Reprogramming of Human Fibroblasts to Induced Pluripotent Stem Cells under Xeno-free Conditions

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ABSTRACT

The availability of induced pluripotent stem cells (iPSCs) has created extraordinary opportunities for modeling and perhaps treating human disease. However, all reprogramming protocols used to date involve the use of products of animal origin. Here, we set out to develop a protocol to generate and maintain human iPSC that would be entirely devoid of xenobiotics. We first developed a xeno-free cell culture media that supported the long-term propagation of human embryonic stem cells (hESCs) to a similar extent as conventional media containing animal origin products or commercially available xeno-free medium. We also derived primary cultures of human dermal fibroblasts under strict xeno-free conditions (XF-HFF), and we show that they can be used as both the cell source for iPSC generation as well as autologous feeder cells to support their growth. We also replaced other reagents of animal origin (trypsin, gelatin,

matrigel) with their recombinant equivalents. Finally, we used vesicular stomatitis virus G-pseudotyped retroviral particles expressing a polycistronic construct encoding Oct4, Sox2, Klf4, and GFP to reprogram XF-HFF cells under xeno-free conditions. A total of 10 xeno-free human iPSC lines were generated, which could be continuously passaged in xeno-free conditions and maintained characteristics indistinguishable from hESCs, including colony morphology and growth behavior, expression of pluripotency-associated markers, and pluripotent differentiation ability in vitro and in teratoma assays. Overall, the results presented here demonstrate that human iPSCs can be generated and maintained under strict xeno-free conditions and provide a path to good manufacturing practice (GMP) applicability that should facilitate the clinical translation of iPSC-based therapies. *STEM CELLS* 2010;28:36–44

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The generation of induced pluripotent stem cells (iPSCs), pioneered in the mouse by the laboratory of Shinya Yamanaka [1, 2], has led to the creation of human pluripotent cell lines [3–6] and enabled the production of disease-specific iPSCs for modeling human disease [7–10], as well as patient-specific iPSCs with potential for cell therapy applications [11]. The clinical translation of iPSC-based strategies, however, will require satisfactorily addressing safety-related issues. Chief among them is their cancer risk potential, which has been reportedly reduced by omitting c-Myc from the reprogramming factors [12]. Moreover, a variety of reprogramming strategies have been recently developed that result in iPSCs

with minimal or no genetic modifications, as they achieve efficient excision of the reprogramming transgenes [13, 14], do not rely on transgene integration [15–17], or utilize transient delivery of proteins, rather than DNA [18]. However, all reprogramming protocols reported to date involve the use of animal-derived products at several steps, making them unsuitable for the generation of clinical-grade iPSCs [19]. Exposure of human cells to animal origin products may increase the risk of non-human pathogen transmission (reviewed in 20) and immune rejection of grafted cells [21], in addition to being a roadblock for the achievement of good manufacturing practice (GMP) standards (reviewed in 22).

Xenogenic products are present at multiple steps in currently available protocols for iPSC generation and maintenance. First, primary cultures of human somatic cells to be

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reprogrammed are typically established using fetal bovine serum (FBS)-containing media and passaged with animal-derived enzymes such as trypsin. A second possibility of xenogenic contamination may occur when using viruses to transduce somatic cells with the reprogramming factors, since most protocols use viral supernatants produced by cell lines maintained with FBS and trypsin. Finally, the selection of reprogrammed iPSC colonies and their maintenance and expansion are usually carried out using feeder layers of mitotically-inactivated mouse embryonic fibroblasts plated on gelatin of animal origin, culture media containing serum substitutes such as knockout serum replacement (KO-SR), which includes bovine serum albumin (BSA) in its formulation, and cell dissociation enzymes (such as trypsin, dispase, or collagenases) from animal sources. Xeno-free alternatives to these animal-derived products have been used for the derivation and/or maintenance of human embryonic stem cell (hESC) lines [23–30]. In addition, immortalized human fibroblasts have been very recently shown to support iPSC generation [31]. However, no successful attempts at reprogramming human somatic cells under completely xeno-free conditions have been reported to date. This is particularly relevant inasmuch as iPSC generation from human somatic cells, even using optimized animal-derived products, is an extremely inefficient process [3–6], and the lower performance of xeno-free substitutes [28] may thwart reprogramming success to levels incompatible with practical application.

Here, we show that reprogramming of human fibroblasts to pluripotency can be achieved under xeno-free conditions at efficiencies similar to those obtained using animal-derived products. For this purpose, we established a primary culture of xeno-free human foreskin fibroblasts, which were used as both the source of cells for reprogramming as well as autologous feeder cells for the generation and maintenance of iPSCs. We also developed a xeno-free culture medium for hESC/iPSC based on a human plasma-derived serum substitute and show that it supports their long-term culture with a performance similar to those of commercially-available xeno-free hESC media and conventional KO-SR-based media. Finally, after replacing all other animal-derived products with their human or recombinant counterparts, we developed a xeno-free protocol for the generation and maintenance of *bona fide* iPSC using concentrated and purified retroviral particles expressing the reprogramming factors Oct4, Sox2, and Klf4 from a polycistronic construct.

