Generation of disease-free hematopoietic progenitors from Fanconi anemia-specific induced pluripotent stem cells

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The generation of induced pluripotent stem (iPS) cells by ectopic expression of a defined set of factors[1-5] has enabled the derivation of patient-specific pluripotent cells and provided valuable experimental platforms to model human disease[6-8]. Patient-specific iPS cells are also thought to hold great therapeutic potential, although direct evidence for this is still lacking. Here we show that somatic cells from Fanconi anemia (FA) patients, upon correction of the genetic defect, can be reprogrammed to pluripotency to generate patient-specific iPS cells. These cell lines appear indistinguishable from human embryonic stem cells and iPS cells from healthy individuals in colony morphology, growth properties, expression of pluripotency-associated transcription factors and surface markers, and differentiation potential in vitro and in vivo. Most importantly, we show that corrected FA-specific iPS cells can give rise to hematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal, i.e. disease-free. These data offer proof-of-concept that iPS cell technology can be used for the generation of disease-corrected, patient-specific cells with potential value for cell therapy applications.

The possibility of reprogramming mature somatic cells to generate iPS cells[1-5] has opened new perspectives in regenerative medicine. The generation of iPS cells may have a wide range of applications in cell and gene therapy, and could be particularly relevant for the treatment of inherited bone marrow failure (BMF) syndromes, where the progressive decline in hematopoietic stem cell numbers limits the production of peripheral blood cells. In these cases, the generation of disease-free hematopoietic progenitor cells from genetically-corrected reprogrammed cells from other tissues may open new therapeutic options not previously considered.

Among the different inherited BMF syndromes, Fanconi anemia is the most common[9]. FA is a rare recessive, autosomal or X-linked, chromosomal instability disorder caused by mutations in any of the 13 genes so far identified in the FA/BRCA pathway[10]. Cells from these patients display typical chromosomal instability and hypersensitivity to DNA cross-linking agents, characteristics that are used to make the diagnosis of FA[11]. Most FA patients develop BMF, being the cumulative incidence of 90% by 40 years of age[12]. Additionally, FA patients are prone to develop malignancies, principally acute myeloid leukemia and squamous cell carcinomas[12]. Currently, the therapy of choice for FA patients is transplantation of hematopoietic grafts from HLA-identical siblings, since the output of transplants from non-related donors is poor[13,14]. Although the genetic correction of autologous HSCs with integrative vectors may constitute a good therapeutic option for FA patients, gene therapy trials conducted so far have not been clinically successful[15,16]. The paucity of hematopoietic stem cells in the bone marrow of FA patients[16-18] not only accounts for the BMF occurring in FA patients[12], but also constitutes one of the main factors limiting the efficacy of FA gene therapy[15,16]. The generation of genetically corrected FA-specific iPS cells by the reprogramming of non-hematopoietic somatic cells would result in the production of large numbers of autologous hematopoietic stem cells that may be used to restore the hematopoietic function in these patients.

In this study, we obtained samples from 6 FA patients, 4 of which are from the FA-A complementation group (patients FA5, FA90, FA153, and FA404) and 2 from the the FA-D2 complementation group (FA430 and FA431). Samples from patients FA5, FA90, FA153, FA430, and FA431 were cryopreserved primary dermal fibroblasts that had undergone an undetermined number of passages. From patient FA404 we obtained a skin biopsy, from which we established primary cultures of dermal fibroblasts and epidermal keratinocytes. Current protocols of induced reprogramming are highly inefficient for

human fibroblasts, especially adult human fibroblasts. Successful reprogramming of human adult fibroblasts with retroviruses encoding OCT4, SOX2, KLF4 and c-MYC has been achieved by prior lentiviral transduction with the mouse receptor for retroviruses[2], co-transduction with hTERT and SV40 large T[4], or by using VSVg-pseudotyped retroviruses[6,7]. Even under those conditions, the reprogramming efficiency of human adult fibroblasts is as low as 0.01-0.02%. Similarly, lentiviral delivery of OCT4, SOX2, NANOG, and LIN28 has been reported to reprogram human adult fibroblasts, although at even lower efficiencies (~0.001%, ref. 8). For this reason, we first attempted to optimize the reprogramming protocol using primary dermal fibroblasts from a foreskin biopsy of a healthy donor (see Supplementary Text and Supplementary Fig. 1). Our improved reprogramming protocol consisted of 2 rounds of infection with murine stem cell virus- (MSCV) based retroviruses encoding N-terminal FLAG-tagged versions of OCT4, SOX2, KLF4 and c-MYC, performed 6 days apart. Transduced fibroblasts were passaged after 5 days onto a feeder layer of mitotically-inactivated primary human fibroblasts and then switched to human embryonic stem (hES) cell medium the next day. We also included a selection step based on the combined inhibition of MEK1 and GSK3 with inhibitors PD0325901 and CT99021 (a combination termed 2i that enhances derivation and growth of mouse ES cells[19]) for 1 week, starting 1 week after plating onto feeders.

Because of the genetic instability and apoptotic predisposition of FA cells[20], skin cells from FA-A and FA-D2 patients were reprogrammed, either directly or after genetic correction with lentiviral vectors encoding FANCA or FANCD2, respectively. We have previously shown that genetic complementation of human and mouse FA cells with these vectors efficiently corrects the FA phenotype[21]. We were not successful at obtaining iPS-like colonies from fibroblasts of patients FA5, FA153 or FA430, either unmodified or corrected, after at least 5 reprogramming attempts, probably owing to the cells having

accumulated too many passages and/or karyotypic abnormalities (Supplementary Table 1).
However, from patient FA90 we readily obtained iPS-like colonies when using genetically
corrected fibroblasts (Fig. 1a). Overall, we obtained 10-15 iPS-like colonies in each of 3
independent experiments. Of these, we randomly picked 10 colonies, all of which could
successfully be expanded on feeder layers or Matrigel-coated plates and grew as flat
colonies of tightly packed cells with a high nucleus-to-cytoplasm ratio (Fig. 1b),
morphologically indistinguishable from hES cells, and stained strongly positive for alkaline
phosphatase (AP) activity (Fig. 1c). Five of these lines (cFA90-44-1, -11, -14, -20, and -21)
were selected for further characterization. All of them displayed a normal karyotype (46
XX) at passages 12-16 and could be maintained in culture for, at least, 20 passages. At the
time of the writing, cFA90-44-14 had undergone 43 passages without signs of replicative
crisis, while maintaining a normal karyotype (Supplementary Fig. 2). Immunofluorescence
analyses of the 5 lines revealed expression of high levels of transcription factors (OCT4,
SOX2, NANOG) and surface markers (SSEA3, SSEA4, TRA1-60, TRA1-81) characteristic
of pluripotent cells (Fig. 1d-f and Supplementary Fig. 3). These results indicated that we
had successfully generated patient-specific iPS cell lines from fibroblasts of a FA-A
patient.

In another series of experiments, we attempted to reprogram somatic cells from
another FA-A patient, patient FA404, and obtained similar results. Fibroblasts that had
been transduced with lentiviruses encoding FANCA (Fig. 1g) were readily reprogrammed
to generate iPS-like cells (Fig. 1h). We established 2 cell lines (cFA404-FiPS4F1 and
cFA404-FiPS4F2), which were expanded and analyzed in detail. These cell lines displayed
typical hES-like morphology and growth characteristics, stained positive for AP activity,
and expressed all the pluripotency-associated markers tested (Fig. 1i-l and Supplementary
Fig. 4). From patient FA404 we also derived primary epidermal keratinocytes, which we

attempted to reprogram using an efficient protocol recently set up in our laboratory[22]. We were successful at generating iPS cells from keratinocytes transduced with FANCA-expressing lentiviruses, but not from uncorrected keratinocytes. The overall reprogramming efficiency of keratinocytes from patient FA404 was much lower (~20-fold) than that of early-passage primary juvenile keratinocytes from healthy donors[22]. Nevertheless, the 3 iPS cell lines that we established from these experiments (cFA404-KiPS4F1, -KiPS4F3, and -KiPS4F6) displayed all the main characteristics of *bona fide* iPS cells and hES cells (Supplementary Fig. 4) and a normal 46 XY karyotype (Supplementary Fig. 2).

