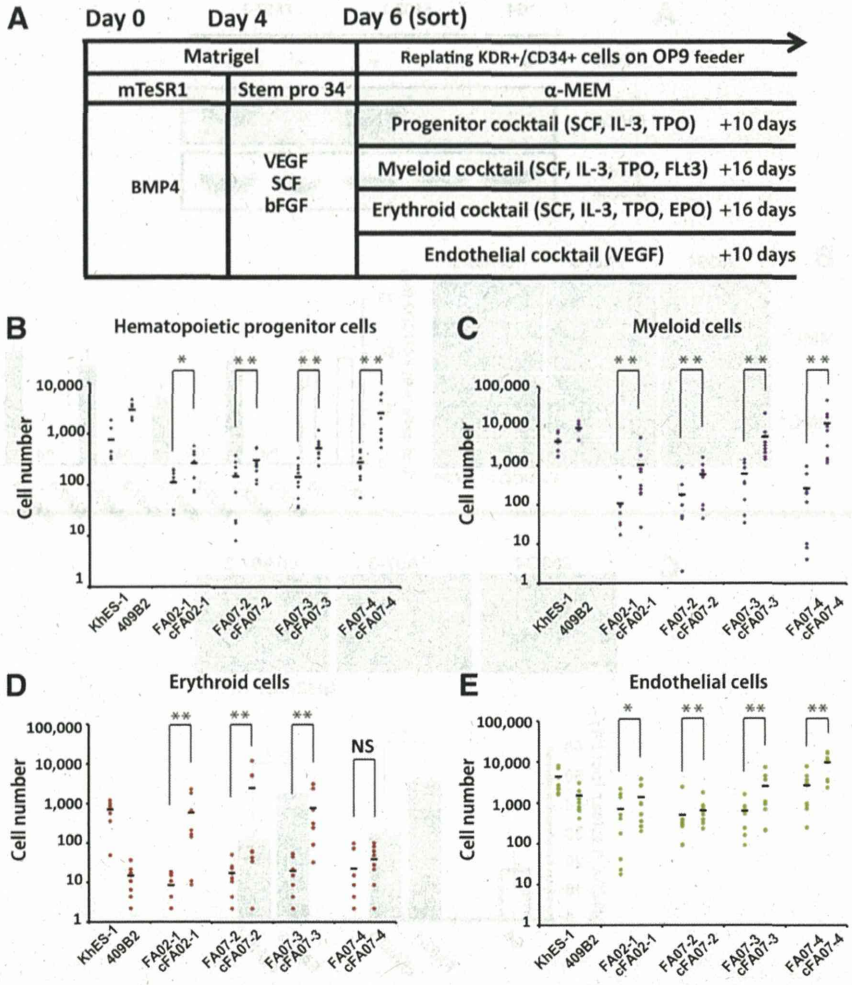

Figure 3. The defective differentiation propensity in Fanconi anemia induced pluripotent stem cell-derived hemoangiogenic progenitors. KhES1 and 409B2 are control embryonic and induced pluripotent stem cell lines, respectively. **(A):** A schematic diagram of hematopoietic differentiation. **(B–E):** The number of differentiated cells derived from 5,000 sorted KDR^+CD34^+ cells. **(B)**: $CD34^+CD45^+$ hematopoietic progenitors. **(C):** $CD33^+CD45^+$ myeloid cells. **(D):** $CD45^-CD235\alpha^+$ erythroid cells. **(E):** $CD31^+CD34^+$ endothelial cells. All data are presented as mean ± SD and are representative of three independent experiments. All *p* values were determined by Wilcoxon rank sum test. *, $p < .05$; **, $p < .01$; ***, $p < .001$ ($n = 3$). Abbreviations: α-MEM, alpha Minimum Essential Medium; bFGF, basic fibroblast growth factor; EPO, erythropoietin; IL-3, interleukin 3; NS, not significant; SCF, stem cell factor; TPO, thyroid peroxidase; VEGF, vascular endothelial growth factor.

Consequently, we selected one and three FA-iPSC lines of the TKFA02 and TKFA07 cells, respectively (Fig. 1A).

The FA-iPSC lines showed human pluripotent cell-like morphology, expressed pluripotent markers, and differentiated into three germ layers in the teratoma formation assay (Fig. 1B). A short tandem repeated analysis confirmed that the patient identity was conserved throughout the reprogramming process (supplemental online Fig. 1). The FA-iPSC lines had residual transgene expressions, as reported previously [16] (supplemental online Fig. 2A). Consistent with this, these FA-iPSC clones, including their complemented counterparts (discussed below), showed reduced p53 expression (supplemental online Fig. 2B). Although the fibroblasts from both patients had normal karyotypes, both FA-iPSC lines had aberrant karyotypes (Fig. 1C), which is compatible with a previous report [16] and indicates that the FA pathway has an important role in determining chromosomal stability through reprogramming events. Collectively, by combining the reprogramming factors with modulation of the p53 pathway, we were able to develop a robust reprogramming strategy that enabled us to establish iPSC-like colonies from FA patient-derived somatic cells.