MATERIALS AND METHODS

Establishment of Xeno-Free Primary Cultures of Human Foreskin Fibroblasts

Studies were approved by the authors' Institutional Review Board and conducted under the Declaration of Helsinki. A foreskin biopsy from a 3-year old individual was used after obtaining his parents' written informed consent. The sample was collected in sterile saline solution, divided into small pieces, and allowed to attach to cell culture dishes before adding xeno-free human foreskin fibroblast growth medium (XF-HFFm), which was Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 10% human serum (HS) (Sigma, St. Louis, MO, <http://www.sigmaaldrich.com>) and penicillin/streptomycin (0.5X) (Invitrogen). After 10 days of culture at 37°C, 5% CO₂, fibroblast outgrowths were dissociated and split 1:4 using a recombinant trypsin-like enzyme (TrypLE Select, Invitrogen). XF-HFFs were used for reprogramming at passages 2–3. For the generation of feeder layers, XF-HFFs were expanded

five passages and then irradiated at 55 Gy and cryopreserved as 4-million-cell vials in 90% HS, 10% dimethyl sulfoxide (iXF-HFF).

Human Plasma Source and F44 Production Process

All human plasma products were a kind gift from Instituto Grifols, S.A. (Barcelona, Spain, <http://www.grifols.com>). F44 is a human plasma-derived cell culture additive, developed by Instituto Grifols, S.A., obtained through cold-ethanol industrial plasma fractionation, according to previously described procedures [32]. Briefly, human plasma was collected from healthy donors at U.S.A.-based FDA-licensed plasmapheresis centers (Biomat USA, Los Angeles, CA, <http://www.biomatusa.grifols.com> or Plasmacare, Cincinnati, OH, <http://www.plasmacare.com>) and shipped to Instituto Grifols for processing. Individual plasma donations were tested for the presence of virological markers according to United States and European Pharmacopoeia regulations for plasma intended for manufacture of medicinal products through industrial fractionation. In addition, all plasma were subjected to nucleic acid testing, for the presence of RNA or DNA from relevant transfusion transmissible agents [33]. After testing, plasma pools were prepared containing donations from over 1,000 different donors and subjected to cryoprecipitation followed by cold-ethanol fractionation [32] with minor proprietary modifications. After purification, F44 was bottled under sterile conditions into glass vials, freeze-dried, and γ -irradiated.

Media for hESC/iPSC Culture

hESm is composed of KO-DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 20% KO-SR, 2 mM Glutamax, 50 μ M 2-mercaptoethanol, penicillin/streptomycin (0.5X, all from Invitrogen), non-essential amino acids (Cambrex, Walkersville, MD, <http://www.cambrex.com>), and 10 ng/ml of bFGF (Peprotech, Rocky Hill, NJ, <http://www.peprotech.com>). F44m is composed of KO-DMEM supplemented with 10% F44 (Grifols), Xeno-free KO-SR Growth Factor Cocktail (0.5X, Invitrogen), 2 mM Glutamax, 50 μ M 2-mercaptoethanol, penicillin/streptomycin (0.5X), nonessential amino acids, and 20 ng/ml of bFGF. F44m without Xeno-free KO-SR Growth Factor Cocktail can also maintain the culture of undifferentiated hESCs for at least 60 passages (data not shown). XF-hESm is composed of KO-DMEM supplemented with 15% Xeno-free KO-SR (Invitrogen), Xeno-free KO-SR Growth Factor Cocktail (1X), 2 mM Glutamax, 50 μ M 2-mercaptoethanol, penicillin/streptomycin (0.5X), nonessential amino acids, and 20 ng/ml of bFGF.

pMXs-OSKG Plasmid Construction

The mouse *Oct4* cDNA was amplified using a reverse primer eliminating the *Oct4* stop codon and adding a BspEI site and cloned into pCRII (Invitrogen) to give pCRII-Oct4-Bsp (oriented NotI-5'cDNA3'-Acc65I). The mouse *Sox2* cDNA was amplified using a forward primer containing an AgeI site followed by P2A peptide sequence and a reverse primer eliminating the *Sox2* stop codon and containing a BspEI site; this fragment was cloned in pCRII to give pCRII-Age-Sox2-Bsp (oriented NotI-5'cDNA3'-Acc65I). pCRII-Age-Sox2-Bsp was digested with AgeI and Acc65I and cloned into pCRII-Oct4-Bsp digested with BspEI-Acc65I, producing pCRII-Oct4-P2A-Sox2-BspEI. The same cloning strategy was repeated twice, to incorporate the *Klf4* and *eGFP* coding sequences (producing pCRII-OSKG). The last ORF was amplified in order to preserve the stop codon. pCRII-OSKG was digested with EcoRI, and the OSKG inserted was cloned into a unique EcoRI site located in the polylinker of the pMXs vector, generating pMXs-OSKG.

Retroviral Production

Retroviral supernatants of FLAG-tagged OCT4, SOX2, KLF4, c-MYC^{T58A}, or pMXs-OSKMG were produced in Phoenix Amphotropic packaging cells as previously described [34]. High-titer vesicular stomatitis virus G (VSV-G)-pseudotyped retroviruses

Figure 1. Human plasma fractions for human embryonic stem cell (hESC) culture. (A) Flow diagram of the albumin purification process from human plasma. Main proteins found in Fraction I: fibrinogen, fibronectin, factor VIII, C1q, C1r, C1s; in Fraction IV1+IV4: α - and β -globulins, α 1-antitrypsin, IgM, antithrombin III, ceruloplasmin, haptoglobin, transferrin. (B) AP staining of ES[2] cells after two passages in 100% of xeno-free protein source: human plasma cryoprecipitate supernatant (B), S/N Fr I (C), F44 (D), S/N Fr IV1+IV4 (E), human albumin (F), and control in hESm (G). Large areas of differentiation are evidenced as negative AP staining in (B), (E), and (F), whereas uniformly undifferentiated colonies are present in (C), (D), and (G). In all cases, hESC were plated onto feeder layers of irradiated human foreskin fibroblasts in gelatin-coated dishes. Scale bars, 250 μ m ((A)–(G)).

expressing the OSKG polycistron were produced in 293T cells by transient transfection of pMXs-OSKG, the VSV-G-expressing construct pMD2.G, and the retroviral *gag/pol*-expressing plasmid pUMVC [35] (Addgene plasmid 8449), and purified by ultracentrifugation as described [36].