We were also successful at reprogramming fibroblasts from patient FA431 (Supplementary Fig. 5a), a FA-D2 patient. In this case, iPS-like colonies appeared in roughly equal numbers from either unmodified or genetically-corrected fibroblasts (Supplementary Table 1). We picked 2 iPS-like colonies from either condition, which grew as iPS-like colonies after passaging and stained positive for AP activity (Supplementary Fig. 5c, g). However, whereas those derived from corrected fibroblasts (cFA431-44-1 and cFA431-44-2) could be maintained in culture for extended periods of time (18 passages at the time of writing) and showed expression of pluripotency-associated transcription factors and surface markers (Supplementary Fig. 5d-f and data not shown), those derived from unmodified fibroblasts experienced a progressive growth delay and could not be maintained over the third passage (Supplementary Fig. 5g). The observation that uncorrected FA-D2 fibroblasts from patient FA431 could be reprogrammed, while we only obtained iPS cells from FANCA-complemented fibroblasts from patients FA90 or FA404, could be explained by the fact that FA-D2 patients, in particular FA431, carry hypomorphic mutations compatible with the expression of residual FANCD2 protein[23].

Of the 19 patient-specific iPS cell lines generated in these studies, we selected 10 for a more thorough characterization (Supplementary Table 1). We confirmed the presence of the reprogramming transgenes integrated in their genome by PCR of genomic DNA (Fig. 2a and Supplementary Fig. 6), as well as the origin of the iPS cell lines by comparing their HLA type and DNA fingerprint with those of patients' somatic cells (Supplementary Table 2). Next, we analyzed whether the expression of retroviral reprogramming transgenes had been silenced by quantitative RT-PCR analyses using transgene-specific primers. In all lines tested, transgenic expression of the 4 factors was reduced to low or undetectable levels, compared to an iPS cell line (KiPS4F3) that was previously shown not to have silenced the retroviral expression of *OCT4* and *c-MYC*[22] (Fig. 2b). Furthermore, all the patient-specific iPS cell lines tested showed re-activation of endogenous *OCT4* and *SOX2* expression, as well as that of other pluripotency-associated transcription factors such as *NANOG*, *REX-1*, and *CRIPTO* (Fig. 2c). Taking advantage of the fact that the retroviral transgenes used in our studies were FLAG-tagged, we confirmed, by immunofluorescence, that iPS cells displayed negligible anti-FLAG immunoreactivity (Fig. 2d-g). Finally, the promoters of the pluripotency-associated transcription factors *OCT4* and *NANOG*, heavily methylated in patients' fibroblasts, were demethylated in FA-specific iPS cells (Fig. 2h), indicating epigenetic reprogramming to pluripotency.

We next analyzed the ability of patient-specific iPS cells to differentiate into cell derivatives of all three embryonic germ layers. In vitro, iPS-derived embryoid bodies readily differentiated into endoderm, ectoderm and mesoderm derivatives as judged by cell morphology and specific immunostaining with α-fetoprotein/FoxA2, TuJ1/GFAP, and α-actinin, respectively (Fig. 3a-c, and Supplementary Fig. 7). Following specific in vitro differentiation protocols, iPS cells gave rise to specialized mesoderm-derived cell types such as rhythmically beating cardiomyocytes (Supplementary Movie 1) and hematopoietic

progenitor cells (see below). We also subjected our patient-specific iPS cells to the most stringent test available to assess pluripotency of human cells, the formation of *bona fide* teratomas[24]. For this purpose, we injected cells from 8 different lines into the testes of immunocompromised mice. In all cases, teratomas could be recovered after 8-10 weeks that were composed of complex structures representing the three main embryonic germ layers, including glandular formations that stained positive for definitive endoderm markers, neural structures that expressed neuroectodermal markers, and mesoderm derivatives such as muscle and cartilage (Fig. 3d-f, Supplementary Fig. 8, and data not shown). Using comparable assays, we have recently characterized the in vitro differentiation and teratoma induction abilities of a variety of normal human pluripotent stem cell lines, including hES cells[25] and iPS cells generated from healthy donors[22]. Overall, we did not detect conspicuous differences in the capacity of FA patient-specific iPS cell lines to differentiate into any of the cell lineages tested, when compared to that of either hES cells or normal iPS cells.

The generation of indefinitely self-renewing iPS cells from patients with monogenic diseases provides a unique opportunity for controlled ex vivo gene therapy. Our FA patient-specific iPS cell lines were generated from somatic cells that had been previously transduced with FA-correcting lentiviruses. Indeed, we could detect the presence of integrated copies of the gene therapy vectors by quantitative PCR of genomic DNA in all FA-iPS cell lines tested (Supplementary Fig. 9a). A concern with gene therapy strategies is the silencing of the correcting transgene. Even though lentiviral transgenes are particularly resistant to silencing in hES cells[26], this appears to be promoter-dependent[27] and nearly complete silencing has been recently observed in the context of induced reprogramming[3,8]. In our experiments, a partial degree of silencing of the correcting transgene occurred in FA-iPS cells. The FANCA-expressing lentiviruses used to infect patients' cells carried an

EGFP reporter after an IRES element that conferred a weak, but detectable, fluorescence to transduced cells (Supplementary Fig. 9b). However, EGFP expression was no longer detectable in FA-iPS or FA-iPS-derived cells (data not shown), indicating that the transgene had been, at least, partially silenced. To check the extent of transgene silencing, we analyzed the expression of FANCA, absent in uncorrected fibroblasts from patients FA90 or FA404 (Fig. 4a and data not shown). All the FA-iPS cell lines analyzed expressed lentiviral-derived FANCA, showing that none of them had completely silenced the transgene (Fig. 4a). While the majority of FA-iPS cell lines expressed FANCA at levels comparable to those of hES cells, the expression in some lines was much lower. In this respect, we have recently shown that weak expression of FANCA is enough to restore the FA pathway in FA-A cells[21]. To confirm the disease-free phenotype of FA-iPS cells we performed a battery of functional tests. When the FA pathways is functional, FANCD2 is activated and subsequently relocated to stalled replication forks in a process that depends on FANCA[10]. We induced subnuclear accumulation of stalled replication forks by high-energy local UV-irradiation across a filter with 5 μm pores and checked whether FANCD2 relocated to the locally-damaged subnuclear areas, visualized by immunofluorescence with antibodies against cyclobutane pyrimidine dimers (CPD)[28]. In those experiments, FANCD2 relocated to stalled replication forks in normal or complemented FA fibroblasts, as well as in fibroblast-like cells derived from FA-iPS cells, but not in uncorrected FA fibroblasts (Fig. 4b). In addition, we induced replication fork collapse by treating FA-iPS-derived cells with the DNA replication inhibitor hydroxyurea (HU). Stalled and broken replication forks were detected by phosphorylated histone H2AX (γ-H2AX) immunoreactivity. Also in this case, we found that FANCD2 normally relocated to HU-induced stalled replication forks in normal fibroblasts, genetically complemented FA fibroblasts, or FA-iPS-derived cells, but failed to do so in uncorrected FA fibroblasts (Supplementary Fig. 10). These results,

together with the persistent FANCA expression in FA-iPS cells, clearly show that iPS cells generated from genetically corrected FA somatic cells maintain a fully-functional FA pathway and are, thus, phenotypically disease free.

Our findings that successful reprogramming of FA cells only occurred in those that had been transduced with FANCA-expressing lentiviruses (in spite of only 35-50% of cells being actually transduced with the correcting lentiviruses; see Supplementary Fig. 9b), and that lentiviral transgenes were not completely silenced in FA-iPS cells, suggest that a functional FA pathway confers a strong selection advantage for iPS cell generation and/or maintenance. This would be consistent with the marked proliferation advantage observed in hematopoietic stem cells from mosaic FA patients that have spontaneously reverted a pathogenic mutation in one of the affected alleles[29-31] or from FA mice genetically treated with therapeutic lentiviruses[32]. To directly address this possibility, we knocked down the transgenic expression of *FANCA* in FA-iPS cells by lentiviral delivery of *FANCA*-shRNAs. Of the 5 different shRNAs tested, 3 achieved greater than 70% downregulation of FANCA expression in cFA404-KiPS4F3 cells (Fig. 4c). Notably, iPS cells with the lowest FANCA levels failed to proliferate after 1 passage (Fig. 4d). We repeated these experiments with cFA90-44-14 cells and obtained very similar results (data not shown). As a complementary approach, we induced transient downregulation of *FANCA* expression in FA-iPS-derived cells by siRNA transfection, which led to a marked decrease in cell proliferation (~ 7 fold) compared to scramble siRNA-transfected cells (Fig. 4e). The decrease in cell proliferation induced by *FANCA* siRNA was even more pronounced (> 15 fold) in response to diepoxybutane-induced DNA damage (Fig. 4e). These results provide further evidence for the FA disease-free status of our FA patient-specific iPS cells and, importantly, unveil a previously unsuspected role of the FA pathway as a critical player in the maintenance of pluripotent stem cell self-renewal. It is conceivable that the FANCA requirement for

normal iPS cell proliferation may have played an important part in ensuring that FA patient-specific iPS cells are maintained disease-free; for instance, by positively selecting iPS cells that have not completely silenced the correcting transgene and express *FANCA* above threshold levels.

