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Generation of induced pluripotent stem cells from virus-free *in vivo* reprogramming of BALB/c mouse liver cells

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ABSTRACT

The *in vivo* cell reprogramming of terminally differentiated somatic cells to a pluripotent state by the ectopic expression of defined transcription factors has been previously shown in the BALB/c mouse liver upon plasmid DNA injection with no teratoma formation in the host tissue. Here, we hypothesized that the reprogrammed cells could be extracted from the tissue and cultured *in vitro*. We called these cells *in vivo* induced pluripotent stem (i^2PS) cells because they showed pluripotent characteristics equivalent to a standard mouse ES cell line (E14TG2A). The pluripotent character of i^2PS cells was determined by a battery of morphological, molecular and functional assays, including their contribution to adult tissues of chimeric mice upon blastocyst injection. These observations further confirm that terminally differentiated somatic cells in wild type, adult animals can be reprogrammed *in vivo* using virus-free methodologies. The reprogrammed cells can generate *in vitro* stem cell colonies that exhibit pluripotency similar to ES cells with numerous implications for the application of *in vivo* reprogramming for tissue regenerative purposes.

1. Introduction

Induced pluripotent stem (iPS) cells with properties similar to those of embryonic stem (ES) cells were first generated *in vitro* from mouse embryonic and adult fibroblasts by the ectopic expression of four transcription factors, Oct3/4, Klf4, Sox2, and cMyc, which are also known as Yamanaka or OKSM factors [1]. After this initial discovery, transcription factor-mediated reprogramming technology was shown to be applicable also to human fibroblasts [2,3] as well as to other mouse and human cell types [4,5]. These findings have opened new ways of cell reprogramming toward the pluripotent state and are posed to resolve many of the immunological and ethical concerns raised by the generation and use of human ES cells. However, the limited efficiency of the reprogramming methodologies along with the safety of the iPS cells generated still question the clinical translation of this approach. Concerns on the risk of insertional mutagenesis due to integrating vectors and tumorigenesis mediated by the reactivation of cMyc have motivated the search for different *in vitro* techniques to generate iPS cells by avoiding these drawbacks [6]. Also, reports on spontaneous teratoma formation upon subcutaneous injection of the *in vitro* generated iPS cells, while reaffirming the pluripotency of the cells, have raised concerns on the safety of the iPS-derived cells [4,7].

An alternative approach developed to overcome some of these issues involves transdifferentiation strategies that reprogram cells directly from one type to another, without the intermediate pluripotent state [8,9]. Such transdifferentiation strategies, also termed 'direct reprogramming', have been reported for different tissues *in vivo*. *In vivo* transdifferentiation avoids the possible aberrations that can be triggered by the maintenance of the cells *in vitro*, as well as benefits from the influence of the *in vivo* microenvironment to help direct differentiation into the desired cell type. However, this approach still relies strongly on the usage of

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viral vectors for the delivery of the reprogramming factors and is quite limited on the type and number of cells that can be reprogrammed [10–17].

Our laboratory has recently reported that virus-free ectopic expression of the OKSM factors *in vivo* can transcriptionally reprogram terminally differentiated somatic cells in their *in vivo* environment [18]. Reprogramming of BALB/c mouse liver cells was rapid, efficient and transient [19]. Cell clusters staining positive for pluripotency markers (*e.g.* NANOG) were observed in the liver 4 days after hydrodynamic tail vein (HTV) injection of the reprogramming plasmids with no manifestation of carcinogenesis or teratoma formation detected up to three months. In the present study, we aimed to isolate, culture, and characterize the in vivo reprogrammed cells from the total hepatocyte population extracted, as further proof that cell reprogramming was indeed taking place in the animal liver. We hypothesized that reprogrammed cells extracted from their *in vivo* microenvironment (liver in this case) could be cultured under standard mouse ES (mES) conditions and possess ES cell-like characteristics such as self-renewal, proliferation in colonies and potential to differentiate into cells from all three different developmental lineages (i.e. pluripotency). We provide morphological, molecular and functional evidence of the pluripotent character of these cells, which were named *in vivo* induced pluripotent stem (i^2PS) cells in reference to their origin. The ability of i^2PS cells to form teratomas upon subcutaneous injection in nude mice contrasted with the absence of *in situ* tumorigenesis following *in vivo* reprogramming observed in our previous study [18], which further highlights the critical role of the tissue microenvironment in the control of pluripotency and differentiation.

2. Materials and methods

2.1. Plasmids

Reprogramming plasmids pCX-OKS-2A encoding *OCT3/4, KLF4, SOX2* and pCX-cMyc encoding *CMYC* (as previously described by Okita et al. [20]) were obtained from Addgene (USA) as bacterial stabs. Research grade plasmid production was performed at Plasmid Factory, Germany.

2.2. Hydrodynamic tail vein (HTV) injection of plasmid DNA

All experiments were performed with prior approval from the UK Home Office (PPL 80/2296). Female BALB/c mice, 6 weeks old, (4 mice/group) were purchased from Harlan, UK. Mice were allowed one week to acclimatize prior to use. Mice were warmed in a 37 °C heating chamber, anesthetized with isofluorane and injected via tail vein in 5–7 s with 1.5 ml of 0.9% saline solution including 75 μg of pCX-OKS-2A and 75 μg of pCX-cMyc plasmids or no plasmid, as previously described [18,19]. Mice were culled 2 days after HTV injection.

2.3. Isolation of hepatocyte cell population

Mice livers were perfused as previously described [21,22] with some modifications. In brief, livers were first perfused with Ca^{2+} and Mg^{2+} free HBSS (Sigma–Aldrich, UK) and then with Liver Digest Medium (Gibco Life technologies, UK) at 37 °C. After digestion, liver was washed with Hepatocyte Wash Medium (HWM, Gibco Life technologies, UK) at 4°C and cell suspension was passed through a 100 μm cell strainer. Cells were centrifuged at 50 g for 5 min to separate parenchymal cells (including hepatocytes) which were collected in pellet and non-parenchymal cells (including Kupffer and epithelial cells) which stayed in the supernatant. The hepatocyte fraction was collected after washing twice with HWM.