Generation of iPS Cells

The generation of human iPS cells was done following a protocol approved by the Spanish competent authorities (Commission on Guarantees Concerning the Donation and Use of Human Tissues and Cells of the Carlos III Health Institute). Fibroblasts were cultured in XF-HFFm at 37°C, 5% CO₂ and used between 2 and 3 passages. For reprogramming experiments, about 100,000 fibroblasts were seeded per well of a 6-well plate and infected with the indicated mixture of retroviral supernatants encoding FLAG-tagged OCT4, SOX2, KLF4, and c-MYC^(T58A) (1 ml each), or with 4 ml of retroviral supernatant encoding the OSKM polycistron, or with 10 μ l of high-titer VSV-G-pseudotyped retroviruses expressing the OSKG polycistron, in the presence of 5 μ g/ml of polybrene. Infection consisted of a 45-minute spinfection at 750 $\times$ g after which supernatants were left in contact with the cells for 24 h at 37°C, 5% CO₂. Two rounds of infections were then performed on consecutive days. The following day, fibroblasts were dissociated and seeded onto confluent iXF-HFF in 10-cm plates coated with CELLStart (Invitrogen) in F44m or XF-hESm. The medium was then replaced daily. After 10 days, one million iXF-HFF were added to each plate. Colonies were picked based on morphology 25–35 days after the initial infection and plated onto fresh iXF-HFF coated with CELLStart. Xeno-free iPS cell lines were maintained by mechanical dissociation of colonies and splitting 1:3 onto iXF-HFF coated with CELLStart in the corresponding XF media.

For assaying the reprogramming efficiency of different culture media, infected fibroblasts were plated onto irradiated HFF in 10-cm gelatin-coated dishes and cultured with hESm, XF-hESm, or F44m. After 24 days, the plates were fixed and stained for alkaline phosphatase activity.

DNA Fingerprinting, HLA Typing, and Quantitative Reverse Transcription-Polymerase Chain Reaction

Molecular typing of cell lines was performed by Banc de Sang i Teixits (Barcelona, Spain, <http://www.bancsang.net>). Microsatellite DNA fingerprinting was performed using multiplex polymerase chain reaction of nine microsatellites and short tandem repeats plus amelogenin gene using AmpliFISTR Profiler Plus Kit (Applied Biosystems, Foster City, CA, [\[com\]\(http://www.appliedbiosys.com\)\). Expression of retroviral transgenes and endogenous pluripotency-associated transcription factors was analyzed by quantitative reverse transcription-polymerase chain reaction \(RT-PCR\) as previously reported \[34\]. Expression of the retroviral transgenes was analyzed using the oligonucleotides forward 5' CACCCACACTTGTGACTAT GCA and Reverse 5' TGTGCCTTGAGATGAGAACTCTTG.](http://www.appliedbiosys-</p>
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Promoter Methylation Analysis

Genomic DNA from xeno-free iPS cell lines or XF-HFF was extracted from about 1,000,000 cells using QIA AMP DNA Mini Kit (Qiagen). Purified DNA (500 ng) was mutagenized with Epigentek (Bionova) according to the manufacturer's specifications. The promoter sequences of interest were amplified by two subsequent PCR reactions using primers previously described [37]. The resulting amplified products were cloned into pGEM T Easy plasmids, amplified in TOP10 cells, purified, and sequenced.

Southern Blot

Genomic DNA from each cell line was isolated using All Prep DNA/RNA columns (Qiagen), following the manufacturer's guidelines. Each lane of the Southern blot corresponds to 4 mg of genomic DNA digested with 40 U of BamHI restriction enzyme (New England Biolabs, Ipswich, MA, <http://www.neb.com>), electrophoresed on a 1% agarose gel, transferred to a neutral nylon membrane (Amersham, Hybond-N, GE Healthcare Europe GmbH, Cerdanyola, Spain, <http://www.gelifsciences.com>), and hybridized with a GFP DIG-dUTP labeled probe generated by polymerase chain reaction (PCR) using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland, <http://www.roche-applied-science.com>). The probe was detected by an AP-conjugated DIG-Antibody (Roche Diagnostics) using CDP-Star (Sigma-Aldrich) as a substrate for chemiluminescence. Conditions were as per the instructions of the manufacturer. The GFP probe was generated using an eGFP template with the following primers (F, forward; R, reverse): GFP F 5'- CGCACCATCTTCTTCAAGGAC-3'; GFP R 5'- CATGCCGAGAGTGATCCCG-3'.