The most prominent feature of FA is BMF arising from the progressive decline in the numbers of functional hematopoietic stem cells[16-18]. Therefore, we tested whether patient-specific iPS cells could be used as a source of hematopoietic cells for potential cell therapy applications. Embryoid bodies from 6 different patient-specific iPS cell lines (cFA90-44-11 and -44-14, cFA404-FiPS4F2, -KiPS4F1, -KiPS4F3, and -KiPS4F6) were used in differentiation experiments based on co-culture with OP9 stromal cells[33] in the presence of hematopoietic cytokines. In all cases, we could detect CD34$^+$ cells by flow cytometry starting at day 5 and peaking at day 12 (7.23 ± 2.57%, n = 7). We could also detect CD45$^+$ cells in those cultures from day 10, which reached 0.95 ± 0.38% (n = 6) by day 12 (Fig. 5a). The timing of appearance and frequency of hematopoietic progenitors obtained from patient-specific iPS cells were similar to those obtained using iPS cells from healthy individuals (7.24 ± 3.43% of CD34$^+$ cells at day 12, n = 5 from 2 independent iPS cell lines) and hES cells (6.62 ± 1.03% of CD34$^+$ cells at day 12, n = 5 from 2 independent hES cell lines; see also ref. 34). These results show that FA patient-specific iPS cells display normal potential to undergo early hematopoiesis in vitro.

We purified FA-iPS-derived CD34$^+$ cells at day 12 of the differentiation protocol by 2 rounds of magnetic activated cell sorting (MACS) to test their hematopoietic differentiation ability in clonogenic progenitor assays. We could observe that FA-iPS cell-derived CD34$^+$ cells generated erythroid (burst forming unit-erythroid [BFU-E]) and myeloid (colony forming unit-granulocytic, monocytic [CFU-GM]) colonies after 14 days

in methylcellulose culture (Fig. 5b-c). The myeloid nature of CFU-GM colonies was confirmed by the expression of the CD33 and CD45 markers in these colonies (Fig. 5d). The hematopoietic potential of FA-iPS cell-derived CD34$^+$ cells was robust and the numbers of colony-forming cells (CFCs) obtained in clonogenic assays were comparable to those obtained from CD34$^+$ cells derived from hES cells or control iPS cells (Fig. 5e, solid bars). These results indicate that patient-specific iPS cells successfully differentiated into hematopoietic progenitors of the erythroid and myeloid lineages. In some experiments, iPS-derived CD34$^+$ cells were maintained with hematopoietic growth factors for 7 days. In these cases, the number of CFCs increased very significantly (about 60 fold), suggesting a progressive hematopoietic differentiation in these cultures. We also attempted to generate blood cells in NOD/SCID mice transplanted with CD34$^+$ cells derived from genetically corrected FA-iPS cells, but no engraftments of human hematopoietic cells were observed in those animals, in agreement with previous data showing current technical limitations to repopulate immunodeficient mice with in vitro-differentiated hES cells[35].

To test whether hematopoietic progenitors derived from genetically corrected and reprogrammed FA-A cells maintained the disease-free phenotype of FA-iPS cells, we incubated hematopoietic colonies in the presence of mitomycin C, since hypersensitivity to DNA cross-linking agents is a hallmark of FA cells[11]. The proportion of mitomycin C-resistant colonies obtained from FA-iPS-derived CD34$^+$ cells was similar to that obtained from mononuclear bone marrow cells from a healthy donor, or from CD34$^+$ cells derived from either hES cells or iPS cells generated from somatic cells of a healthy donor, and contrasted sharply with the hypersensitivity to mitomycin C shown by FA mononuclear bone marrow cells (Fig. 5e, white bars). Moreover, FA-iPS cell-derived CD34+ cells were able to localize FANCD2 to foci of mitomycin C-induced DNA damage (Fig. 5f), demonstrating a functional FA pathway. The maintenance of the disease-free phenotype in

FA-iPS-derived hematopoietic progenitors is further supported by the sustained expression of the lentiviral FANCA transgene during the process of in vitro hematopoietic differentiation (data not shown).

Our results demonstrate that iPS cell technology can be used for the generation of patient-specific, disease-corrected cells with potential value for cell therapy applications. However, a number of caveats need to be solved before the clinical application of iPS-based strategies is realized. Retroviral transduction of adult somatic cells with OCT4, SOX2, KLF4, and c-MYC, while currently the most efficient method for generating human iPS cells, results in permanent undesirable transgene integrations. Although the retroviral transgenes become silenced during reprogramming, their re-activation during cell differentiation (particularly that of the oncogene c-Myc) has been associated with tumor formation[36]. Human iPS cells can be generated without c-MYC, but reprogramming efficiency in this case is drastically reduced[22,37]. To ascertain whether FA patient-specific iPS cells could be generated without retroviral transduction with c-MYC, we used primary keratinocytes from patient FA404. After 3 reprogramming attempts, we generated 1 iPS cell line (cFA404-KiPS3F1), which expanded robustly and showed all the characteristics and differentiation ability of iPS cells generated with 4 factors, and gave rise to hematopoietic progenitors in vitro (Supplementary Fig. 11). As expected, the genome of cFA404-KiPS3F1 cells did not contain integrations of the *c-MYC* retrovirus, as revealed by Southern hybridization with probes specific for the reprogramming factors (Supplementary Fig. 12) and PCR of genomic DNA (data not shown). These results demonstrate that "safer" patient-specific iPS cells can be generated with just 3 factors, although at efficiencies that may not be compatible with practical application. Moreover and ideally, permanent modification of the genome of iPS cells should be avoided, and integrating retroviruses omitted altogether. The recent implementation of reprogramming protocols that

do not rely on viral integration[38,39], if their applicability to human cells was confirmed, would bring the realization of this possibility closer.

The availability of patient-specific iPS cells could overcome the main limitation of current gene therapy strategies, due to risks of insertional oncogenesis[40], as genetically corrected iPS cells lend themselves to the screening of safe integration sites of the therapeutic transgenes. In addition, the generation of iPS cells from patients with genetic diseases offers the possibility of correcting these cells using gene targeting approaches based on homologous recombination[41]. Our studies, thus, may represent a step forward in the potential application of iPS technology for regenerative medicine.

Methods

Patients. Studies were approved by the authors' Institutional Review Board and conducted under the Declaration of Helsinki. Patients were encoded to protect their confidentiality, and written informed consent obtained. The generation of human iPS cells was done following a protocol approved by the Spanish competent authorities (Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III). Fanconi anemia patients were diagnosed on the basis of clinical symptoms and chromosome breakage tests of peripheral blood cells using a DNA cross-linker drug. Patients FA5, FA90 and FA153 have been previously described[42]; patients FA430 and FA431 correspond to patients #2 and #10, respectively, in ref. 23. Patient FA404 was subtyped by analyzing the G2-phase arrest of dermal fibroblasts transduced with EGFP and FANCA retroviral vectors and then exposed to mitomycin C, as previously described[42].

Cell lines. 293T and HT1080 cells (ATCC CRL-12103) were used for the production and titration of lentiviruses, respectively. These cell lines were grown in Dulbecco´s modified medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Biowhitaker). The ES[2] and ES[4] lines of hES cells were maintained as originally described[25]. The control iPS cell lines KiPS4F1 and KiPS3F1 and the partially-silenced KiPS4F3 cell line were cultured as reported[22].

