NANOG PRIMING BEFORE FULL REPROGRAMMING MAY GENERATE GERM CELL TUMOURS

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Abstract

Reprogramming somatic cells into a pluripotent state brings patient-tailored, ethical controversy-free cellular therapy closer to reality. However, stem cells and cancer cells share many common characteristics; therefore, it is crucial to be able to discriminate between them. We generated two induced pluripotent stem cell (iPSC) lines, with NANOG pre-transduction followed by OCT3/4, SOX2, and LIN28 overexpression. One of the cell lines, CHiPS W, showed normal pluripotent stem cell characteristics, while the other, CHiPS A, though expressing pluripotency markers, failed to differentiate and gave rise to germ cell-like tumours *in vivo*. Comparative genomic hybridisation analysis of the generated iPS lines revealed that they were genetically more stable than human embryonic stem cell counterparts. This analysis proved to be predictive for the differentiation potential of analysed cells. Moreover, the CHiPS A line expressed a lower ratio of p53/p21 when compared to CHiPS W. NANOG pre-induction followed by OCT3/4, SOX2, MYC, and KLF4 induction resulted in the same tumour-inducing phenotype. These results underline the importance of a re-examination of the role of NANOG during reprogramming. Moreover, this reprogramming method may provide insights into primordial cell tumour formation and cancer stem cell transformation.

Keywords: Induced pluripotent stem cells, stem cells, cancer stem cells, NANOG, cancer, germ-cell tumour, reprogramming, pluripotency.

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Introduction

Pluripotent stem cells have attracted much attention from the scientific community in recent years. They hold promise for various therapeutic applications, from regenerative therapies to pharmacological screening. Technical difficulties and ethical issues associated with the isolation of adult or embryonic stem cells have redirected interest towards induced pluripotent stem cells (iPSCs). Obtained from lineage-specific cells via reprogramming with a maximum of four reprogramming genes (RGs), *POU5F1 (OCT3/4)*, *SOX2* and *MYC*, *KLF4*, or NANOG and LIN28 (Takahashi *et al.*, 2007; Yu *et al.*, 2007), iPSCs seem to be a very promising area for future stem cell research. Despite obvious advantages such as donor compatibility and an unlimited supply, there are still numerous challenges to be met before iPSCs become routinely used in regenerative medicine or standardised for patient-specific drug research (reviewed in: Stadtfeld and Hochedlinger, 2010). One major obstacle is the capability of pluripotent stem cells to form tumours *in vivo*. At first, human embryonic stem cells (hESC) were believed to produce only benign teratomas; however, recent studies have demonstrated that hESC lines can also develop malignant features after being kept in culture (Hovatta *et al.*, 2010; reviewed in: Blum and Benvenisty, 2009).

There is increasing evidence of the existence of a distinct cell sub-population within a tumour, called cancer stem cells (CSCs), which shares many characteristics with normal stem cells (Bonnet and Dick, 1997). CSCs are believed to be responsible for the tumour's ability to undergo epithelial-mesenchymal transition (EMT), and may play a role in relapse, metastasis, and treatment resistance (reviewed in: Bomken *et al.*, 2010; Tysnes, 2010). It remains controversial as to whether CSCs originate from stem cells that have undergone aberrant transformation or from terminally differentiated cells that have reversed their cellular program. Very limited evidence indicates that normal stem cells and cancer stem cells differ (Guzman *et al.*, 2005; Yilmaz *et al.*, 2006). Therefore, it is crucial to understand the carcinogenic aspect of stem cells before they can be implemented

therapeutically. Thus, research towards finding ways to limit the tumour formation risk of iPSC, for instance by developing strategies allowing accurate sorting of potentially carcinogenic cells, is essential. In the present study, we generated and analysed two iPSC lines. One of the lines, the CHiPS W, displayed normal pluripotent stem cell characteristics and gave rise to a teratoma expressing components of the three germ layers during an *in vivo* differentiation assay. The other line, the CHiPS A, despite its iPSC-like morphology and marker expression, resulted in germ cell-like tumours when injected into immunoincompetent mice. The analysis of *OCT3/4* and *NANOG* expression and the methylation status of their promoters showed their re-activation in the CHiPS A line. Since it has been previously demonstrated that genomic instability in hESC lines might lead to neoplastic progression (Werbowetski-Ogilvie *et al.*, 2009), we used comparative genomic hybridisation and single nucleotide polymorphism assays to detect genomic signatures in the derived iPSC lines. We also estimated the relative expression of TP53 (p53) and CDKN1A (p21) in both lines. Finally, we showed that the resulting NANOG-induced tumourigenic phenotype is independent of the other RGs used. These results underline the importance of a re-examination of the role of NANOG during reprogramming and the need for detailed iPSC characterisation prior to clinical use. Moreover, as the generated lines were derived from a uniform genetic background, they may serve as a model of cancer stem cell formation.