Immunofluorescence and Alkaline Phosphatase Activity Analyses

Xeno-free iPS cells were co-cultured with XF iHFF on plastic chamber slides and fixed with 4% paraformaldehyde. The following antibodies were used: TRA-1-60 (MAB4360, 1:200), TRA-1-81 (MAB4381, 1:200), and SOX2 (AB5603, 1:500) from Chemicon (Temecula, CA, <http://www.chemicon.com>); SSEA-4 (MC-813-70, 1:2) and SSEA-3 (MC-631, 1:2) from Developmental Studies

Table 1. Main components measured in F44 supernatant

Component	Identity/concentration
Proteins	Albumin
	Transferrin
	Ceruloplasmin
	Hepatoglobin
	Anti-proteases
Fatty acids	Palmitic acid
	Stearic acid
	Oleic acid
	Linoleic acid
Sodium	137 mM
Glucose	64 mg/dl
Osmolality	235 mOsm/kg
Endotoxins	<0.5 UE/ml

Hybridoma Bank at the University of Iowa (<http://www.dshb.biology.uiowa.edu>); Tuj1 (1:500; Covance, Princeton, NJ, <http://www.covance.com>); α -fetoprotein (1:400; Dako, Glostrup, Denmark, <http://www.dako.com>); α -actinin (1:100; Sigma); OCT4 (1:100; C-10, SantaCruz, Santa Cruz, CA, <http://www.scbt.com>); NANOG (1:100; Everest Biotech EB06860, Oxfordshire, U.K., <http://www.everestbiotech.com>); and Anti-FLAG (Sigma M2). Images were taken using a Leica SP5 confocal microscope. Direct alkaline phosphatase activity was analyzed using an Alkaline Phosphatase Blue or Red Membrane substrate solution kit (Sigma) according to the manufacturer's guidelines.

In Vitro Differentiation

Embryoid body (EB) formation was induced from colony fragments mechanically collected and then maintained in suspension in the presence of xeno-free medium for 24 hours. We performed a preconditioning culture where EBs were maintained for 2–3 days in ultralow attachment plates in differentiation media. In particular, for endoderm differentiation, EBs were cultured in the presence of KO-DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM 2-*b*-mercaptoethanol, nonessential amino acids, and penicillin/streptomycin. For mesoderm differentiation, we used the same medium described above but added ascorbic acid (0.5 mM). For ectoderm induction, EBs were cultured in N2/B27 medium. After the preconditioning step, EBs for endoderm and mesoderm differentiation were transferred to 0.1% gelatin-coated plastic chamber slides and cultured in differentiation medium and differentiation medium plus acid ascorbic (0.5 mM), respectively, for 2 weeks. For the ectoderm differentiation, EBs were co-cultured with the stromal cell line PA6 in the presence of N2/B27 medium for 2 weeks. The medium for each condition was changed every other day.

Teratoma Formation Assays

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. Severe combined immunodeficient beige mice (Charles River Laboratories, Wilmington, MA, <http://www.crivier.com>) were anesthetized, and approximately 0.5×10^6 XF-iPS cells, resuspended in 20 ml of media, were injected into the testes. Mice were euthanized 8 weeks after cell injection, and tumors were processed and analyzed following conventional immunohistochemistry protocols (Masson's trichrome stain) and immunofluorescence.

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Figure 2. F44m and xeno-free human embryonic stem medium (XF-hESm) support long-term culture of undifferentiated human embryonic stem cell (hESC). The ES[2] hESC line cultured for 70 and 20 passages in F44m and XF-hESm, respectively, maintains expression of the transcription factors OCT4 ((A), (G)), SOX2 ((B), (H)), and NANOG ((C), (I)) and the surface markers SSEA3 ((A), (G)), SSEA4 ((B), (H)), TRA1-81 ((C), (I)), and TRA1-60 ((H)). Immunofluorescence analyses show expression of markers for neuroectoderm (TuJ1, green) ((D), (J)), endoderm (α -fetoprotein, green; FOXA2, red) ((E), (K)), and mesoderm (sarcomeric α -actinin, red) ((F), (L)). (M), Percentage of undifferentiated (SSEA4⁺/TRA1-60⁺) hESC (line ES[4]) detected in cultures maintained with hESm, F44m, or XF-hESm at the indicated passages. In all cases, hESC were plated onto feeder layers of irradiated human foreskin fibroblasts (HFFs) in gelatin-coated dishes. Scale bars, 250 μ m (A) and 100 μ m ((B)–(L)).

RESULTS

Development of Xeno-Free hESC/iPSC Culture Media

The original culture medium for the maintenance of undifferentiated hESC used 20% FBS as the main source of protein [38]. Most researchers have now replaced FBS with a more defined serum replacement, typically 20% KO-SR, which contains BSA as the main protein source (hES medium, or hESm). The concentration of KO-SR can be reduced by half if supplemented with the appropriate amount (0.5%) of

Figure 3. Establishment of a xeno-free primary culture of human foreskin fibroblasts (XF-HFF). (A): Passage three of XF-HFF showing typical fibroblast morphology. (B): Flow cytometry analyses of CD44 and CD90 expression in XF-HFF. Antibody-specific signals are shown in green, and the isotype control is shown in red. (C)–(F): Colonies of iPSC generated from XF-HFF under conventional, non-xeno-free conditions. Image pairs show pre- and post-alkaline phosphatase staining of a representative induced pluripotent stem cell (iPSC) colony obtained with three reprogramming factors (OCT4, SOX2, and KLF4; (C)–(D)), or four reprogramming factors (OCT4, SOX2, KLF4, and c-MYC; (E)–(F)). Scale bars, 250 μ m ((A), (C)–(F)).

purified human serum albumin (HSA) in a medium commonly referred to as HUES medium [39]. Since HSA is readily available as a highly standardized, clinical-grade product (Fig. 1A), our laboratory routinely uses HUES medium for the derivation and maintenance of hESC and iPSC lines [11, 34, 40]. Therefore, with the aim of removing all xenogenic components from hESC/iPSC culture medium, we first attempted to completely substitute HSA for KO-SR as the main protein source but failed to maintain undifferentiated hESC for more than two passages. Since it has been reported that human serum (HS) supports the derivation of hESC lines [27], we next tested the ability of a similar product, the supernatant of human plasma cryoprecipitate, to support hESC propagation, but again in this case, hESC colonies showed a strong tendency to differentiate and could not be maintained in culture for extended periods of time (Fig. 1B).