Generation of iPS cells. Fibroblasts were cultured in DMEM supplemented with 10% FBS (all from Invitrogen) at 37ºC, 5% CO_2, 5% O_2 and used between 2-6 passages. For reprogramming experiments, about 50,000 fibroblasts were seeded per well of a 6-well plate and infected with a 1:1:1:1 mix of retroviral supernatants of FLAG-tagged OCT4, SOX2, KLF4, and c-MYC^{T58A} (ref. 22) in the presence of 1μg/ml polybrene. Infection consisted of a 45-min spinfection at 750 x g after which supernatants were left in contact

with the cells for 24 h at 37ºC, 5% CO_2. One or two rounds of 3 infections on consecutive

days were performed at the times indicated in Supplementary Text. Five days after

beginning the last round of infection, fibroblasts were trypsinized and seeded onto feeder

layers of irradiated human foreskin fibroblasts in the same culture medium. After 24 h, the

medium was changed to hES cell medium, consisting on KO-DMEM (Invitrogen)

supplemented with 10% KO-Serum Replacement (Invitrogen), 0.5% human albumin

(Grifols, Barcelona, Spain), 2 mM Glutamax (Invitrogen), 50 µM 2-mercaptoethanol

(Invitrogen), non-essential aminoacids (Cambrex), and 10 ng/ml bFGF (Peprotech).

Cultures were maintained at 37ºC, 5% CO_2, with media changes every other day. Starting 1

week after plating onto feeders, medium was supplemented with 1 µM PD0325901 and 1

µM CT99021 (both from Stem Cell Sciences) for 1 week. Colonies were picked based on

morphology 45-60 d after the initial infection and plated onto fresh feeders. Lines of

patient-specific iPS cells were maintained by mechanical dissociation of colonies and

splitting 1:3 onto feeder cells in hES cell medium or by limited trypsin digestion and

passaging onto Matrigel-coated plates with hES cell medium pre-conditioned by mouse

embryonic fibroblasts (MEFs). Other inhibitors were used as indicated in Supplementary

Text, at the following concentrations: 10 µM U0126 (Calbiochem), 25 µM PD098059

(Calbiochem), 5 µM BIO (Sigma), 10 µM Y27632 (Calbiochem). Generation of patient-

specific KiPS cells was essentially as previously reported[22], except that primary epidermal

keratinocytes were derived from small biopsy explants in the presence of irradiated

fibroblasts in DMEM/Hams-F12 (3:1) supplemented with 10% FBS, 1µg/ml EGF

(BioNova), 0.4µg/ml hydrocortisone, 5µg/ml Transferrin, 5µg/ml Insulin, $2x10^{-11}$ M

Liothyronine (all from Sigma), and 10^{-10} M cholera toxin (Quimigen).

Quantitative RT-PCR, transgene integration, and promoter methylation analyses.

Expression of retroviral transgenes and endogenous pluripotency-associated transcription

factors, integration of retroviral transgenes by genomic PCR or Southern blot, and methylation status of *OCT4* and *NANOG* promoters were assessed as previously reported[22].

HLA typing and DNA fingerprinting. Molecular typing of cell lines was performed by Banc de Sang i Teixits (Barcelona, Spain). HLA typing hES cell lines used sequence-based typification (SBT) with the AlleleSEQR HLA Sequencing Kit (Atria Genetics). Microsatellite DNA fingerprinting was performed using multiplex polymerase chain reaction of 9 microsatellites/short tandem repeats (STRs) plus *Amelogenin* gene using AmplFlSTR Profiler Plus Kit (Applied Biosystems).

Analysis of proviral copy number and transgene expression. Quantification of proviral copy number per cell was analyzed by qPCR in a Rotor Gene RG-3000 (Corbett Research Products) using primers against *FANCA* transgene: *hFANCA*-F: 5'-GCTCAAGGGTCAGGGCAAC-3' and *hFANCA*-R: 5'-TGTGAGAAGCTCTTTTTCGGG-3' and detected with the Taqman probe *FANCA*-P: 5'-FAM- CGTCTTTTTCTGCTGCAGTTAATACCTCGGT- BHQ1-3'. To quantify the number of cells, ®-actin primers were used: DNA-RNA-*β Actin*-F: 5´-ATTGGCAATGAGCGGTTC C-3´ and DNA- *β Actin* -R: 5´-ACAGTCTCCACTCACCCAGGA-3´ and detected with the probe DNA-RNA- *β Actin* -P: 5´-Texas Red-CCCTGAGGCACTCTTCCAGCCTTCC-BHQ1-3´. To measure the average proviral DNA per transduced cell a standard curve of LV: (FANCA-IRES-EGFP) and *β Actin* DNA amplification was made. Next, the average proviral number per cell was estimated by interpolation of the *hFANCA β Actin* ratio from each DNA sample in the standard curve. The expression of the human *FANCA* transgene was analyzed by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) on cDNA obtained from total RNA. Samples from a healthy donor and a FA patient were used as controls. To

distinguish between endogenous expression of *hFANCA* and the expression due to the transgene, total *hFANCA* expression was analyzed using *hFANCA* primers and probe and the endogenous expression was analyzed using *h3'FANCA*-F: TCTTCTGACGGGACCTGCC and *h3'FANCA*-R: AAGAGCTCCATGTTATGCTTGTAATAAAT and detected with Taqman probe: *h3'FANCA*-P: 5'-FAM-CACACCAGCCCAGCTCCCGTGTAA-BHQ1-3'. For housekeeping control expression *β Actin* was analyzed using DNA-RNA-*β Actin*-F primer, RNA-*β Actin*-R primer: 5′- CACAGGACTCCATGCCCA-3′ and Taqman probe DNA-RNA-*β Actin*-P. Differences between the expression obtained with the *hFANCA* and the *h3'FANCA* indicate the expression of the integrated provirus.

Western blot. Cell extracts were prepared using standard RIPA buffer. Briefly, harvested cells were washed three times with PBS and then resuspended in RIPA buffer. The total protein concentration in the supernatant was then measured using the Bio-Rad Protein Assay (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. 40 µg of total proteins were then loaded on a 6% SDS–PAGE and subjected to standard Western blot procedure followed with immunodetection with an anti-human FANCA antibody kindly provided by the Fanconi Anemia Research Fund, Eugene, Portland, USA. Vinculin (Abcam, Cat. No. ab18058; 1:5000) was used as internal loading control.

Functional studies of the FA pathway in iPS-derived cells. Subnuclear accumulation of stalled replication forks was induced by local UVC irradiation essentially as described[28] with some minor modifications. Briefly, cells (primary fibroblasts or iPS-derived cells) were seeded on 22x22 mm sterile coverslips. Prior to irradiation, the medium was aspirated and the cells were washed with PBS. Cells were then covered with an isopore polycarbonate filter with pores of 5 µm diameter (Millipore, Badford, MA, USA) and

exposed to 60J/m^2 UVC from above with a Philips 15W UV-C lamp G15-T8. Subsequently, the filter was removed and fresh pre-warmed medium added back to the cells, which were returned to culture conditions and processed for immunofluorescence 6h later. In parallel experiments, primary fibroblast and iPS-derived cells were exposed to hydroxyurea (HU, 2mM) for 24h and then fixed and processed for immunofluorescence as described below.

For FANCD2 detection at UV-induced stalled replication forks, cells were fixed with PBS containing 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature (RT), washed with PBS and incubated with PBS, 0.5% Triton (Sigma-Aldrich) for 10 min at RT. Next, cells were washed with PBS and subsequently rinsed with a washing buffer (WB) consisting of 5% bovine albumin (Sigma-Aldrich) and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then treated with 1M HCl for 5 min at 37 ºC and incubated for 1h at 37° C with a primary rabbit antibody against FANCD2 (Abcam, Cambridge, UK; 1:1000) mixed with an anti CPD antibody (Kamiya Biomed, MC-062; 1:500). Cells were then washed for 15 min in WB with gentle agitation and incubated with secondary antibodies anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, Oregon, USA) and anti-rabbit Alexa Fluor 555 (Molecular Probes) diluted in WB for 30min at 37° C followed by a 15 min washing step in WB with gentle agitation, rinsed in distilled water, air dried and mounted in anti-fading medium containing 4'-6'-diamidino-2-phenylindole (DAPI, Sigma). In the HU experiments, immunodetection was identical with the exception that the HCl washing step and a primary mouse antibody against anti-γH2AX (Upstate; 1:3000) was used instead of an anti-CPD to visualize nuclei foci representing stalled and broken replication forks. Using this color combination, nuclei were visualized in blue color, the site of stalled replication forks (UV irradiation spot or HU-induced foci) in green color, and FANCD2 in red color. Microscopic analysis and image capturing were performed in

identical optical and exposure conditions for all cell types using a Zeiss Axio Observer A1 epifluorescence microscope equipped with a AxioCam MRc 5 camera and the AxioVision, Rel. 4.6 software.