Materials and Methods

Cell lines

The hESC line H1 came from the WiCell Research Institute (Madison, WI, USA). HS401 was derived as previously described (Inzunza *et al.* 2005; Ström *et al*., 2010). iPSC lines were derived as follows: the CHiPS A line was derived by transducing 50,000 human fibroblasts (ATCC, CRL-2429) with 5 MOI of pSin-EF2-Nanog-Pur. After 24 h, cells were re-transduced with three viruses (MOI 5): pSin-EF2-Sox2-Pur, pSin-EF2-Oct4-Pur, and pSin-EF2-Lin28-Pur (kindly donated by J.A. Thomson). The CHiPS W line was derived by transducing 50,000 human fibroblasts with 5 MOI of pSin-EF2-Nanog-Pur. The cells were treated with puromycin for 5 days to select for transformed cells and then re-transduced with three viruses: pSin-EF2-Sox2-Pur, pSin-EF2-Oct4-Pur, and pSin-EF2-Lin28-Pur (MOI 5). The CHiPS 22 line was derived by first transducing 50,000 human fibroblasts with 5 MOI of pSin-EF2-Nanog-Pur; after 24 h, cells were re-transduced with the Stem Cell Cassette (STEMCCA) polycistronic lentiviral vector, carrying the four Yamanaka RGs (Sommer *et al.*, 2009) (kindly donated by G. Mostoslavsky). All iPSC and hESC lines were cultured on irradiated human foreskin fibroblasts and passaged mechanically. Cells were grown in iPSC medium: Knockout Dulbecco's Minimal Essential Medium (DMEM) supplemented with 20 % Serum Replacement, 2 mmol/L Glutamax, 40 µg/mL gentamycin and 100 µmol/L β-mercaptoethanol (all Gibco/Invitrogen, Carlsbad, CA, USA). For the H1 cell line, medium was supplemented with 10 ng/mL bFGF (fibroblast growth factor). All iPSC lines were initially cultured according to the protocol established by Thomson's group (Yu *et al*., 2007), and supplemented with 100 ng/mL bFGF; however, the CHiPS 22 and CHiPS A lines grew robustly, so we decided to lower the concentration of bFGF to 10 ng/mL for those lines to decrease the cost of culture. To exclude the influence of the different bFGF concentrations on the experimental results, prior to DNA, RNA or protein extraction, cells were cultured for one passage (at least 48 h) under feeder-free culture conditions on Matrigel (Becton Dickinson AG, Basel, Switzerland) coated plates in NutriStem medium (Stemgent, Cambridge, MA, USA) supplemented with 10 ng/mL bFGF. Matrigel was diluted 1:30 with KO DMEM and after plating allowed to gel at room temperature for 1 h before use. The fibroblast feeders (ATCC, Manassas, VA, USA; CRL-2429) were cultured in IMDM supplemented with 10 % foetal calf serum, 2 mmol/L Glutamax and 1 % penicillin/streptomycin (all Gibco/Invitrogen). Feeder cells were mitotically inactivated by irradiation at 35 Gy before seeding on a gelatin-coated 35 mm dish at 3.5×10^5 cells/dish. The iPSC and hESC culture medium was changed daily.

Immunohistochemistry

Cells were passaged manually onto a GFP-positive feeder layer (Unger *et al.*, 2009). After fixation in 4 % formaldehyde, they were washed three times with phosphate buffered saline (PBS), blocked with 1 % bovine serum albumin (BSA) and stained with antibodies against OCT3/4 (Santa Cruz Laboratories, Santa Cruz, CA, USA; sc-5279), NANOG (Santa Cruz Laboratories, sc-33759), TRA-1-60 (Santa Cruz Laboratories, sc-21705), and SSEA4 (Santa Cruz Laboratories, sc-21704) overnight at 4 °C. After three washes with PBS, cells were incubated with fluorescent probe-labelled secondary antibodies and mounted with UltraCruz™ Mounting Medium containing DAPI. Ki-67 immunostaining was performed as follows: after standard deparaffinisation, antigen retrieval was performed with 0.01 M citrate (pH 6) at 100 °C. Endogenous peroxidase was blocked with HP-Blocking solution (S2023; Dako, Glostrup, Denmark) and the slides were incubated with a Ki-67 antibody (mib1, Dako, M7240). Secondary antibody incubation and signal development was performed in DAB solution according to the manufacturer's instruction (Dako). All staining were performed three times independently.