We next tested whether intermediate products in the process of industrial HSA fractionation could support the long-term growth and expansion of hESC. After replacing KO-SR with the relevant HSA fractionation product in a stepwise fashion for 1 month, only the media supplemented with 10% of Fraction I supernatant or with 10% of F44 supernatant maintained cultures with undifferentiated hESC colony morphology (Fig. 1C,1D), whereas those supplemented with the supernatants of Fraction IV1+IV4, or with HSA could not maintain undifferentiated hESC for more than two passages (Fig. 1E,1F).

Since the supernatants of Fraction I and F44 showed a similar ability to support undifferentiated hESC growth, with the latter being a further purification step still containing proteins involved in iron metabolism such as transferrin and ceruloplasmin (Table 1), known to support hESC cell growth [41], we selected the F44 supernatant-supplemented hESC culture medium (F44m) for further analyses. First, we tested whether F44m could support undifferentiated hESC culture for extended periods of time. After 70 passages or 16 months of culture in F44m, hESC (ES[2] line) remained undifferentiated and pluripotent, as judged by expression of the pluripotency-associated markers OCT4, NANOG, and SOX2, and surface markers SSEA3, SSEA4, TRA1-81 (Fig. 2A–2C), and

TRA1-60 (data not shown), and the ability to differentiate in vitro into derivatives of the three main embryonic germ layers (Fig. 2D–2F).

During the course of the experiments above, a new xeno-free KO-SR-based hESC culture medium (XF-hESm) became commercially available, which we tested along with F44m for comparison. At the time of writing, ES[2] hESC have been maintained for 20 passages with XF-hESm and retained undifferentiated morphology and expression of pluripotency-associated markers OCT4, NANOG, and SOX2, and surface markers SSEA3, TRA1-60, TRA1-81 (Fig. 2G–2I), and SSEA4 (data not shown), as well as their pluripotent in vitro differentiation ability (Fig. 2J–2L). Overall, these analyses could not detect any conspicuous differences in the ability of F44m and XF-hESm to support the propagation of undifferentiated hESC. As a quantitative assay of this ability, we kept feeder-free cultures of hESC (ES[4] line) in parallel with XF-hESm, F44m, or hESm for five passages, and analyzed by flow cytometry the percentage of undifferentiated cells, which co-expressed SSEA4 and TRA1-60, under each condition (Fig. 2M). The results of these analyses clearly indicate that both xeno-free culture media, F44m and XF-hESm, can support the undifferentiated growth of hESC to a similar extent and are also comparable to conventional KO-SR-based hESC culture media.

Establishment of Xeno-Free Primary Culture of Human Fibroblasts

Since commonly available primary human cell cultures are potentially xeno-contaminated as they were derived and/or maintained with media containing FBS, we next established a xeno-free primary culture of human foreskin fibroblasts (XF-HFF) from a 3-year old individual using human serum (Fig. 3A). XF-HFF cells displayed typical fibroblast morphology and grew robustly under xeno-free conditions in uncoated tissue culture plates with media containing human serum and recombinant trypsin for routine passaging. We also characterized the immunophenotype of XF-HFF cells, which displayed

a typical fibroblast pattern with high expression of the surface markers CD90 and CD44 [25] (Fig. 3B).

To test whether XF-HFF cells were amenable to induced reprogramming, we infected 100,000 cells with combinations of retroviral supernatants expressing four (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) or three (without *c-MYC*) reprogramming factors, and 2 days later, infected XF-HFF cells were plated onto feeder layers of irradiated human foreskin fibroblasts (HFF) and cultured with conventional hESm. Between 3 and 6 weeks after plating, colonies of tightly packed cells with a high nucleus-to-cytoplasm ratio and hESC-like morphology appeared in those cultures. We picked two colonies from each condition (infected with 3 or 4 factors), all of which expanded robustly in culture as colonies with hESC-like morphology, strong alkaline phosphatase staining, expression of pluripotency associated transcription factors and surface markers, and

pluripotent differentiation ability in vitro (Fig. 3C–3F and data not shown).

We next tested the ability of xeno-free hESC culture media (F44m and XF-hESm) to support reprogramming of XF-HFF cells, in comparison with that of conventional hESm. For this purpose, we infected XF-HFF cells with retroviral supernatants encoding the same four reprogramming factors as above, split equal numbers of infected cells onto feeder layers of irradiated HFFs in 10-cm dishes, and cultured each dish with a different medium. After 24 days, we scored the number of iPSC-like colonies formed under each condition by morphology and alkaline phosphatase staining and found that all three media supported iPSC generation to a similar extent (Fig. 4A). Moreover, we repeated the comparison using XF-HFF cells infected with retroviral supernatants expressing the four reprogramming factors and GFP linked by porcine teschovirus-1 2A (P2A) peptide sequences from a single polycistronic construct. Also in this case, all three media tested supported iPSC generation to a comparable degree, although we noticed that the use of XF-hESm resulted in faster growth of iPSC colonies (Fig. 4A). In similar experiments, we tested the ability of xeno-free media to support the reprogramming of fibroblasts from adult individuals. For this purpose, we used dermal fibroblasts obtained from skin biopsies of two healthy adults (aged 47 and 48). Also in this case, the numbers of iPSC-like, alkaline phosphatase-positive colonies were similar in dishes cultured with conventional hESm (9 and 15 colonies, respectively), F44m (12 and 10 colonies, respectively) and XF-hESm (7 and 12 colonies, respectively). Our results so far show that the xeno-free culture media F44m and XF-hESm are comparable to conventional hESC culture medium containing animal-derived products in their ability to support the propagation of undifferentiated hESC and the generation of iPSC. These results also suggest that