Immunofluorescence and AP analyses. Patient-specific iPS cells were grown on plastic coverslide chambers and fixed with 4% paraformaldehyde (PFA). The following antibodies were used: Tra-1-60 (MAB4360, 1:100), Tra-1-81 (MAB4381, 1:100), and Sox2 (AB5603, 1:500) from Chemicon, SSEA-4 (MC-813-70, 1:2) and SSEA-3 (MC-631, 1:2) from the Developmental Studies Hybridoma Bank at the University of Iowa, Tuj1 (1:500; Covance), TH (1:1000;Sigma), α-fetoprotein (1:400; Dako), α-actinin (1:100; Sigma), Oct-3/4 (C-10, SantaCruz, 1:100), Nanog (Everest Biotech; 1:100), GFAP (1:1000; Dako), Vimentin (1:500, Chemicon), FoxA2 (1:100; R&D Biosystems). Secondary antibodies used were all the Alexa Fluor Series from Invitrogen (all 1:500). Images were taken using Leica SP5 confocal microscope. Direct AP activity was analysed using an Alkaline Phosphatase Blue/Red Membrane Substrate solution kit (Sigma) according to the manufacturer guidelines. For FANCD2 inmunofluorescence assays, cells were grown in plastic coverslide chambers and treated with 30 nM mitomycin C. After 16 h cells were fixed with 3.7% PFA in PBS for 15 minutes followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After blocking for 30 minutes in blocking buffer (10% FBS, 0.1% NP-40 in PBS), cells were incubated with polyclonal rabbit anti-FANCD2 antibody (Novus Biologicals, NB 100-182, 1/250). Anti-rabbit Texas red conjugated antibody (Jackson Inmunoresearch Laboratories) was used as secondary antibody (1:500). Slides were analyzed with a fluorescence microscope Axioplan2 (Carl Zeiss, Göttingen, Germany) using a 100x/1.45 Oil working distance 0.17 mm objective.

In vitro differentiation. Differentiation towards endoderm, cardiogenic mesoderm, and neuroectoderm was carried out essentially as described[25]. Differentiation towards fibroblast-like cells was accomplished by plating embryoid bodies (EBs) onto gelatin-coated plates in 90% DMEM, 10% FBS and repeated passaging of differentiated cells with fibroblast-like morphology. For hematopoietic differentiation, EBs were produced by scraping of confluent iPS wells and cultured in suspension in EB medium (90% DMEM, 10% FBS) for 24-48hrs. EBs were then placed over a feeder layer of confluent OP9 stromal cells and allowed to attach. The medium used for the first 48h of differentiation was 50% EB medium and 50% hematopoietic differentiation medium. The hematopoietic differentiation medium was StemSpan Serum Free Medium (StemCell Technologies) supplemented with cytokines BMP4 (10 ng/ml), VEGF (10 ng/ml), SCF (25 ng/ml), FGF (10 ng/ml), TPO (20 ng/ml), and Flt ligand (10 ng/ml). After 48h, cells were cultured with hematopoietic differentiation medium, with medium changes every 48h until the end of the differentiation protocol, day 13 after EB plating. At day 13, OP9 and EBs were collected by trypsinization (0.25% trypsin), washed and labeled with anti CD34-beads conjugated antibody (Miltenyi Biotec) according to manufacturer's specification. The CD34^{+} fraction was purified by MACS, and fraction purity was increased by a second round of MACS. Final purity of the collected cells for CD34 was checked on a fraction of the MACS eluate by flow cytometry. The remaining CD34^{+} cells were frozen in medium IMDM containing 10%DMSO and 20% FBS and stored in liquid nitrogen until further use. For the assessment of colony forming cells (CFCs), samples were cultured in triplicates, in Methocult H4434 (Stem Cell Technologies) at 37ºC, in 5% CO2, 5% O2 and 95% humidified air. Colonies were scored after two weeks in culture. To analyze the mitomycin C-resistance of the hematopoietic progenitors, CFC cultures were treated with 10 nM mitomycin C (Sigma). In some experiments, iPS-derived CD34^{+} cells were cultured for 7 days in StemSpan Serum

Free Medium (StemCell Technologies) supplemented with hematopoietic growth factors SCF (Amgen, 300 ng/ml), TPO (R&D Systems, 100 ng/ml), and Flt ligand (BioSource, 100 ng/ml).

Flow cytometry analyses. For surface phenotyping the following fluorochrome (phycoerythrin [PE], or allophycocyanin [APC])–labeled monoclonal antibodies were used (all from Becton Dickinson Biosciences): anti-CD34 PE (581/CD34), anti-CD45 APC (HI30). Gating was done with matched isotype control mAbs. Hoechst 33528 (H258) was included at 1 μg/mL in the final wash to exclude dead cells. All analyses were performed on a MoFlo cell sorter (DakoCytomation) running Summit software. To analyze the phenotype of hematopoietic progenitors, CFU-GM colonies were picked and washed with PBS. Cells were stained with antihuman CD45-PECy5 mAb (Clone J33, Immunotech) in combination with antihuman CD33-PE mAb (D3HL60.251, Immunotech). Cells were then washed in PBA (phosphate-buffered salt solution with 0.1% BSA and 0.01% sodium azide), resuspended in PBA + 2 μg/mL propidium iodide, and analyzed using an EPICS ELITE-ESP cytometer (Coulter). Off-line analysis was done with CXP Analysis 2.1 flow-cytometry software (Beckman Coulter Inc).

Teratoma formation. Severe combined immunodeficient (SCID) beige mice (Charles River Laboratories) were used to test the teratoma induction capacity of patient-specific iPS cells essentially as described[22]. All animal experiments were conducted following experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals, in full compliance with Spanish and European laws and regulations.

Genetic correction of FA cells with lentiviral vectors. Lentiviral (LV) vectors carrying the *hFANCA*-IRES-*EGFP* cassette under the control of the internal spleen focus forming virus (*SFFV*) U3 promoter (FANCA-LV; ref. 21) were used to transduce fibroblasts and

keratinocytes from FA-A patients. Fibroblasts from the FA-D2 patient were transduced with a LV carrying the *FANCD2* cDNA under the control of the *vav* promoter(FANCD2-LV, ref. 21). Lentiviral vectors carrying either of these promoters were equally efficient to correct the phenotype of human FA cells[21]. Vector stocks of VSV-G pseudotyped LVs were prepared by four-plasmid calcium phosphate-mediated transfection in 293T cells, essentially as described[43]. Supernatants were recovered 24h and 48h after transfection and filtered through 0.45 µm. Functional titers of infective LVs were determined in HT1080 cells, plated at 3.5x104 cells per well in 24 well-plates and infected overnight with different dilutions of either LV-supernatant. Cells were washed and incubated with fresh medium, and the proportion of EGFP+ cells was determined 5 days later by flow cytometry, or after 8 days by qPCR.

Knockdown of FANCA. Lentiviral vectors expressing scramble shRNA and 5 different *FANCA*-shRNAs (Sigma, MISSION shRNA NM_000135) were used to generate viral particles according to the manufacturer's instructions. For infection, FA patient-specific iPS cells were incubated with viral supernatants in 6-well plates for 24 hours. Puromycin selection (2 µg/ml) was applied for 24 hours 3 days after lentiviral infection and cells were allowed to recover for 3 days before splitting. Transient RNA interference experiments with siRNA were performed as previously described[44]. In brief, cells were grown in OPTI-MEM medium (Gibco, Cat. No. 31985) with 10% FCS without antibiotics and transfected with 10nM *FANCA* siRNA (ref. 45) or *Luciferase* siRNA as a control (5'CGUACGCGGAAUACUUCGA[dT][dT]3'), with Lipofectamin RNAiMAX transfection reagent (Invitrogen, Cat. No. 13778-075) twice over a period of 24h. 24h after the second transfection, cells were left untreated or were treated with diepoxybutane (DEB) at 0.02µg/ml for 3 days and subsequently harvested for protein lysates or processed

following standard cytogenetic methods. Mitotic indexes were calculated by counting the
number of mitotic cells in 500-6000 cells per point in duplicate..