Karyotype

Karyotyping was performed in at least twenty metaphase spreads, using the GTG-banding method, by an independent laboratory (Genetic Service, Geneva University Hospitals, Switzerland). Briefly, iPSCs were incubated in iPSC medium, supplemented with 0.2 mg/mL colcemid (Roche, Basel, Switzerland) at 37 °C for 20 min, and subsequently washed three times with 2 mL PBS containing Ca^{2+} and Mg^{2+}. A minimum of 15 colonies were collected in 2 mL 1× trypsin-EDTA (Invitrogen) and incubated at 37 °C for 5 min. The final mixture of cells was pipetted several times to disaggregate the cells. The trypsin activity was stopped with 4 mL iPSC medium and cells were spun at 300 g

Table 1. Primers used in the study.

quantitative PCR			
Gene	**Forward**	**Reverse**	**Amplicon**
GUSB	CCACCAGGGACCATCCAAT	AGTCAAAATATGTGTTCTGGACAAAGTAA	79
OCT3/4 (POU5F1)	AGTGCCCGAAACCCACACTG	ACCACACTCGGACCACATCCT	81
NANOG	GATTTGTGGGCCTGAAGAAA	TTGGGACTGGTGGAAGAATC	138
LIN28	TGTAAGTGGTTCAACGTGCG	CCTCACCCTCCTTCAAGCTC	145
SOX2	GCGAACCATCTCTGTGGTCT	GGAAAGTTGGGATCGAACAA	145
Brachyury	ACAGCTGTGACAGGTACCCAAC	CATGCAGGTGAGTTGTCAGAAT	109
NKX2.5	CTATCCACGTGCCTACAGCGAC	GCACAGCTCTTTCTTTTCGGC	67
MSI	TTGGGAAGGTGGACGACG	CTCAAACGTGACAAACCCGAAC	80
FOXA2	GGAGCGGTGAAGATGGAAG	TACGTGTTCATGCCGTTCAT	122
REX1 (ZFP42)	GAAGAGGCCTTCACTCTAGTAGTG	TTTCTGGTGTCTTGTCTTTGCCCG	179
semi-quantitative PCR			
Gene	**Forward**	**Reverse**	**Amplicon**
OCT3/4 (POU5F1) endogenous	GCCTTCCTTCCCCATGGC	CCTCAAAATCCTCTCGTTGT	911
OCT3/4 (POU5F1) exogenous	TCAAGCCTCAGACAGTGGTTC	CCTCAAAATCCTCTCGTTGT	976
NANOG endogenous	TCCTCTATACTAACATGAGTG	AACACAGTTCTGGTCTTCTG	319
NANOG exogenous	TCAAGCCTCAGACAGTGGTTC	AACACAGTTCTGGTCTTCTG	385
GAPDH	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG	174
DAZL	ATGTTAGGATGGATGAAACTGAGATTA	CCATGGAAATTTATCTGTGATTCTACT	178
GDF3	AGACTTATGCTACGTAAAGGAGCT	CTTTGATGGCAGACAGGTTAAAGTA	150
KIT	CAGGCAACGTTGACTATCAGT	ATTCTCAGACTTGGGATAATC	288
STELLAR	GTTACTGGGCGGAGTTCGTA	TGAAGTGGCTTGGTGTCTTG	174
SOX2	F AGA TGC ACA ACT CGG AGA TC	GTC ATG GAG TTG TAC TGC AG	438
STEMCCA OCT3/4 -KLF4 JUNCTION	ACCATCTGTCGCTTCGAGGCC	GGCTAGGAGGGCCGGGTTGTT	848

for 10 min. Subsequently, the pellet was resuspended and incubated in 1 mL pre-warmed potassium chloride solution (KCl, 0.075 M) for 10 min at 37 °C. Cells were then pre-fixed with 1 mL Carnoy fixative solution (methanol/acetic acid = 3/1) at -20 °C, and immediately spun at 1800 rpm for 10 min. Finally, the supernatant was discarded, the pellet resuspended again in Carnoy fixative solution and the cells were prepared for analysis.