To establish isogenic FANCA-complemented clones, we introduced exogenous wild-type FANCA cDNA into each clone (Fig. 2A; supplemental online Fig. 3). Mitomycin C-induced FANCD2 foci formation was restored in these FANCA-complemented FA-iPSC clones (designated as cFA-iPSCs) (Fig. 2B). During maintenance of the iPSCs, the FA-iPSCs showed an increased DNA double-strand break rate, as evaluated by the frequency of nuclear foci of phosphorylated H2AX compared with the complemented counterparts (Fig. 2C), but the distribution of the cells in the different phases of the cell cycle was not significantly different (supplemental online Fig. 4). Consequently, the complementation of the FA pathway recovers the in vitro phenotype of FA-iPSCs.

To determine whether the hematopoietic differentiation is affected by disruption of the FA pathway, we next differentiated

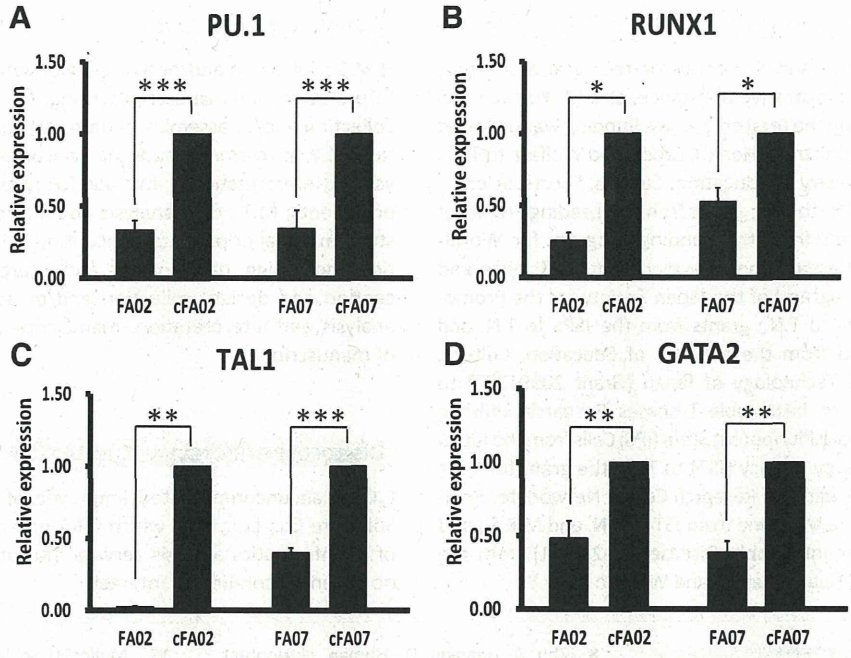

Figure 4. FANCA-deficient hemoangiogenic progenitor cells show the decreased expression of hematopoietic marker genes. **(A–D):** The results of the quantitative polymerase chain reaction analysis for hematopoietic marker genes in KDR^+CD34^+ hemoangiogenic progenitors (day 6). The relative gene expression (*y*-axis) was calculated relative to the expression in corresponding cFA induced pluripotent stem cells after normalization to the expression of glyceraldehyde-3-phosphate dehydrogenase. All data are presented as mean ± SD and are representative of three independent experiments. All *p* values were determined by Student's *t* test. *, $p < .05$; **, $p < .01$; ***, $p < .001$ ($n = 3$). Abbreviation: cFA, FANCA-complemented Fanconi anemia induced pluripotent stem cell clone.

the FA-iPSC lines into hematopoietic progenitor cells through the previously reported serum- and feeder-free monolayer culture system [10, 11]. Under this protocol, the progenitor cells committed to the hematopoietic lineage are obtained as KDR^+CD34^+ early hemoangiogenic progenitor cells (HAPCs), which give rise to both endothelial and hematopoietic cells. To evaluate the differentiation propensity of HAPCs quantitatively, we sorted the KDR^+CD34^+ HAPCs on day 6 and cultured them on OP9 feeder cells with lineage-specific cytokine cocktails (Fig. 3A; supplemental online Fig. 5). The FA-iPSC lines showed a significant reduction of $CD34^+CD45^+$ hematopoietic precursors compared with cFA-iPSC lines (Fig. 3B). Subsequently, the myeloid and erythroid lineage hematopoietic cells were significantly reduced (Fig. 3C, 3D). The number of $CD31^+$ endothelial cells from FA-iPSC-derived HAPCs (FA-HAPCs) was also reduced compared with that in the cFA-iPSC lines (Fig. 3E). The distribution of the cell cycle in FA-iPSC-derived KDR^+CD34^+ HAPCs was comparable to that of the complemented counterparts (supplemental online Fig. 6). We also confirmed that KDR^+CD34^+ HAPCs were not apoptotic (supplemental online Fig. 7). Although the expression level of p53 reduced and varied among the FA- or cFA-iPSC lines (supplemental online Fig. 2B), the ability of these cells to undergo hematopoietic differentiation was dependent on the FANCA status but not on the p53 level, indicating that, at least in this study, the expression level of p53 has no obvious impact on the differentiation propensity of HAPCs. Taken together, these findings indicate that FA-HAPCs showed a defective propensity to differentiate toward both hematopoietic and endothelial lineages.