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Figure legends

Fig. 1. Derivation of patient-specific induced pluripotent stem cells from Fanconi anemia patients. a-f, Successful reprogramming of genetically-corrected primary dermal fibroblasts (**a**) derived from patient FA90. **b,** Colony of iPS cells from the cFA90-44-14 line grown on Matrigel-coated plated showing hES cell-like morphology. **c-f,** The same iPS cell line shows strong AP staining (**c**) and expression of the transcription factors OCT4 (**d**), SOX2 (**e**) and NANOG (**f**) and the surface markers SSEA3 (**d, e**) and SSEA4 (**f**). **g,** Genetically-corrected fibroblasts from patient FA404. **h,** Colony of iPS cells from the cFA404-FiPS4F1 line grown on feeder cells displaying typical hES cell morphology. **i-l,** The same iPS cell line shows strong AP staining (**i**) and expression of the pluripotency-associated transcription factors OCT4 (**j**), SOX2 (**k**) and NANOG (**l**) and surface markers SSEA3 (**j**), SSEA4 (**k**) and TRA1-80 (**l**). Cell nuclei were counterstained with DAPI in **d-f** and **j-l**. Scale bar, 100 μm (**a**, **c-g**, **i-l**) and 250 μm (**b**, **h**).

Fig. 2. Molecular characterization of FA patient-specific iPS cell lines. a, PCR of genomic DNA to detect integration of the indicated retroviral transgenes in the patient-specific iPS cell lines cFA90-44-14 and cFA404-FiPS4F1. Genetically-corrected fibroblasts (Fibr.) from patient FA404 prior to reprogramming were used as negative control. **b, c,** Quantitative RT-PCR analyses of the expression levels of retroviral-derived reprogramming factors (**b**) and of total expression levels of reprogramming factors and pluripotency-associated transcription factors (**c**) in the indicated patients' fibroblasts (fibr.) and patient-specific iPS cell lines. hES cells (ES[4]) and partially-silenced iPS cells (KiPS4F3) are included as controls

Transcript expression levels are plotted relative to GAPDH expression. **d-g,** Colony of cFA90-44-14 iPS cells showing high levels of endogenous NANOG expression (**e**, green channel in **d**) and absence of FLAG immunoreactivity (**f**, red channel in **d**). Cell nuclei were counterstained with DAPI (**g**, blue channel in **d**). **h,** Bisulfite genomic sequencing of the *OCT4* and *NANOG* promoters showing demethylation in the patient-specific iPS cell lines cFA90-44-14 and cFA404-KiPS4F3, compared to patient's fibroblasts. Open and closed circles represent unmethylated and methylated CpGs, respectively, at the indicated promoter positions. Scale bar, 100 μm.
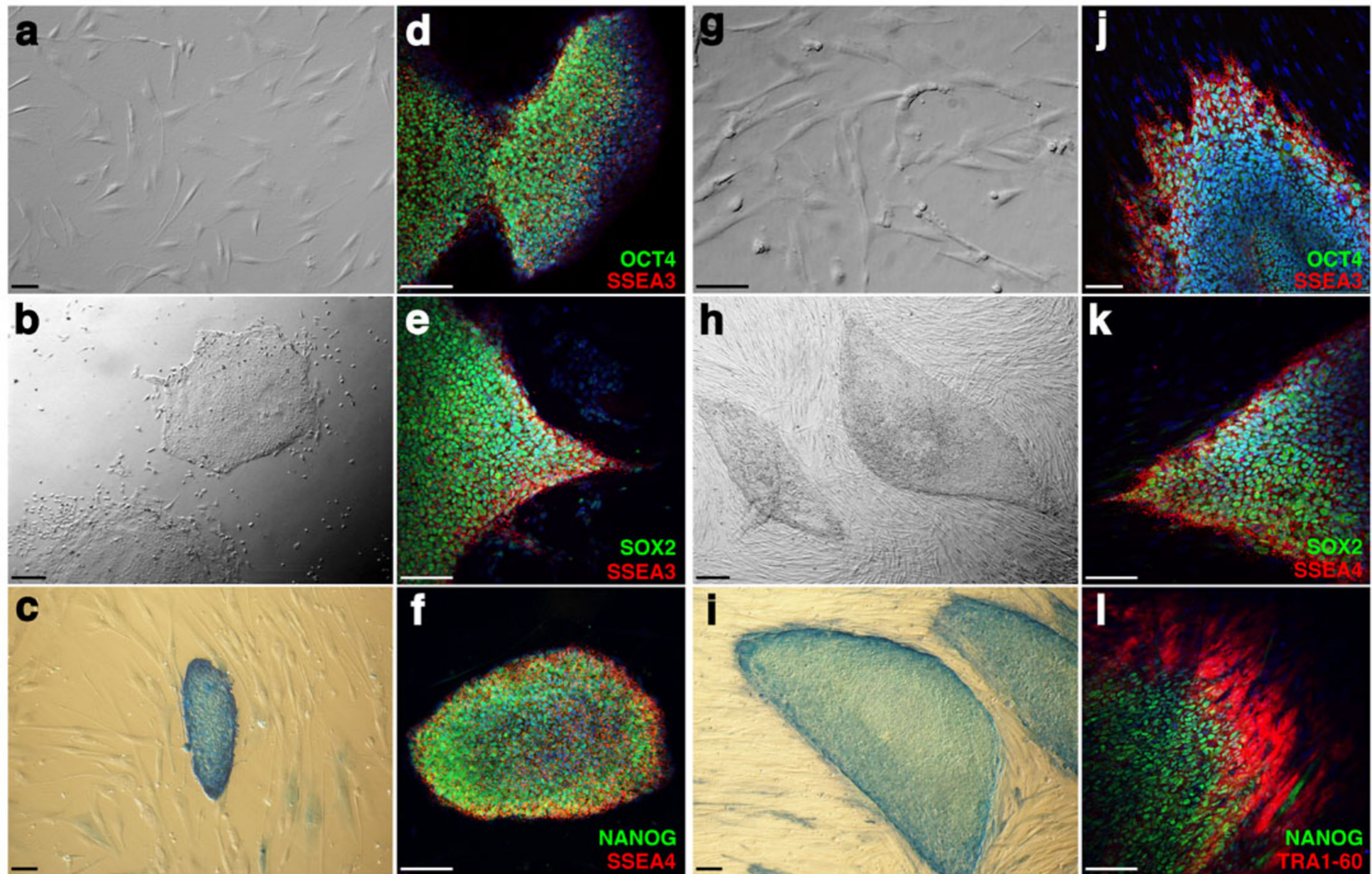
Fig. 3. Pluripotency of FA patient-specific iPS cells. a-c, In vitro differentiation experiments of cFA404-FiPS4F2 iPS cells reveal their potential to generate cell derivatives of all three primary germ cell layers. Immunofluorescence analyses show expression of markers of **a,** endoderm (α-fetoprotein, green; FoxA2, red), **b,** neuroectoderm (TuJ1, green; GFAP, red), and mesoderm (α-actinin, red). **d-f,** Injection of cFA90-44-14 iPS cells under the skin of immunocompromised mice results in the formation of teratomas containing structures that represent the 3 main embryonic germ layers. Endoderm derivatives (**d, e**) include glandular structures that stain positive for endoderm markers (α-fetoprotein, green); ectoderm derivatives (**e**) include structures that stain positive for neuroectoderm markers (TuJ1, red); mesoderm derivatives (**f**) include structures that stain positive for muscle markers (α-actinin, red). All images are from the same tumor. Scale bar, 100 μm (**a**, **b**, **d**, **e**) and 25 μm (**c**, **f**).