Figure 4. Generation of xeno-free induced pluripotent stem cell (iPSC) lines. (A): Alkaline phosphatase staining of a representative experiment comparing the ability of the indicated culture media to support reprogramming of xeno-free human foreskin fibroblast (XF-HFF) by retroviral transduction with four independent retroviruses expressing *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (4F), or a retrovirus expressing the same four factors and GFP from a polycistron (RV-OSKMG). The number of iPSC-like colonies scored by morphology and positive alkaline phosphatase staining is given for each condition as mean $\pm$ s.d. of three independent experiments. (B): Map of the retroviral construct pMXs-OSKMG expressing *Oct4*, *Sox2*, *Klf4*, and *GFP* linked by P2A peptide sequences from a polycistron that was used for the generation of xeno-free iPSC lines. ((C)–(D), (I)–(K)): Both iPS F44 #1 and iPS XF-hES #1 show expression of the transcription factors OCT4 ((C), (I)), SOX2 ((D), (J)), and NANOG ((E), (K)) and the surface markers SSEA3 ((C), (I)), SSEA4 ((D), (J)), and TRA1-81 ((E), (K)). ((F)–(H), (L)–(N)): Immunofluorescence analyses of in vitro differentiation assays demonstrate expression of markers for neuroectoderm (TuJ1, green) ((F), (L)), endoderm (α -fetoprotein, green; FOXA2, red) ((G), (M)), and mesoderm (α -smooth muscle actin, red (N); sarcomeric α -actinin, red, and GATA4, green (H)). ((O)–(Q)): Injection of iPS F44 #1 and iPS XF-hES #1 cells into the testes of immunocompromised mice results in the formation of teratomas containing structures that represent the three main embryonic germ layers: ectoderm derivatives ((I), (R)) including structures that stain positive for neuroectoderm markers (TuJ1, red); endoderm derivatives ((J), (S)), including glandular structures that stain positive for endoderm markers (α -fetoprotein, green; FOXA2, red); and mesoderm derivatives ((K), (T)), including structures that stain positive for muscle markers (α -sarcomeric actinin, red). All images are from the same tumor. Scale bars, 50 μ m ((I)–(K)), 100 μ m ((C), (D), (F)–(H), (M), (N), (P)–(T)), and 250 μ m ((E), (L), (O)).

induced reprogramming of human fibroblasts may be feasible under xeno-free conditions.

Generation of Xeno-Free Human iPSC Lines

We next designed a strategy for the generation of xeno-free iPSC lines based on the use of XF-HFF as source cells for reprogramming as well as autologous feeder cells for iPSC generation, and xeno-free hESC culture media F44m or hESm. To avoid the use of retroviral supernatants to transduce XF-HFF with the reprogramming factors, we pseudotyped retroviruses with vesicular stomatitis virus G (VSV-G), which allows for purification and concentration of retroviral particles. Moreover, since expression of the reprogramming factors from a single polycistronic construct resulted in iPSC generation from XF-HFF (Fig. 4A, see also ref [42]) and using a single retroviral preparation simplified the procedure, we decided to use purified and concentrated VSV-G-pseudotyped retroviral particles expressing the reprogramming factors from a polycistron. In this case, we constructed a polycistronic retrovirus expressing only three reprogramming factors (Oct4, Sox2, and Klf4) and GFP (pMXs-OSKG, Fig. 4B), since the use of c-Myc to generate iPSC has been associated to an elevated cancer risk [2, 12].

To attempt xeno-free iPSC generation, we infected XF-HFF with high-titer, purified retroviral particles expressing the OSKG polycistron. Two days later, infected cells were dissociated with recombinant trypsin, plated onto irradiated autologous XF-HFF cells in 10-cm plates coated with a xeno-free defined substrate (CELLstart), and cultured with F44m or XF-hESm. Both xeno-free culture media supported the reprogramming of XF-HFF cells to pluripotency, and iPSC-like colonies began appearing after 3–4 weeks. From 10^4 infected XF-HFF cells, we obtained 7 and 9 colonies of iPSC-like morphology in F44m and XF-hESm cultures, respectively. We picked 4 and 6 colonies from the F44m and XF-hESm conditions, respectively. All of them were easily propagated under xeno-free conditions by mechanical dissociation and replating onto irradiated XF-HFF feeder cells in CELLstart-coated plates. Two clones from each condition (clones iPS F44 #1 and #2, and iPS XF-hES #1 and #2) were selected for a more thorough characterization. All four clones presented the hallmarks of *bona fide* iPSC, including (1) typical hESC-like morphology and growth characteristics, (2) positive staining for alkaline phosphatase activity, (3) expression of pluripotency-associated markers (OCT4, NANOG, SOX2) and surface markers (SSEA3, SSEA4, TRA1-60, and TRA1-81), (4) the ability to differentiate in vitro into cell derivatives of the three main embryonic germ layers, and (5) the formation of complex teratomas upon injection into immunodeficient mice comprising ectoderm-, mesoderm-, and endoderm-derived structures (Fig. 4C–4T, supporting information Fig. 1, and data not shown). Moreover, following specific in vitro differentiation protocols, iPS XF-hES #1 and iPS F44 #1 cell lines gave rise to specialized mesoderm-derived cell types such as rhythmically beating cardiomyocytes (supporting information movies 1 and 2).