2.4. Cell culture of cells isolated from mouse liver

Liver cells (hepatocyte fraction) were isolated as described above. The final pellet was re-suspended in 5 ml of HWM and left for 15 min on ice to let cells settle down at the bottom of the tube. After discarding the supernatant which contained mainly cells debris, around 1.5–2 ml of cell suspension (loose pellet) could be collected. Hepatocytes from this cell suspension were then enumerated and seeded (2×10^6 cells per well) on 6-well plates coated with Matrigel basement membrane matrix, growth factor reduced (BD biosciences; UK). The culture of liver cells extract on Matrigel was maintained for 2 or 8 days in DMEM/LIF medium [DMEM medium (Gibco Life technologies, UK) supplemented with 15% of heat-inactivated fetal bovine serum (Gibco Life technologies, UK), 1% non-essential aminoacid (Hyclone, ES qualified, ThermoScientific, UK), 1% penicillin-streptomycine (Gibco Life technologies, UK), 50 μM 2-mercaptoethanol (Gibco Life technologies, UK), and 10 ng/ml of mouse leukemia inhibitor factor (mLIF, e-Biosciences, UK)]. Alternatively, extracted cells were also seeded (300,000 hepatocytes for a 35 mm dish) on a mouse embryonic fibroblast feeder layer (MEF, Invitrogen-Life technologies, UK) prepared 48 h before the liver cell isolation as described by the manufacturer. MEF cells were seeded (250,000 cells for a 35 mm dish) on dishes previously coated (2 h, 37 °C) with sterile pig gelatin (0.1% in PBS, pH 7.3, Sigma–Aldrich, UK). The co-culture of primary liver cells extract on MEF cells was maintained for 10 days in DMEM/LIF medium, as described above. Evolution of the cell culture was monitored daily by optical microscopy. The cell culture medium was also refreshed on a daily basis. Primary cell cultures were split (1/2) on fresh MEF feeder cells after 10 days and monitored for another 5 days period with daily fresh medium change. Dome shaped cell colonies of *in vivo* induced pluripotent stem (i^2PS) cells were fixed for staining 48 h after appearing. Co-culture of i^2PS cells on MEF cells (mixed i^2PS-MEF) were frozen in 5% DMSO, 50% FBS, completed with full cell maintenance medium (described above), 48 or 72 h after appearance of colonies for further experiments.

2.5. Cell culture of mouse embryonic stem (mES) cells

The mouse embryonic stem cell line mES-E14TG2A (#CRL-1821, ATCC, USA) (referred as mES) was used as a reference for the characterization of the i^2PS cells. mES cells were seeded (300,000 for a 35 mm dish) on MEF feeder layers prepared as described above, following ATCC recommendations for culture of these cells (DMEM/mLIF medium). The cell culture medium was refreshed daily.

2.6. RNA isolation and reverse transcription-real time PCR (RT-qPCR) analysis

Total RNA was isolated with Nucleospin RNA II kit (Macherey–Nagel, UK). cDNA synthesis from 1 μg of RNA sample was performed using iScript cDNA synthesis kit (Bio-Rad, UK) according to manufacturer's instructions. 2 μl of each cDNA sample were used to perform RT-qPCR reactions with iO SYBR Green Supermix (Bio-Rad, UK). Primer sequences are shown in Supplementary Table 1. Samples were run on CFX-96 Real Time System (Bio-Rad, UK) with the following protocol: 95 °C for 3 min, 1 cycle; 95 °C for 10 s, 60 °C for 30 s, – repeated for 40 cycles. *β-actin* was used as a housekeeping gene and gene expression levels were normalized to different controls according to each particular experiment.

2.7. Global gene expression analysis by DNA microarray

Total RNA was isolated from feeder-free i^2PS and E14TG2a cell cultures using Nucleospin RNA II kit (Macherey–Nagel, UK) and 1 μg of the extracted RNA was used for microarray analysis. This experiment was performed at the Genome Centre (Queen Mary University, London, UK) using a Mouse WG-6 v2.0 Expression Bead Chip (Illumina) that allowed the profiling of 45,200 transcripts. Three biological replicates of each cell type were included in the study and results were analyzed using GenomeStudio software and NCBI database for pathway analysis. The data discussed in this publication is deposited in the NCBI's Gene Expression Omnibus (GEO), accession number GSE55996 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55996).

2.8. Embryoid body (EB) formation and differentiation

Feeder-free i^2PS cell cultures maintained under standard mES cell culture conditions (DMEM/LIF medium, as described above) were used to form embryoid bodies (EBs) as previously described [12,23]. In brief, the cells were detached from the tissue culture vessel with 0.05% trypsin-EDTA and suspended in EB medium [DMEM, FBS (20%), NEAAs (1%), 2-mercaptoethanol (0.1%), penicillin (50 U/ml) and streptomycin (50 μg/ml)]. 10,000 cells were seeded in each well of a 1% agar-coated 96-well plate. After 3 days, the aggregated cells forming EBs were transferred to 0.1% gelatin-coated tissue culture dishes and maintained in the same culture medium. The EBs then attached to the bottom of the dishes and differentiating cells started to spread out of the necrotic cores. A batch of the differentiated cells was fixed after 7 days in culture for immunostaining of differentiation markers as described below. Another batch was left to differentiate for a total of 15 days and the relative gene expression of differentiation markers was analyzed by RT-qPCR. The gene expression levels were normalized against those of the starting i^2PS cells used to form the EBs.