RNA extraction, quantitative and non-quantitative real time polymerase chain reaction

Total RNA was extracted from the cell lines, using the QIAGEN (Hilden, Germany) RNeasy MiniKit according to the manufacturer's protocol (Invitrogen). RNA integrity and quantity were assessed with an Agilent (Santa Clara, CA, USA) 2100 bioanalyser, using RNA 6000 nanochips. 1 µg of total RNAs were reverse transcribed with the Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. One-twentieth cDNA template was used as template for each PCR reaction. cDNA was real time polymerase chain (PCR) amplified in a 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using the Power SYBR Green PCR master mix (Applied Biosystems). Raw threshold-cycle (Ct) values were obtained with the Sequence Detection Systems 2.0 software (Applied Biosystems). Melting curve analysis were automatically performed to monitor production of the appropriate PCR product. Relative quantities (RQs) were calculated with the formula RQ = E-Ct, using efficiencies calculated for each run with the Data Analysis for Real-

Time PCR (DART-PCR) algorithm, as described (Peirson *et al.*, 2003). A mean quantity was calculated from triplicate PCR reactions for each sample, and this quantity was normalised to similarly measured quantity of normalisation gene (*GusB*). The highest normalised relative quantity was arbitrarily designated as a value of 1.0 and the other quantities were recalculated proportionally. Each PCR reaction was performed at least in triplicate with negative controls and the mean quantities were calculated from them. They were expressed as +/- SD. For non-quantitative PCR, reactions were performed in a Biometra (Göttingen, Germany) thermocycler, with RedTaq polymerase mix (Sigma-Aldrich, St. Louis, MO, USA), 250 nM primers and 1 µL of cDNA. In the case of teratoma RT-PCR, we used 3 µL of template cDNA. Primers are listed in Table 1.

Bisulphite sequencing

About 2 µg of DNA, extracted from the iPSC and control cell lines, were bisulphite-converted and purified using Epitect Bisulfite Kits (Qiagen) following the manufacturer's instructions. DNA bisulphite treatment and processing were performed simultaneously for all cell lines. The promoter regions of *OCT4* and *NANOG* were amplified with specific primers as described previously (Freberg *et al.*, 2007) using JumpStart REDTaq DNA Polymerase (Sigma-Aldrich) and KAPA2G Robust DNA Polymerase (Kapa Biosystems, Cambridge, MA, USA). Unincorporated primers and nucleotides were removed by incubation with Exonuclease I and Shrimp Alkaline Phosphatase (New England Biolabs, Ipswich, MA, USA), and the PCR products were cloned into the pCRII-TOPO vector (Invitrogen); transformants were grown on agar plates supplemented with X-Gal. Randomly chosen clones containing an insert were re-amplified by M13 primers and sequenced by SP6 and T7 common primers from each cell line for each gene.

Teratoma formation assay

IPS cells were grown on Matrigel-coated dishes and collected by trypsin digestion. About $5x10^6$ cells mixed with Matrigel (diluted in KO DMEM 5:1) were injected into the hind limb muscle of 8-week-old Nod-SCID mice. Teratomas were dissected after 8-12 weeks and fixed in 4 % paraformaldehyde overnight. Samples were embedded in paraffin, cut, and stained with haematoxylin and eosin.

Affymetrix SNP 6.0 array and DNA copy number variations

Genotyping was carried out using the Affymetrix Genome-Wide Human SNP Array 6.0 (San Diego, CA, USA). Labelling and hybridisation were performed following the protocols and kits provided by the manufacturer (Affymetrix Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0). A set of 50 arrays previously hybridised and genotyped in the same laboratory was used as the copy number neutral reference sample. Copy-number variations (CNVs) and loss of heterozygosity (LOHs) were extracted with the Affymetrix proprietary Genotyping Console Software, using the standard setup recommended by the producer. Variations were annotated using the libraries supplied by Affymetrix (version 29). Copy number neutral LOH regions (UPDs) were detected by determining LOH regions from the genotype calls (dChip, https://sites.google.com/site/dchipsoft/home, 2010) and by comparing the results with the ploidy detected in those regions by the Affymetrix software. Genes were annotated to detect variations using the Biomart database (http://www.biomart.org/, 2010). Gene ontology analysis was performed in R (www.r-project.org, 2010) using the package GO_stats ver. 2.12. The gene's universe was determined from the Affymetrix annotation files (version 29).