The four xeno-free iPSC lines tested displayed a normal karyotype (46, XY) at passage 10 (supporting information Figs. 1 and 2) and were derived from XF-HFF, as demonstrated by identical DNA fingerprint and HLA type (supporting information Table 1). In all lines tested, expression of the reprogramming factors driven by the transgenic polycistron was reduced to low or undetectable levels, as measured by quantitative RT-PCR analysis of transgene-derived *Klf4* mRNA (Fig. 5A). Furthermore, all the xeno-free iPSC lines tested showed re-activation of endogenous *OCT4* and *SOX2* expression, as well as of other pluripotency-associated transcription factors such as *NANOG* and *CRIP1* (Fig. 5A).

Figure 5. Molecular characterization of xeno-free induced pluripotent stem cell (iPSC) lines. (A): Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses of the expression levels of retroviral-derived reprogramming factors (Transgenic) and of endogenous expression levels of reprogramming factors and pluripotency-associated transcription factors in xeno-free human foreskin fibroblast (XF-HFF) and iPS XF-human embryonic stem (XF-hES) #1 and #2 and iPS F44 #1 and #2 cell lines. The expression levels of the indicated transcripts are given relative to GAPDH expression. The expression levels of the retroviral polycistron were measured using primers specific for the transgenic *Klf4*. Note the different scales of endogenous and transgenic transcripts levels. (B): Schematic representation of the sequences inserted by pMXs-OSKG into the host genome, indicating the location of BamHI sites and the GFP probe used for Southern hybridization analyses. (C): Southern hybridization analysis of BamHI-digested genomic DNA from XF-HFF and the indicated xeno-free iPSC lines using a GFP probe. Asterisks mark integration events. (D): Bisulphite genomic sequencing of the *OCT4* promoter showing demethylation in iPS XF-hES #1 and #2 and iPS F44 #1 and #2, compared to parental XF-HFF cells. Open and closed circles represent unmethylated and methylated CpGs, respectively, at the indicated promoter positions.

Consistent with this, the promoter of *OCT4*, heavily methylated in XF-HFF cells, was demethylated in xeno-free iPSC lines, indicating epigenetic reprogramming to pluripotency (Fig. 5D). We finally tested whether the different xeno-free iPSC lines obtained represent independent reprogramming events by Southern hybridization analysis with a GFP-specific probe (Fig. 5B). The pattern of retroviral integrations in the four xeno-free iPSC lines tested demonstrates that they are, indeed, independent and contain a total of 1–5 retroviral

integrations (Fig. 5C). The fully characterized xeno-free iPSC lines generated in these studies have been registered and deposited at the Spanish National Stem Cell Bank for the purpose of distribution to interested researchers

DISCUSSION

The results of our studies show, for the first time to our knowledge, that human iPSC lines can be generated under xeno-free conditions. For this purpose, we first had to identify xeno-free culture media that supported iPSC generation. Several animal product-free culture media have been reported in the past to support the derivation and/or maintenance of hESC, but their performance tends to be lower than that of KO-SR-based media [28]. Similarly, whole human serum has been used to derive and maintain xeno-free hESC lines [27], but the use of human serum would be complicated by availability and batch-to-batch variation issues. Instead, we focused on the process of industrial HSA production. Here, human plasma is subjected to a very well established industrial process of cold-ethanol fractionation, which has remained essentially unchanged for more than 60 years [32]. Moreover, the products of this industrial process show extremely low interbatch variation, as pools of sera from thousands of individuals are used for each batch and are manufactured under strict GMP conditions. The source material for this process is the crude supernatant of plasma cryoprecipitation, which we have shown cannot support the propagation of undifferentiated hESC. We reasoned that differentiation-promoting activities might be present in this material that could be removed in subsequent fractionation steps. Similarly, the final products of industrial HSA production, HSA itself and the supernatant of Fraction IV1+IV4, failed to maintain hESC cultures, indicating that factors essential for the propagation of undifferentiated hESC (growth factors, inhibitors of differentiation, or both) had been removed. However, we identified two intermediate products, the supernatants of Fraction I and F44, which could completely replace KO-SR for the long-term maintenance of undifferentiated hESC. Future analyses of components present in Fraction I and Fraction IV1+IV4 are likely to provide valuable information as to the specific factors responsible for this effect.