2.9. CDy1 live staining

The compound of designation yellow 1(CDy1) used to detect live mES cells and live i^2PS cells [24] was kindly provided by Dr. Young-Tae Chang (National University of Singapore and Agency for Science, Technology and Research (A*STAR) of Singapore), and used according to instructions provided. Briefly, the CDy1 stock solution (10 mM in DMSO) was first diluted to a final concentration of 100 μM in PBS 1× and then diluted to 0.1 μM in the cell culture medium of the cells to stain (standard mES cell culture conditions, DMEM/LIF medium). After 1 h incubation at 37 °C, 5% CO_2, cells were washed three times with full medium, and then incubated for 2 h at 37 °C before live imaging under epi-fluorescence microscope (Zeiss Axio Observer).

2.10. Alkaline phosphatase (ALP) staining

ALP activity staining was performed on mES and i^2PS cell colonies using the BCIP/NBT liquid substrate system (Sigma, UK). Methanol-fixed cell cultures were

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first washed with 25 mM HEPES buffered solution and then incubated at 37 °C for 30 min with the BCIP/NBT liquid substrate system. Color development was stopped by rinsing with distilled water.

2.11. Immuno-cytochemistry (ICC) of cell cultures

Cultures of mES, i^{2}PS cells or cells differentiated from the EB were fixed with methanol, pre-cooled at −20 °C, for 10 min, then air-dried for 15 min and finally washed twice with PBS, 5 min each. Cells were then incubated for 1 h in blocking buffer (5% goat serum-0.1% triton in PBS pH 7.3) at room temperature, followed by two washing steps in PBS-BSA solution (1% BSA-0.1% triton, pH 7.3) before overnight incubation at +4 °C with the different primary antibodies [rabbit polyclonal anti-OCT4 (ab19857, 3 μg/ml, Abcam, UK)/rabbit polyclonal anti-SOX2 (ab97959, 1 μg/ml, Abcam, UK)/rabbit polyclonal anti-NANOG (ab80892, 1 μg/ml, Abcam, UK)/mouse monoclonal anti-SSEA1 (ab16285, 20 μg/ml, Abcam, UK)/rabbit polyclonal anti-beta III tubulin (ab76287, 1/200, Abcam, UK)/rabbit polyclonal anti-α-fetoprotein (N1501, ready to use, DAKO, UK)/mouse monoclonal anti-α-smooth muscle actin (N1584, ready to use, DAKO, UK)]. The next day, cell cultures were washed three times with PBS-BSA solution (2 min each) and incubated 1.5 h at room temperature with secondary antibodies (either goat polyclonal anti-rabbit IgG labeled with Cy3 for OCT4/SOX2/NANOG/β-III tubulin/α-fetoprotein or goat polyclonal anti-mouse IgG labeled with Cy3 for SSEA1/α-smooth muscle actin, 1/250 dilution, Jackson ImmunoResearch Laboratories Inc.). Finally, the cultures were washed three times with PBS-BSA solution and mounted in DAPI − antifading agent containing medium (Vectashield mounting medium, Vector Laboratoriesn, UK). Stained cell cultures were visualized under epi-fluorescence microscope (Zeiss Axio Observer).

2.12. Teratoma formation assay

Primary hepatocytes were extracted and isolated by liver perfusion on day 2 after HTV injection with OKSM plasmids or 0.9% saline as described above and 2×10^6 cells were suspended in 1 ml of DMEM media, followed directly by subcutaneous injection in the dorsal flanks of nude female CD1 mice, 6 weeks old, purchased from Charles River, UK. Feeder-free i^{2}PS and mES cells cultured on 0.1% gelatin and standard mES cell culture conditions (DMEM/LIF medium) were injected in the same conditions. Mice were anesthetized with isofluorane and were subcutaneously injected in dorsal flank with 200 μl of cell suspension. After 5 weeks, tumors were dissected and fixed in 10% buffered formaldehyde. Paraffin-embedded sections were stained with H&E. Images were captured by light microscopy (×10).

2.13. Chimera generation and genotyping

i^{2}PS cells were cultured on MEF feeder layers under standard mES cell conditions, as described above. Upon trypsinization, i^{2}PS cells were separated from feeders and 15–20 of them were injected into 3.5 dpc blastocysts from C57BL/6 background. Blastocysts were then surgically transferred to synchronized pseudo-pregnant CD1 surrogate mothers. Genotyping for the Major Hystocompatibility (MHC) Class I antigens was performed to assess the chimerism of the obtained pups. Primer sequences were designed to differentiate between C57BL/6 H2-Kb and BALB/c H2-Kd, those used in this study are listed in Table S1 and the PCR conditions were: 94 °C for 3 min, 1 cycle; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s– repeated for 32 cycles, 72 °C for 10 min.

2.14. Statistical analysis

Statistical significance of RT-qPCR data was assessed by one-way ANOVA and Tukey's post hoc test. Bonferroni correction was applied to microarray data.