Fig. 4. Functional FA pathway in patient-specific iPS cell lines. **a,** Western blot analysis of FANCA in protein extracts from the indicated cell lines, showing expression of FANCA in FA patient-specific iPS cells. The expression of VINCULIN was used as loading control. **b,** FANCD2 (red channel) fails to relocate to UVC radiation-induced stalled replication forks, visualized by immunofluorescence with antibodies against cyclobutane pyrimidine dimers (CPD, green channel), in fibroblasts from patient FA404, while it shows normal accumulation to damaged sites in wild-type fibroblasts (control), corrected fibroblasts (cFA404) or FA-iPS-derived cells (cFA404-FiPS4F2). **c,** Western blot analysis of FANCA in protein extracts from untransduced cFA404-KiPS4F3 cells or 6 days after transduction with lentiviruses expressing scramble shRNA (Control) or the indicated *FANCA*-shRNAs. The expression of VINCULIN was used as loading control. Values at the bottom represent FANCA expression levels measured by densitometry quantification normalized by VINCULIN expression and referred to untransduced cFA404-KiPS4F3 cells. **d,** Alkaline phosphatase staining of cFA404-KiPS4F3 cells 1 passage after being transduced with lentiviruses expressing scramble shRNA (Control) or the indicated *FANCA*-shRNAs, 1 week after seeding. **e,** Mitotic index values in cFA404-FiPS4F2-derived cells transfected with scramble (Control) or *FANCA* siRNAs and incubated in the absence or in the presence of diepoxybutane (DEB). The inset shows FANCA depletion induced by *FANCA* siRNAs in these experiments, as visualized by Western blot using VINCULIN as loading control.
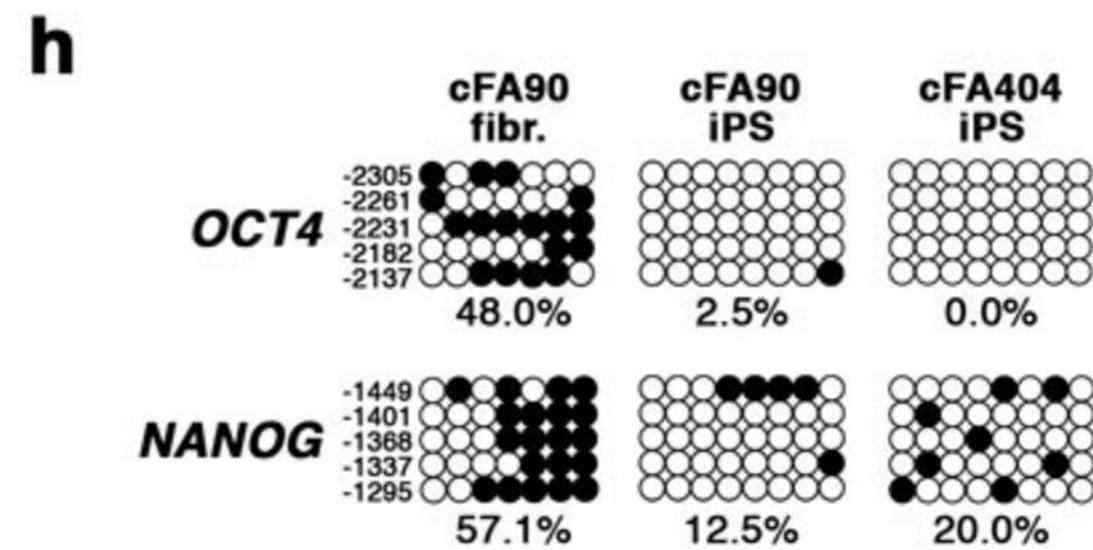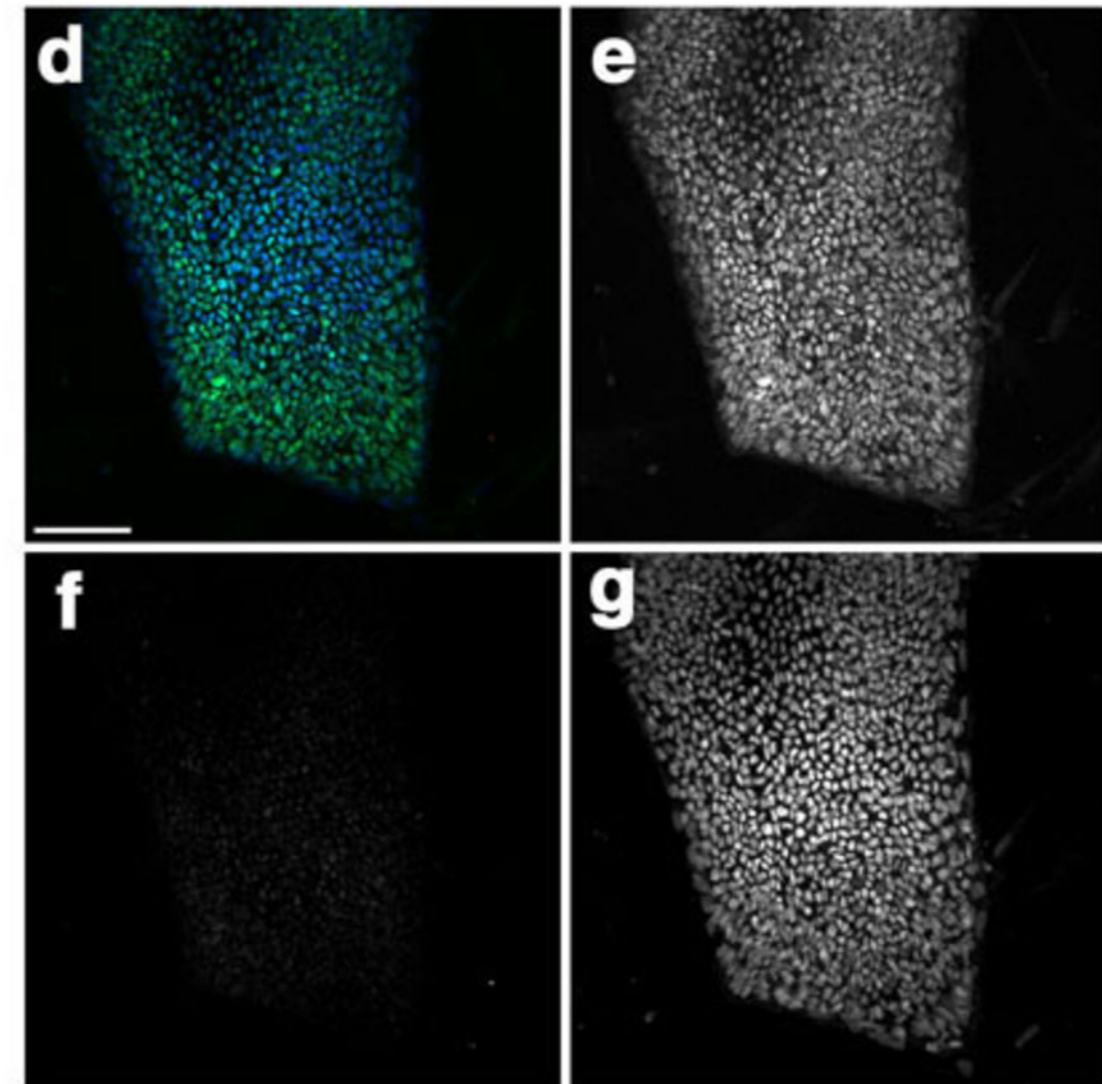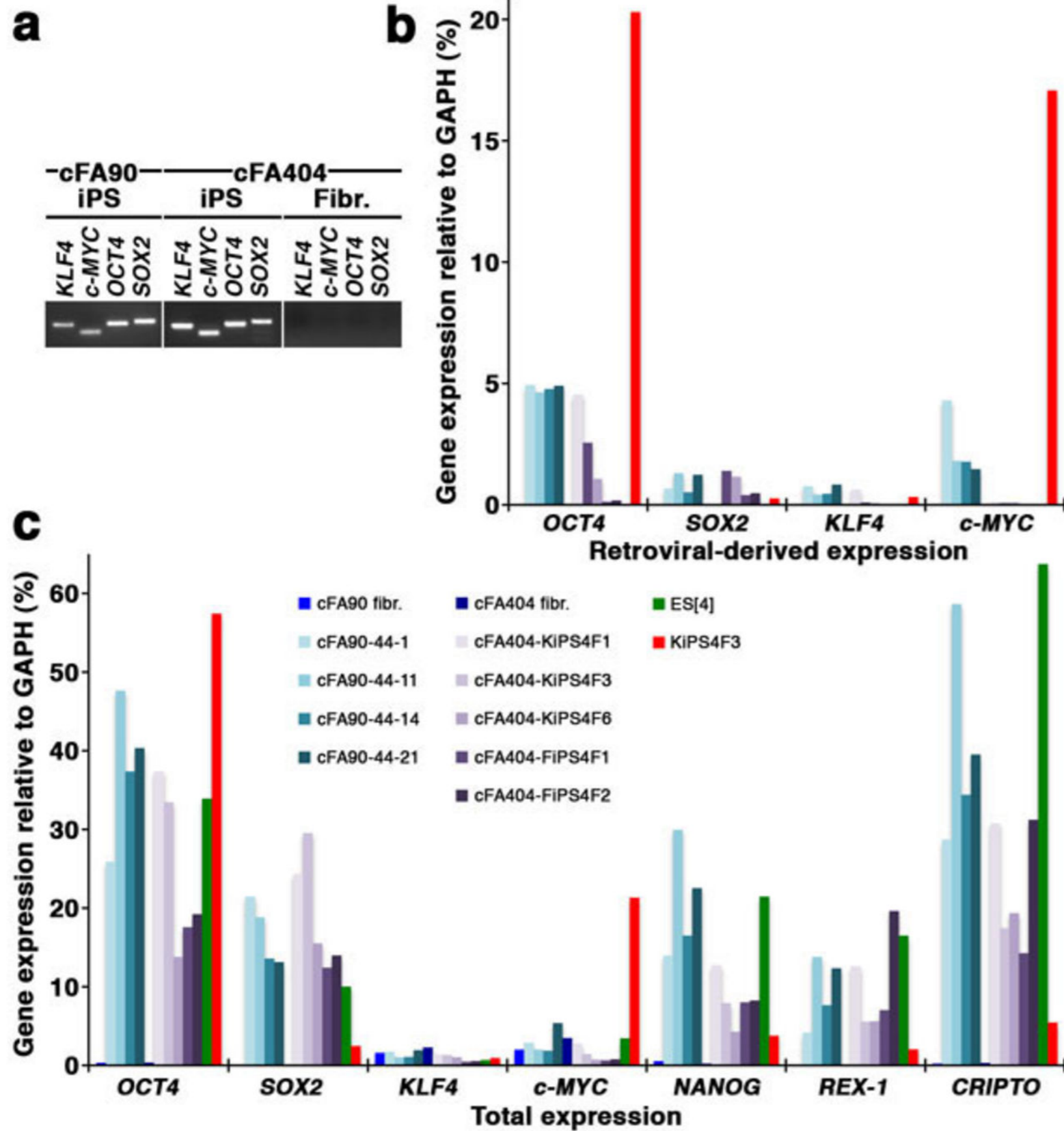
Fig. 5. Generation of disease-free hematopoietic progenitors from patient-specific iPS cell lines. **a,** Expression of CD34 and CD45 markers in iPS cells