***In vitro* differentiation of the pluripotent stem cells**

Whole iPSC and hESC colonies were dissected into ultra low attachment dishes (Corning Costar, Lowell, MA, USA) in KO DMEM medium supplemented with 10 % NCS, 1 mM L-glutamine, 100 µM non-essential amino acids, 100 µM 2-mercaptoethanol, 50 U/mL penicillin and 50 mg/mL streptomycin (Gibco/Invitrogen). Within 24 h, cells aggregated to form embryoid bodies (EBs). Cardiac differentiation was performed using standard procedures, as previously described (Bettiol *et al.*, 2006). Haematopoietic differentiation was carried out as recently described (Chicha *et al.*, 2011). Briefly, collagenased colonies of iPSC were resuspended in StemPro-34 Serum-Free Media (Gibco/Invitrogen) supplemented with 0.5 ng/mL human recombinant BMP-4 (R&D Systems, Minneapolis, MN, USA) in low cluster tissue culture dishes (Corning Costar, Lowell, MA, USA). At day 1 of differentiation, embryoid bodies (EBs) were harvested and transferred to fresh StemPro media supplemented with 10 ng/mL BMP-4 and 5 ng/mL bFGF. After 72 h, EBs were harvested again and transferred to a haematopoietic medium consisting of StemPro media supplemented with 100 ng/mL human recombinant VEGF (R&D Systems), 5 ng/mL bFGF, 100 ng/mL SCF (Amgen), 100 ng/mL FLT3-L (Amgen), and 40 ng/mL TPO (Peprotech, London, UK) for 4 additional days. All differentiation steps were performed under hypoxic conditions (5 % O_2) in a humidified incubator at 37 °C.

Flow cytometry

Cell suspensions were analysed after staining with antibodies specific for CD34 and KDR (BD Biosciences, Allschwill, Switzerland). Dead cells were identified as 7-aminoactinomycin D (7AAD)-positive cells and were excluded. Cells were stained in PBS containing 20 % human AB serum (Sigma-Aldrich) to prevent nonspecific binding. Data were collected using a FACS Calibur device (BD Biosciences) and analysed with Flowjo software (Treestar, Olten, Switzerland).

Immunoblotting

CHiPS A and CHiPS W cells were grown on Matrigel-coated dishes, trypsinised and counted. Protein extracts were prepared by lysing the cells in protein isolation buffer (10 mM Tris-HCl pH 8, 1 % Triton-X100, 2 mM EDTA, 10 % glycerol, 137 mM NaCl, and a proteinase inhibitor cocktail) to a final concentration of about 300,000 cells per 15 µL. The lysates from 300,000 cells were resolved by SDS-polyacrylamide gel electrophoresis. For immunoblotting, the following antibodies were used: a p53-specific polyclonal antibody (Santa Cruz Laboratories,