3. Results

The kinetics of *in vivo* cell reprogramming following HTV injection with plasmid DNA encoding for the OKSM factors have been reported [18] to show that pluripotency within liver is enhanced between 2 and 4 days post-injection. Here, 2 days after HTV injection with reprogramming plasmids (pCX-OKS-2A and pCX-cMyc) or 0.9% saline (negative control), primary hepatocytes were isolated from the liver tissue of BALB/c mice and cultured on either Matrigel-coated plates or a mouse embryonic fibroblast (MEF) feeder layer under standard mES cell culture conditions (Fig. 1a). Gene expression analysis of these cells performed by RT-qPCR revealed an approximately 10-fold higher expression of key pluripotency genes after 1 day in culture of the OKSM group compared to the saline group. Pluripotency markers were further upregulated (between 100 and 1000 times) 8 days after the isolation and start of culture (Fig. 1b). After 12 days in culture, distinct compact and dome shaped cell colonies were formed only from hepatocyte extracts of the OKSM plasmid-injected animals under both culture conditions (on Matrigel and MEFs). The domed shape and refractive edges of the colonies, characteristic of pluripotent cell cultures, were better maintained under the MEFs conditions and hence it was decided to use this support for further culturing (Fig. 1c). These colonies, which were morphologically very similar to those obtained from a standard mES cell line (E14TGa2) (Fig. S1a), were named *in vivo* induced pluripotent stem (i^{2}PS) cells and were further characterized by staining with a series of pluripotency markers. i^{2}PS cell colonies on MEF feeders stained positively for alkaline phosphatase (ALP) activity and the live pluripotent cell-specific dye CDy1 [24], as detected by optical microscopy (Fig. 1d). Various pluripotency markers stained the cultures immunohistochemically (OCT3/4, SOX2, NANOG, SSEA-1) (Fig. 1e). Identical staining patterns were obtained between the control mES cell line cultured on MEFs (Fig. S1b) and the i^{2}PS cells generated.

Once the i^{2}PS cells had been established *in vitro*, the next step was to investigate their gene expression profile in comparison to that of the reference mES cell line (E14TG2a). Feeder-free cell cultures were used to avoid contamination with MEF that would affect the results. First, the relative gene expression of key pluripotency and early differentiation markers was studied by RT-qPCR. Compared to mES cells, i^{2}PS cells showed a moderate but significant upregulation in the expression levels of pluripotency-related genes such as *Nanog, Ecat1, Rex1, Cripto*, endo-*Oct3/4*, endo-*Sox2* and endo-*Klf4* (Fig. 2a). When early differentiation markers representative of each of the germ layers were investigated, an upregulation of *Afp* (endoderm) was observed in the i^{2}PS cells, whereas the expression levels of *T* (mesoderm) and *Fgf-5* (ectoderm) were significantly lower than in E14TG2a cells (Fig. 2b).

The global gene expression profile of i^{2}PS and E14TG2a cells was then compared in a 45,200-probe microarray analysis (accession number in GEO database GSE55996). A scatter plot representing the expression levels of all the probes in the two cell types is illustrated in Fig. 2c. Key pluripotency genes such as *Nanog*, *Rex1* and *Ecat1* are highlighted and showed a very similar expression in i^{2}PS and E14TG2a cells. Moreover, when 274 genes known to participate in the induction, maintenance, amelioration and loss of pluripotency [25] were clustered and studied together, no noteworthy differences in the expression profiles were observed between the two cell types (Fig. 2d). Similarly, no remarkable differences in gene expression were detected among genes involved in endoderm (Fig. S2a), mesoderm (Fig. S2b) or ectoderm development (Fig. S2c). Finally, ten genes that are characteristically upregulated at different stages of hepatocyte differentiation, from the early endoderm to the adult liver, were investigated. All these genes were expressed at background levels both in i^{2}PS and mES cells (Fig. 2e). Overall, these results indicated the pluripotent character of i^{2}PS cells at the molecular level.

Pluripotency is routinely assessed *in vitro* through the formation of cell aggregates known as embryoid bodies (EBs) that resemble the gastrulating embryo. For the cells to be considered functionally pluripotent, the generated EBs must contain cells differentiating into all three lineages of development [23,26,27]. i^{2}PS cells were seeded on non-adherent surfaces (*i.e.* agar-coated culture dishes) and LIF was removed from the cell culture medium to allow the formation of EBs. Loose aggregates started to form only 1 day after seeding and evolved into more compact spheroids after 3 days. The floating EBs were then transferred onto gelatin-coated dishes and, after attachment, cells started to spread out from the edges of the EBs and to differentiate. Immunostaining for differentiation markers characteristic of each of the three germ layers was performed on cells that were left to differentiate for 7 days. Anti α-fetoprotein antibody was used to detect differentiation into the endodermal lineage, anti-α-smooth muscle actin antibody for