In addition to F44m, we have also shown that XF-hESm, recently made commercially available, can support the maintenance of undifferentiated hESC/iPSC and the reprogramming of human fibroblasts to pluripotency. In this way, two different GMP-compliant culture media supplements are available for the generation of xeno-free iPSC lines. In our studies, we generated such lines by reprogramming XF-HFF, which

were also used as feeder cells in our cultures. Whereasthis is certainly not a requirement and allogeneic cells (as long as they were generated and maintained under xeno-free conditions) could also be used as feeders, we consider the use of autologous feeders to be an advantage as it simplifies and streamlines the protocol of xeno-free iPSC generation. Finally, we have shown that *bona fide* iPSC lines can be generated under xeno-free conditions using three reprogramming factors. We chose to use three factors, at the expense of a much lower reprogramming efficiency, because the integration of c-Myc transgenes has been associated with high-risk of cancerogenesis from iPSC [2, 12]. Moreover, the use of polycistronic retroviruses resulted in iPSC lines with as few as one integration event, which should considerably reduce the cancer risk due to insertional mutagenesis. However, integration-free reprogramming strategies [15–18] would clearly be preferable for the clinical application of iPSC. The results of our studies should facilitate the safe clinical translation of iPSC-based therapies, as they set the bases for xeno-free human iPSC generation and provide a path to GMP applicability of iPSC technology.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- 1 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;126:663–676.
- 2 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;448:313–317.
- 3 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.
- 4 Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;318:1917–1920.
- 5 Park IH, Zhao R, West JA et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008;451:141–146.
- 6 Lowry WE, Richter L, Yachechko R et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A* 2008;105:2883–2888.
- 7 Park IH, Arora N, Huo H et al. Disease-specific induced pluripotent stem cells. *Cell* 2008;134:877–886.
- 8 Dimos JT, Rodolfa KT, Niakan KK et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 2008;321:1218–1221.
- 9 Ebert AD, Yu J, Rose FF, Jr. et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 2009;457:277–280.
- 10 Soldner F, Hockemeyer D, Beard C et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 2009;136:964–977.
- 11 Raya A, Rodríguez-Piza I, Guenechea G et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* 2009;460:53–59.

- 12 Nakagawa M, Koyanagi M, Tanabe K et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 2008;26:101–106.
- 13 Kaji K, Norrby K, Poca A et al. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009;458:771–775.
- 14 Woltjen K, Michael IP, Mohseni P et al. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 2009;458:766–770.
- 15 Stadtfeld M, Nagaya M, Utikal J et al. Induced pluripotent stem cells generated without viral integration. *Science* 2008;322:945–949.
- 16 Okita K, Nakagawa M, Hyenjong H et al. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008;322:949–953.
- 17 Gonzalez F, Barragan Monasterio M, Tiscornia G et al. Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci U S A* 2009;106:8918–8922.
- 18 Kim D, Kim CH, Moon JI et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 2009;4:472–476.
- 19 Ahrlund-Richter L, De Luca M, Marshak DR et al. Isolation and production of cells suitable for human therapy: Challenges ahead. *Cell Stem Cell* 2009;4:20–26.
- 20 De Sousa PA, Galea G, Turner M. The road to providing human embryo stem cells for therapeutic use: The UK experience. *Reproduction* 2006;132:681–689.
- 21 Martin MJ, Muotri A, Gage F et al. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 2005;11:228–232.
- 22 Unger C, Skottman H, Blomberg P et al. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum Mol Genet* 2008;17:R48–53.
- 23 Hovatta O, Mikkola M, Gertow K et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum Reprod* 2003;18:1404–1409.
- 24 Inzunza J, Gertow K, Stromberg MA et al. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. *Stem Cells* 2005;23:544–549.
- 25 Stojkovic P, Lako M, Stewart R et al. An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells* 2005;23:306–314.
- 26 Li Y, Powell S, Brunette E et al. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol Bioeng* 2005;91:688–698.
- 27 Ellerström C, Strehl R, Moya K et al. Derivation of a xeno-free human embryonic stem cell line. *Stem Cells* 2006;24:2170–2176.
- 28 Rajala K, Hakala H, Panula S et al. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. *Hum Reprod* 2007;22:1231–1238.
- 29 Meng G, Liu S, Krawetz R et al. A novel method for generating xeno-free human feeder cells for human embryonic stem cell culture. *Stem Cells Dev* 2008;17:413–422.
- 30 Swistowski A, Peng J, Han Y et al. Xeno-free defined conditions for culture of human embryonic stem cells, neural stem cells and dopaminergic neurons derived from them. *Plos One* 2009;4:e6233.
- 31 Unger C, Gao S, Cohen M et al. Immortalized human skin fibroblast feeder cells support growth and maintenance of both human embryonic and induced pluripotent stem cells. *Hum Reprod* 2009;24:2567–2581.
- 32 Cohn EJ, Strong LE, Hughes WL, Jr. et al. Preparation And Properties Of Serum and Plasma Proteins Iv A System For The Separation Into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. *J Am Chem Soc* 1946;68:459–475.
- 33 José M, Gajardo R, Jorquera JI. Stability of HCV, HIV-1 And Hbv Nucleic Acids In Plasma Samples Under long-term Storage. *Biologicals* 2005;33:9–16.
- 34 Aasen T, Raya A, Barrero MJ et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol* 2008;26:1276–1284.
- 35 Stewart SA, Dykxhoorn DM, Palliser D et al. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 2003;9:493–501.
- 36 Follenzi A, Ailles LE, Bakovic S et al. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 2000;25:217–222.
- 37 Freberg CT, Dahl JA, Timoskainen S et al. Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. *Mol Biol Cell* 2007;18:1543–1553.
- 38 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–1147.
- 39 Cowan CA, Klimanskaya I, McMahon J et al. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004;350:1353–1356.
- 40 Raya A, Rodriguez-Piza I, Aran B et al. Generation of cardiomyocytes from new human embryonic stem cell lines derived from poor-quality blastocysts. *Cold Spring Harb Symp Quant Biol* 2008;73:127–135.
- 41 Lu J, Hou R, Booth CJ et al. Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci U S A* 2006;103:5688–5693.
- 42 Carey BW, Markoulaki S, Hanna J et al. Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A* 2009;106:157–162.

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