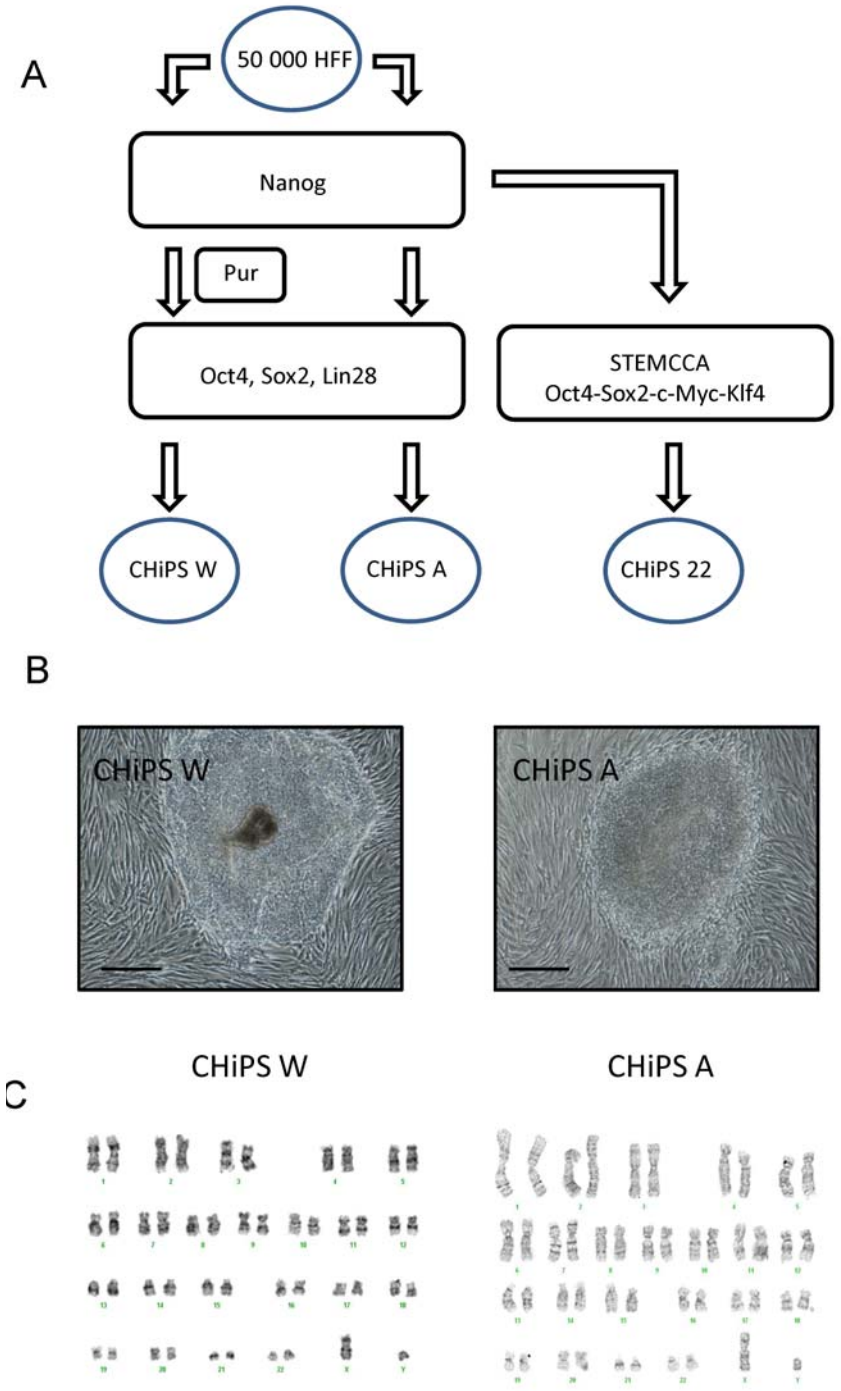

Fig. 1. Generation of induced pluripotent stem cell lines from human fibroblasts (**A**) Schematic representation of the reprogramming of the lines used in the study. Pur - puromycin. (**B**) Phase contrast images of the induced pluripotent stem cell lines CHiPS W and CHiPS A, growing on feeders. Scale bar = 300 μm. (**C**) CHiPS W and CHiPS A karyotype.

sc-126), and a monoclonal p21-specific antibody (Santa Cruz Laboratories, sc-397). The experiment was repeated three times independently.

Results

Generation and characterisation of iPS cells from human fibroblasts

It has been demonstrated that the introduction of four reprogramming factors (OCT3/4, SOX2, NANOG, and LIN28) into a somatic cell results in a return to the pluripotent state, albeit with low efficiency (Takahashi *et al*., 2007; Yu *et al*., 2007). Given that Nanog overexpression in mouse ESC increases reprogramming efficiency (Silva *et al*., 2006), and that re-induction of the reprogramming transgenes leads to higher iPSCs generation (Maherali *et al*., 2008), we wanted to check whether two-step reprogramming would increase the effectiveness of the process. Therefore, human fibroblasts were reprogrammed to pluripotency first by using only a NANOG-expressing lentiviral vector (Yu *et al*., 2007). After 24 h, one part of