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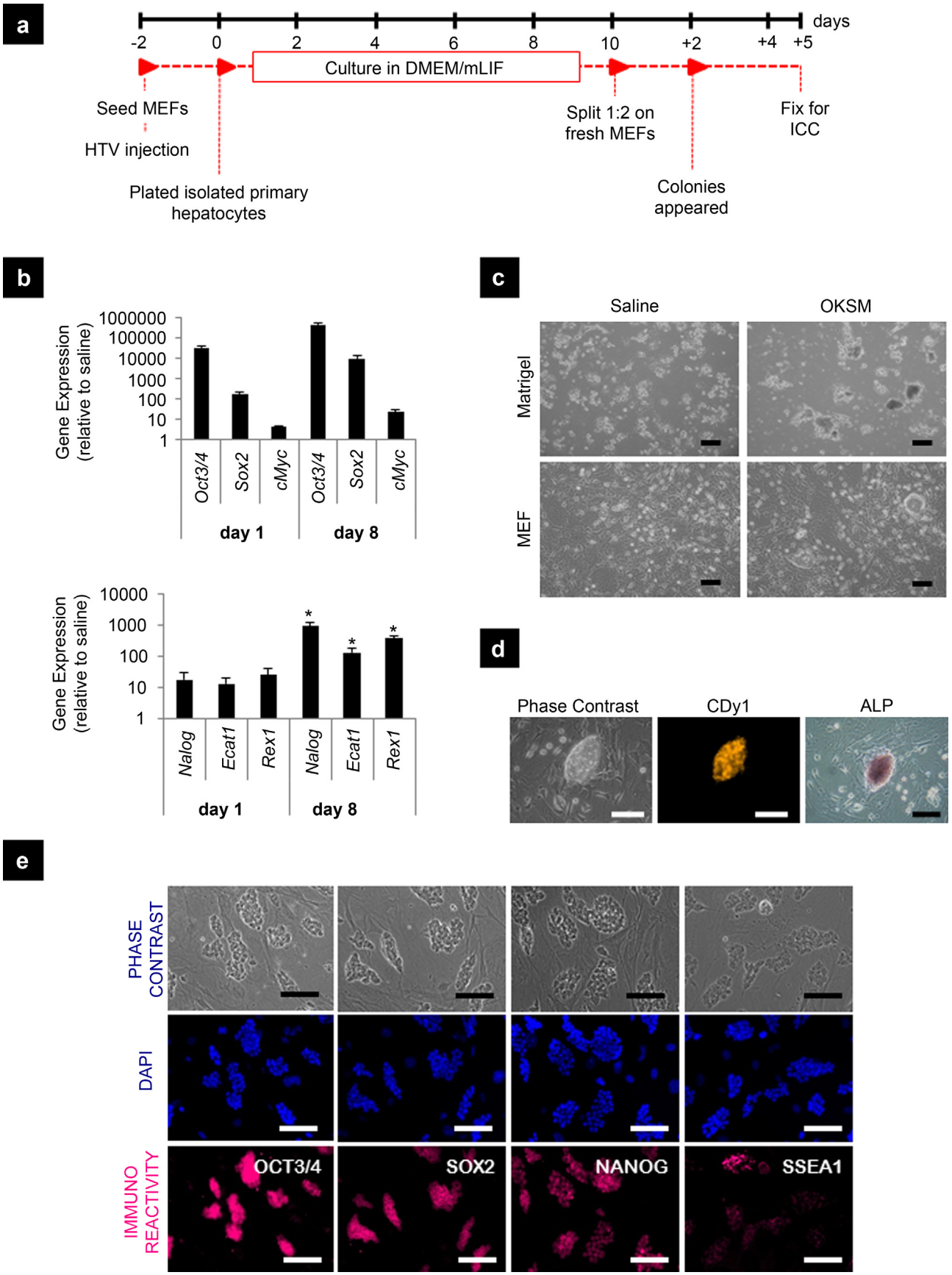

Fig. 1. Isolation and culture of i^{2}PS cells. (a) Protocol for the generation of i^{2}PS cells from *in vivo* reprogrammed hepatocytes. (b) Relative gene expression of transfected reprogramming factors and endogenous pluripotency markers (RT-qPCR, normalized to saline group, $N = 3$, $^*p < 0.05$ designates statistically significant differences between expression levels on days 1 and 8 using one-way ANOVA); (c) Cell cultures, day 12 after seeding (10×); (d) CDy1 live and ALP staining of the same i^{2}PS cell colony; (e) Immunostaining for OCT3/4, SOX2, NANOG and SSEA1; scale bars represent 200 μm.

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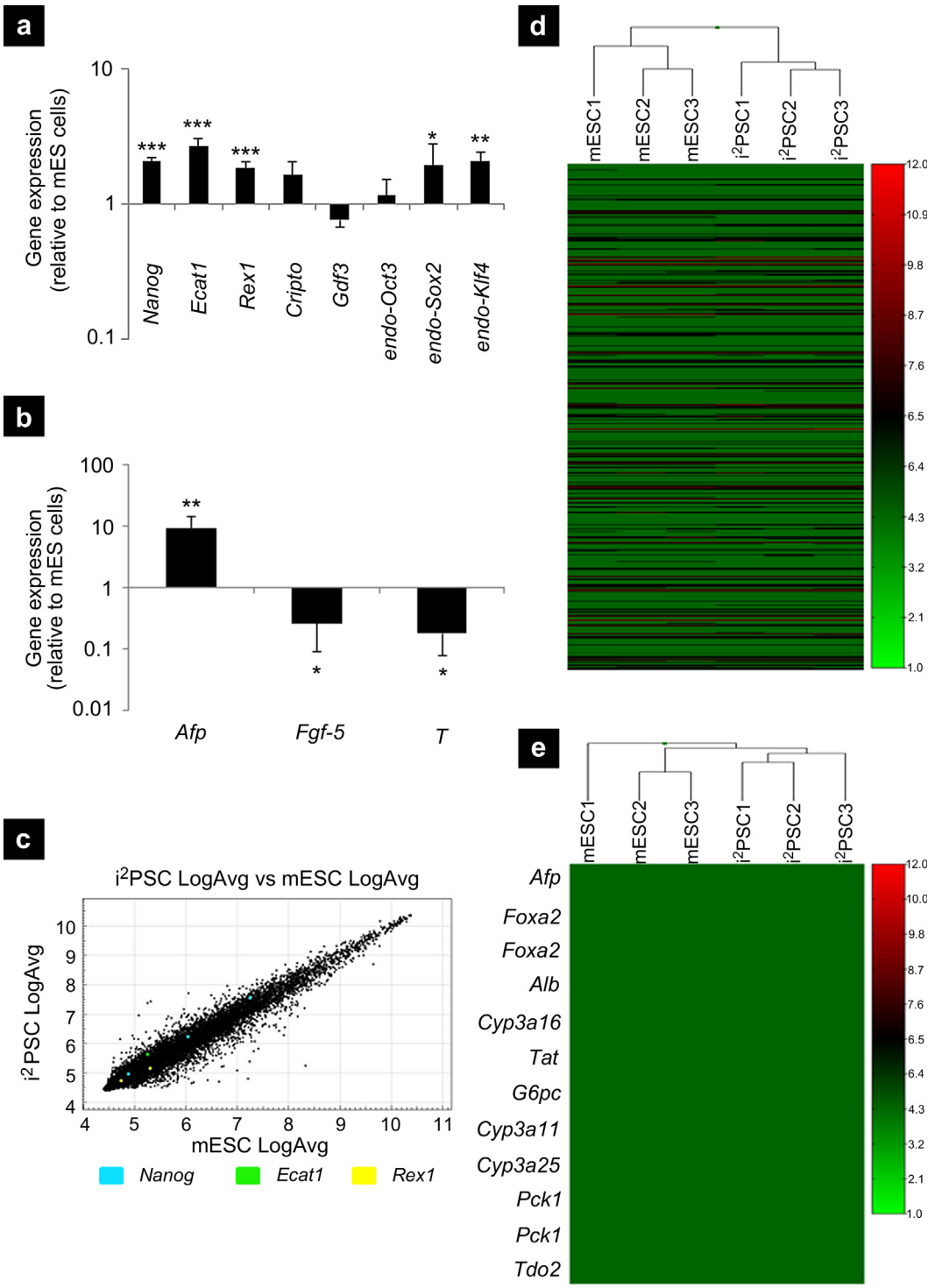