To further elucidate the mechanism underlying the defective hematopoietic differentiation, we next quantified the expression levels of critical transcription factors requird for hematopoietic differentiation in HAPCs [17–20]. HAPCs from FA-iPSC lines showed significant downregulation of these transcription factors (Fig. 4A–4D), indicating that the FA pathway might be involved in maintaining the transcriptional network critical for determining the differentiation propensity of HAPCs. We also compared the global expression profiles of HAPCs from FA- and cFA-iPSCs (supplemental online Fig. 8A, 8B). We identified that 227 genes were significantly upregulated and 396 genes were significantly downregulated in FA-HAPCs (supplemental online Fig. 8C; supplemental online Table 2). A gene ontology enrichment analysis revealed that genes associated with mesodermal differentiation, vascular formation, and hematopoiesis were extensively downregulated in FA-HAPCs (supplemental online Fig. 8D).

Conclusion

In summary, we successfully established FA-iPSCs from FA-A patients. We found that an early pathological phenotype was detected in HAPCs as a defective differentiation propensity into both hematopoietic and endothelial lineages. Interestingly, during hematopoiesis, the expression of FANCA and FANCC were specifically upregulated in KDR^+CD34^+ HAPCs (supplemental online Fig. 9), indicating the stage-specific requirement of these genes in HAPCs. Because the expression of hematopoietic and angiogenic genes was affected, FANCA may have an important role in regulating these genes in FA-HAPCs. Conducting a comprehensive analysis of patient-derived affected progenitors is not feasible without iPSC technology, which provides an unprecedented opportunity to gain further insight into the pathogenesis of BMF in patients with FA.

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Author Contributions

N.M.S.: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; A.N., C.O., N.A., and A.W.: collection and/or assembly of data, data analysis and interpretation; M.Y.: provision of study material or patients; A.H.: data analysis and interpretation; K.-I.W. and T.H.: provision of study material or patients; M.T.: data analysis and interpretation, provision of study material or patients, conception and design; T.N.: conception and design, data analysis and interpretation; M.K.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

C.O. is an uncompensated employee of the Mitsubishi Space Software Co., Ltd., with which CiRA entered into an agreement of bioinformatics analysis service. The other authors indicated no potential conflicts of interest.

References

1 Deans AJ, West SC. DNA interstrand crosslink repair and cancer. Nat Rev Cancer 2011;11:467–480.

2 Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature 2013;493:356–363.

3 Kim H, D'Andrea AD. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes Dev 2012;26:1393–1408.

4 Takata M, Yamamoto K, Matsushita N et al. The Fanconi anemia pathway promotes homologous recombination repair in DT40 cell line. Subcell Biochem 2006;40:295–311.

5 Ceccaldi R, Parmar K, Mouly E et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. Cell Stem Cell 2012;11:36–49.

6 Kamimae-Lanning AN, Goloviznina NA, Kurre P. Fetal origins of hematopoietic failure in a murine model of Fanconi anemia. Blood 2013;121:2008–2012.

7 Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.

8 Zhu Z, Huangfu D. Human pluripotent stem cells: An emerging model in developmental biology. Development 2013;140:705–717.

9 Okita K, Matsumura Y, Sato Y et al. A more efficient method to generate integration-free human iPS cells. Nat Methods 2011;8:409–412.

10 Niwa A, Heike T, Umeda K et al. A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors. PLoS ONE 2011;6:e22261.

11 Yanagimachi MD, Niwa A, Tanaka T et al. Robust and highly-efficient differentiation of functional monocytic cells from human pluripotent stem cells under serum- and feeder cell-free conditions. PLoS ONE 2013;8:e59243.

12 Seki T, Yuasa S, Oda M et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 2010;7:11–14.

13 Raya A, Rodriguez-Piza I, Guenechea G et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 2009;460:53–59.

14 Muller LU, Milsom MD, Harris CE et al. Overcoming reprogramming resistance of Fanconi anemia cells. Blood 2012;119:5449–5457.

15 Muller LU, Schlaeger TM, DeVine AL et al. Induced pluripotent stem cells as a tool for gaining new insights into Fanconi anemia. Cell Cycle 2012;11:2985–2990.

16 Yung SK, Tilgner K, Ledran MH et al. Brief report: Human pluripotent stem cell models of Fanconi anemia deficiency reveal an important role for Fanconi anemia proteins in cellular reprogramming and survival of hematopoietic progenitors. Stem Cells 2013;31:1022–1029.

17 Scott EW, Simon MC, Anastasi J et al. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994;265:1573–1577.

18 Okuda T, van Deursen J, Hiebert SW et al. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 1996;84:321–330.

19 Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 1995;373:432–434.

20 Tsai FY, Keller G, Kuo FC et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 1994;371:221–226.

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