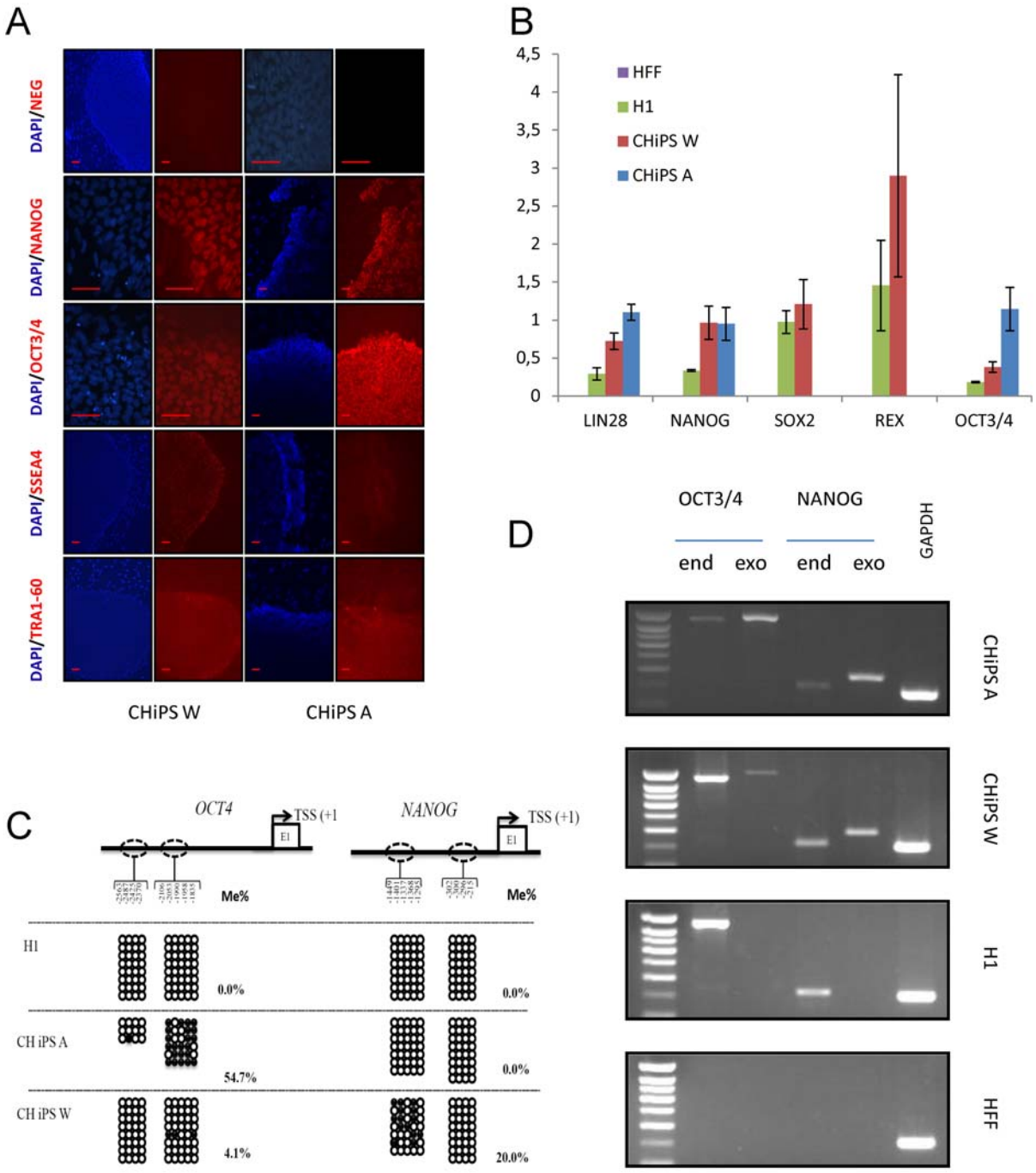

Fig. 2. Characterisation of CHiPS W and CHiPS A lines. (**A**) Immunohistochemistry of pluripotency markers NANOG, OCT3/4, SSEA4, TRA1-60 (red) and DAPI (blue). NEG-negative control. Scale bar = 20 µm. (**B**) qRT-PCR analysis of Lin28, NANOG, SOX2, REX1 and OCT3/4 expression in parental fibroblasts, the human embryonic stem cell line H1 and the induced pluripotent stem cell lines CHiPS W and CHiPS A. (**C**) Bisulphite genomic sequencing of OCT4 and NANOG promoters in the CHiPS A, CHiPS W and H1 lines. Each row represents an individual sequencing reaction for a given amplicon. Open and filled circles indicate unmethylated and methylated CpGs dinucleotides, respectively. The percentage of methylation for each cell line is shown on the right. (**D**) RT-PCR analysis of exogenous and endogenous OCT3/4 and NANOG transcripts. HFF - human feeder fibroblasts.

the cells were re-transduced with three separate lentiviral vectors overexpressing the transcription factors OCT3/4, SOX2, and LIN28 (Yu *et al*., 2007), resulting in the CHiPS A line (Fig. 1A). The other part of the cells was treated with puromycin for 5 days before re-transduction with the same lentivectors as the CHiPS A line, resulting in the CHiPS W line (Fig. 1A). After three weeks, reprogrammed colonies were manually selected based on morphological criteria and expanded. The selected clones had typical ESC morphology (Fig. 1B) and displayed a normal karyotype as assessed by metaphase spreads (Fig. 1C); however, the CHiPS A line proliferated significantly