Fig. 2. Analysis of the gene expression profile of i^{2}PS cells. Relative gene expression of (a) pluripotency markers and (b) early differentiation markers (qRT-PCR, normalized to E14TG2a cells, $N = 3$, $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant differences in the expression levels between i^{2}PS and E14TG2a cells, obtained by one-way ANOVA). DNA Microarray analysis ($N = 3$) (c) Scatter plot shows the gene expression levels of 45,200 probes in i^{2}PS (*Y* axis) and E14TG2a (*X* axis) cells represented as the logarithm of the average signal intensity. Heatmaps comparing the expression of (d) 274 genes involved in the induction, maintenance, amelioration and loss of pluripotency and (e) 10 genes highly expressed at different stages of hepatocyte differentiation. (Microarray data can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55996).

mesoderm and anti β-III tubulin antibody for ectoderm. Positive cells for all three lineages were reproducibly found in different dishes (Fig. 3a).

These results were confirmed by assessing the relative gene expression levels of differentiation and pluripotency markers by RT-qPCR. The gene expression profile of cells that were left to differentiate from EBs for 15 days was compared to that of the starting i^{2}PS cells used to generate the EBs. Fibroblasts were also included as a control group (characteristic ectoderm committed cells). Fig. 3b shows the gene expression levels of *Afp* (endoderm), *T* (mesoderm) and *Fgf-5* (ectoderm). In the control fibroblast cells, the ectodermal marker *Fgf-5* was 100 fold upregulated compared to

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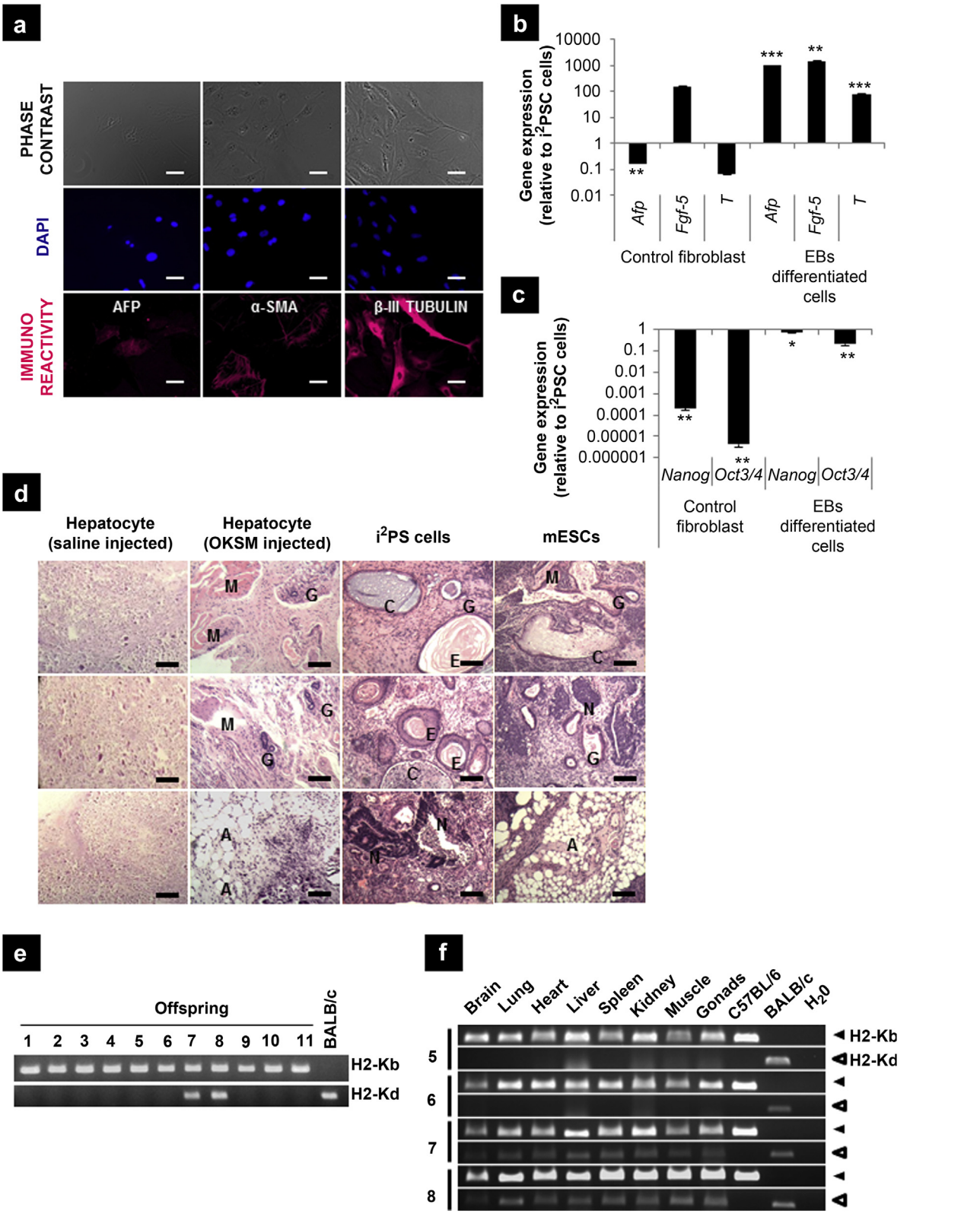