subjected to hematopoietic differentiation. **b, c,** Representative erythroid (BFU-E) and myeloid (CFU-GM) colonies generated 14 days after the incubation of iPS-derived CD34$^+$ cells in semisolid cultures. **d,** The myeloid nature of CFU-GM colonies was confirmed by the co-expression of the CD33 and CD45 markers in CFU-GM colonies. **e,** Total number of colony-forming cells (CFC) generated in the absence and the presence of 10nM mitomycin C (MMC) from CD34$^+$ cells derived from the indicated FA-iPS cell lines. For comparison, clonogenic assays were also performed using hematopoietic progenitors from healthy donors (purified CD34$^+$ cord blood cells from 2 independent donors, CB CD34$^+$; and mononuclear bone marrow cells, BM MNC), from a FA patient, and from CD34$^+$ cells derived from control human pluripotent stem cells, including ES[2] cells (hES) and KiPS4F1 cells (KiPS). **f,** Immunofluorescence analysis showing FANCD2 foci in mitomycin C-treated CD34$^+$ cells derived from FA-iPS cells (line cFA90-44-14).
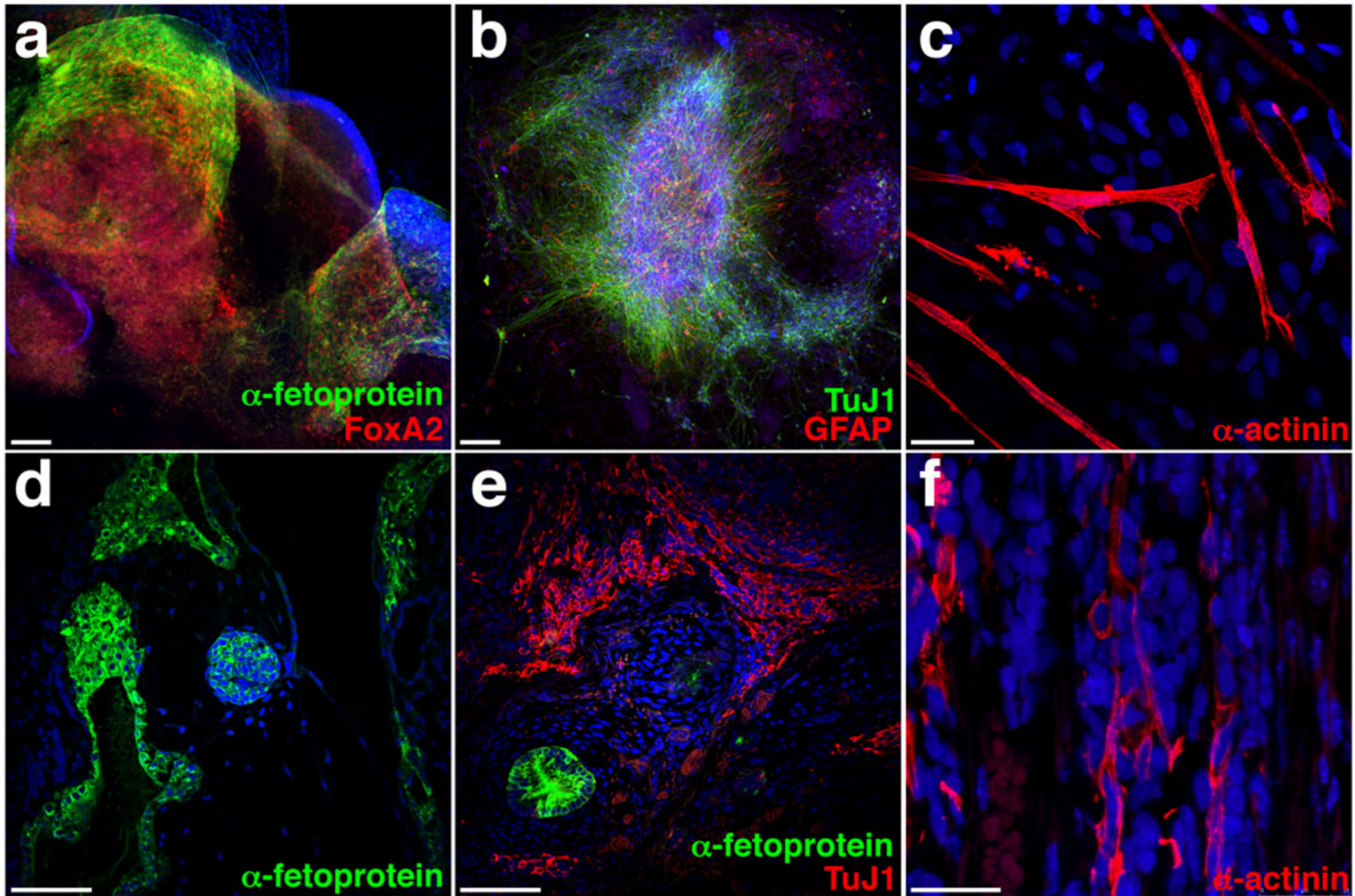
Raya et al. Fig. 2

a — α-fetoprotein / FoxA2

b — TuJ1 / GFAP

c — α-actinin

d — α-fetoprotein

e — α-fetoprotein / TuJ1

f — α-actinin

Raya et al. Fig. 4

Raya et al. Fig. 5