Fig. 3. Functional pluripotency of i^{2}PS cells. (a) Immunostaining for α-fetoprotein (endoderm), α-smooth muscle actin (mesoderm), and β-III tubulin (ectoderm) performed after 7 days of differentiation from EBs. Relative gene expression of (b) early differentiation markers and (c) pluripotency markers in the cells differentiated from EBs and control fibroblast (RT-qPCR, normalized to the starting i^{2}PS cells used to form the EBs, $N = 3$, $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant differences in gene expression in comparison to i^{2}PS cells, obtained by one-way ANOVA). (d) Teratoma assay. M, G, N, C, E and A indicate muscle, gland, neural, cartilage, epidermis and adipocyte tissue, respectively in H&E images (10×). (e) MHC Class I antigen genotype in offspring after microinjection of i^{2}PS cells in C57BL/6 blastocyst (H2-Kb − C57BL/6 and H2-Kd − BALB/c) (f) Contribution of i^{2}PS cells to different tissues in chimeric and non-chimeric offspring.

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i^{2}PS cells, whereas *Afp* and *T* were downregulated. In contrast, the cells differentiated from the EBs showed significant upregulation for all markers. The gene expression levels of two key pluripotency markers were also investigated. As shown in Fig. 3c, dramatic downregulation in both *Nanog* and *Oct3/4* expression was found in the fibroblast control group. These genes were also downregulated in the cells differentiated from the EBs (compared to the starting i^{2}PS cells), however not as pronounced as for the fibroblasts.

The capacity of pluripotent cells to form teratomas containing tissue types from all three developmental lineages upon subcutaneous injection in immunodeficient mice is often used as another assay to validate the differentiation potential of candidate pluripotent cells *in vivo* [28]. For this purpose, the whole primary hepatocyte fraction was isolated from the liver tissue 2 days after HTV injection with OKSM reprogramming factors or 0.9% saline as negative control. 2×10^6 cells were subcutaneously injected into the dorsal flank of CD1 nude mice, bifocally. The same number of i^{2}PS cells cultured under feeder-free conditions were collected and subcutaneously implanted following the same procedure and nude mice injected with E14TG2a cells (2×10^6) were used as positive control for the generation of teratomas (Fig. 3d). After 5 weeks, all animals injected with i^{2}PS cells and mES cells developed teratomas in which the presence of tissues from all three developmental lineages was histologically observed. Most importantly, the animals that were implanted with the same number of cells directly from the primary hepatocyte extract of animals injected with OKSM plasmids (without any culturing) also formed teratomas. In contrast, no teratomas were obtained from subcutaneous implantation of cells from the primary hepatocyte fraction of saline injected animals.

Generation of chimeras upon blastocyst injection is considered a hallmark and requisite for the confirmation of functional pluripotency [29]. i^{2}PS cells cultured on MEF feeder layers under standard mES cell conditions were injected in 3.5 dpc embryos from C57BL/6 background and the genotype for the Major Histocompatibility Complex (MHC) Class I haplotype was investigated in the viable adult offspring as a mean to assess chimerism. Approximately 15–20 i^{2}PS cells were microinjected in each of the 22 C57BL/6 blastocysts and these were surgically transferred into 2 synchronized pseudopregnant surrogate CD1 mothers. While all 11 viable mice obtained showed positive genotype for the H2-Kb haplotype, characteristic of C57BL/6 strain, two of the pups were also positive for the BALB/c haplotype, H2-Kd (Fig. 3e). This indicated contribution of both C57BL/6 and BALB/c derived cells in these two mice. Next, the contribution of i^{2}PS derived cells to various tissues of different developmental origin was investigated (Fig. 3f). As expected, no i^{2}PS cell contribution was observed in mice 5 and 6, which had shown pure C57BL/6 genotype in the previous experiment. Interestingly, chimerism was widespread in mice 7 and 8, with i^{2}PS derived cells distributed in all the tissues analyzed. Moreover, all the offspring that was viable at birth survived for six months, until they were sacrificed to investigate the i^{2}PS cell contribution in different organs. No tumors were detected in the post mortem evaluation in any of the animals.

4. Discussion

In our previous study [18] we reported for the first time the occurrence of *in vivo* reprogramming toward pluripotency in adult, mammalian (BALB/c mice) tissue. We induced this effect in the liver of those animals by targeting the overexpression of transcripts in that tissue using HTV administration of plasmids encoding for the OKSM reprogramming factors. Cell reprogramming was evidenced by an upregulation of pluripotency-related markers in the tissue, both at the mRNA and protein level, and downregulation of hepatocyte-specific genes without any observation of teratoma formation. Vivien et al. had reported the occurrence of *in vivo* reprogramming of somatic tissues to a pluripotent state upon the forced expression of reprogramming transcription factors in an amphibian model [30]. More recently, Abad et al. confirmed that *in vivo* reprogramming to pluripotency in somatic mouse tissues was feasible, however by use of a transgenic strain in which the expression of OKSM factors was switched on ubiquitously upon administration of doxycycline resulting in widespread teratoma formation [31]. Despite the fact that both studies by Vivien et al. and Abad et al. bear no clinical relevance mainly due to the limitations posed by the species used, the *in vivo* reprogrammed cells were isolated from the tissue, cultured and found to attain pluripotent and totipotent characteristics (respectively) and a differentiation capacity equivalent to ES and *in vitro* generated iPS cells. We have hypothesized that virus-free and transient induction of pluripotency in terminally differentiated tissues *in vivo* may offer a more clinically-relevant approach toward tissue regeneration. In this work, we attempted to isolate and culture the cells that had been reprogrammed within the mouse liver *in vivo* in order to interrogate their pluripotent character. Our aim was to fully characterize these cells and compare their differentiation potential to that of a standard mES cell line.

Optimum culture conditions were achieved by isolation of primary hepatocytes 48 h after HTV-injection of BALB/c mice with the reprogramming plasmids. Culture of these primary extracts on MEF feeder layers under standard mES cell culture conditions (DMEM/LIF medium) led first to significantly higher expression levels in key pluripotency-related genes compared to extracts from the saline-injected control animals (Fig. 1 b). Cell colonies morphologically indistinguishable from those of a standard mES cell line (E14TG2a) were obtained only in the cultures extracted from liver tissue that had been administered with the reprogramming plasmids (Fig. 1c). This suggested that cell reprogramming in the hepatocyte fraction occurred in their *in vivo* microenvironment and not as a result of the *in vitro* culture conditions.

The extraction and culture of colonies from the primary hepatocyte fraction containing reprogrammed cells was followed with the characterization of these cells, that we named *in vivo* induced pluripotent stem (i^{2}PS) cells in a reference to their origin. A series of positive immunohistochemical markers for the pluripotent phenotype, such as CDy1, ALP, NANOG, OCT3/4, SOX2 and the mES cell-specific antigen SSEA1 indicated the pluripotent character of i^{2}PS cell colonies at the molecular level (Fig. 1d and e). That was further confirmed by gene expression analysis using RT-qPCR and a DNA microarray analysis (Fig. 2). These studies demonstrated the similarity of i^{2}PS cells to the standard mES cell line in terms of the expression of genes involved in the induction, maintenance, amelioration and loss of pluripotency, as well as markers characteristic of the differentiation toward all three developmental lineages. An upregulation of the endoderm-specific marker *Afp* was observed in i^{2}PS cells by RT-qPCR. Given that hepatocytes originate from the differentiation of the endoderm lineage, this could imply the maintenance of epigenetic marks from the tissue of origin in i^{2}PS cells, as has been observed by others in certain *in vitro* generated iPS cell clones [32]. However, this difference in gene expression could not be confirmed with the microarray analysis when a wider spectrum of endoderm and hepatocyte-specific genes were investigated.

The assessment of the differentiation potential of i^{2}PS cells, both *in vitro* through the generation of EBs and *in vivo* with the teratoma assay, confirmed their capability to differentiate into tissues derived from all germ layers at the mRNA, protein and histological level and did not suggest preferential differentiation towards the endodermal (or any) developmental lineage (Fig. 3a–d).

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Importantly, even the primary hepatocyte fraction freshly isolated 48 h after injection of the reprogramming plasmids was able to form teratomas, which further reinforced the observations that the somatic cells were reprogrammed toward pluripotency in their *in vivo* environment and not as a result of the culture conditioning.

One of the most stringent hallmarks for the assessment of functional pluripotency was fulfilled with the generation of chimeric mice upon blastocyst injection of i^2PS cells. Upon injection of 22 C57BL/6 embryos with i^2PS cells, 11 pups were obtained of which 2 were chimeras. The contribution of cells from BALB/c background was demonstrated making use of the differences in MHC Class I haplotype between the two mice strains (Fig. 3e) and was found to be widespread in several tissues of different developmental origins (Fig. 3f). Their contribution to the germline is currently being investigated in our laboratory. The low efficiency of chimera generation with BALB/c derived i^2PS cells agrees with reports in the literature in which ES cells isolated from BALB/c mice are described to generate chimeras with lower efficiency as compared to other mice strains and to result in poor contribution to the fur coat color and germline [33–35]. Moreover, many factors other than the genetic background of the mice can dramatically influence the generation of chimeras. Among them, technical aspects of the microinjection and quality parameters of the cells (i.e. morphology, size and differentiation status) [36], culturing conditions [37], chromosomal abnormalities [38,39], length of the telomeres [40] and epigenetic signatures present in the injected cells [35] play a crucial role and should be thoroughly investigated for i^2PS cells.

In our previous work we showed that the reprogramming of hepatocytes to pluripotency *in vivo* did not lead to any histopathological or functional adverse reactions or side effects in the liver, nor did it lead to any manifestation of carcinogenesis or teratoma formation (up to 120 days post-injection of the OSKM plasmids) [18]. In the present study, we have demonstrated that the i^2PS cells generated from the reprogrammed hepatocytes led to the development of teratomas upon subcutaneous injection in nude mice, in a similar way to that of the mES cell line. This not only confirmed the pluripotent character of i^2PS cells, but also suggested that the i^2PS cells isolated from the host tissue environment and cultured under conventional mES cell culture conditions were able to maintain their ES cell-like pluripotent characteristics, whereas their pluripotent character was maintained in the host liver tissue only transiently. We attribute this to the highly reversible relationship between the enhanced pluripotent character induced *in vivo* by somatic cell reprogramming and re-differentiation to the original host cell phenotype rapidly driven by endogenous cues and the importance of the tissue microenvironment [41,42]. More studies are necessary to further elucidate the prevailing mechanisms, interactions and cues present in the *in vivo* microenvironment that might be rapidly driving the re-differentiation of the induced pluripotent cells within the tissue. We speculate that the transiency and duration in which reprogrammed cells remain at a state of enhanced pluripotency within the tissue may also determine the risk-benefit balance between teratoma formation and tissue regeneration.

5. Conclusions

We demonstrated here that the forced expression of reprogramming factors leads to the generation of pluripotent cells *in vivo* and that these cells can be isolated and cultured *in vitro* to exhibit similar pluripotent characteristics to those of mES cells. We also hypothesize that when these reprogrammed cells remain within their tissue of origin they are rapidly driven to re-differentiate into tissue-specific cells under the influence of the tissue microenvironment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.05.086.

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