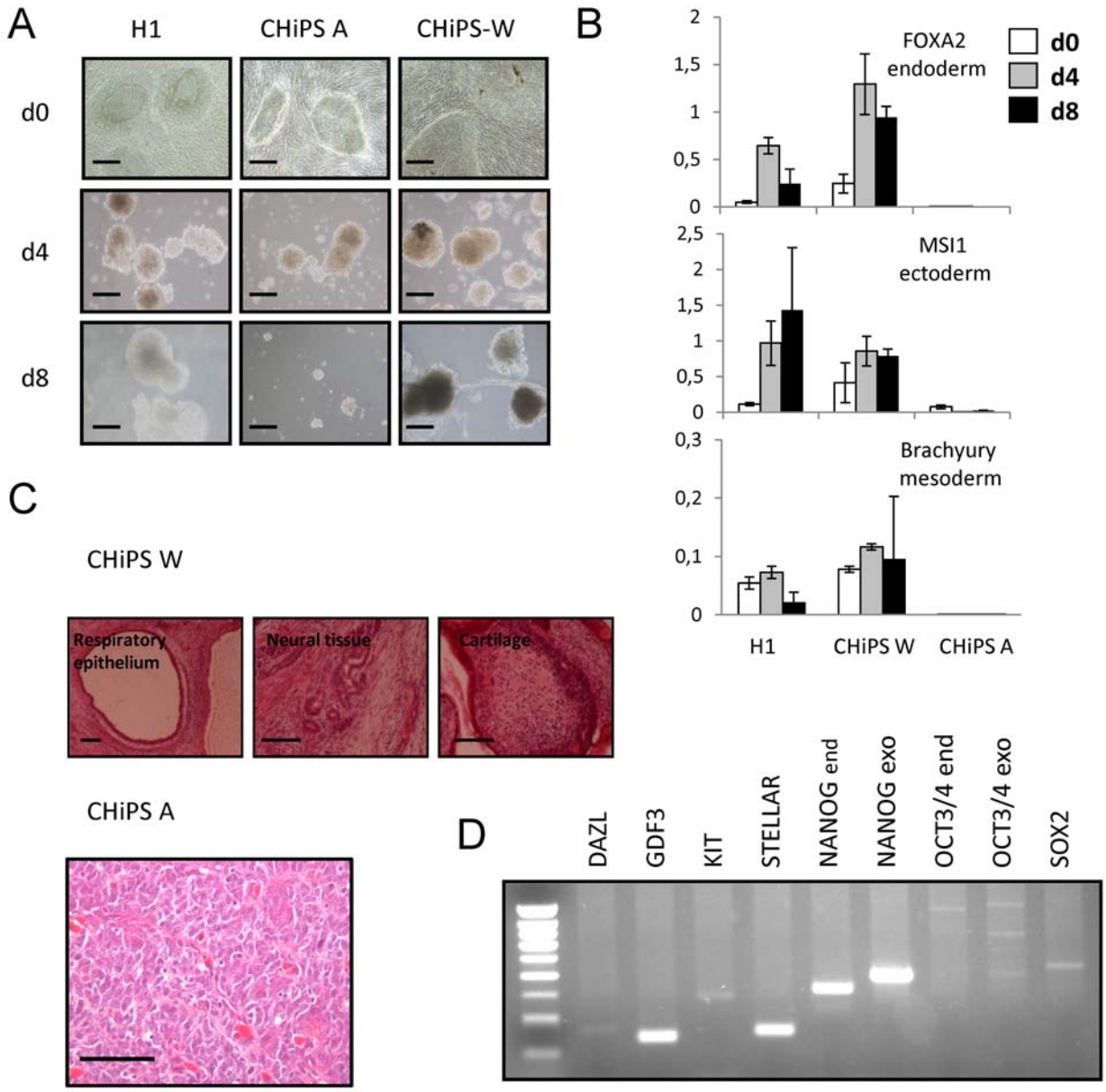

Fig. 3. *In vitro* and *in vivo* differentiation of CHiPS W and CHiPS A lines. (**A**) Phase contrast images of CHiPS A, CHiPS W and hESC H1 lines after spontaneous differentiation as embryoid bodies in suspension culture, at day 0, 4 and 8. Scale bar = 300 μm. (**B**) Quantitative expression of three germ layer markers in CHiPS A, CHiPS W and H1 lines. (**C**) Haematoxylin and eosin staining of tumours derived from intramuscular injection of iPSCs in NOD-SCID mice. CHiPS W line formed teratoma with all embryonic germ layers mesoderm (cartilage) ectoderm (neural tissue) and endoderm (respiratory epithelium) whereas CHiPS A line formed germ cell-like tumour. The scale bar corresponds to 200 μm for CHiPS W, 100 μm for CHiPS A. (**D**) Expression of the seminoma (DAZL, GDF3, KIT, STELLAR) and the pluripotency (NANOG, OCT3/4) markers in CHiPS A-derived tumour by RT-PCR.

faster than the CHiPS W line (data not shown). The iPSC lines expressed the human ESC pluripotency antigens OCT3/4, NANOG, SSEA4 and TRA-1-60 as demonstrated by immunohistochemistry (Fig. 2A). We analysed the expression of pluripotency markers *ZFP42 (REX1)*, *SOX2*, *NANOG*, *LIN28*, and *OCT3/4* by quantitative PCR (Fig. 2B). The analysis showed that, contrary to the CHiPS W line, the CHiPS A line did not express *SOX2* and *REX1* but overexpressed *OCT3/4* instead. In addition, the levels of *LIN28* in the CHiPS A line were also higher than in the CHiPS W line. To clarify the origin of the transcript, we performed bisulphite sequencing, which revealed a surprising methylation status of the *OCT3/4* and *NANOG* promoters in the CHiPS A and W lines (Fig. 2C). Despite overexpression of *OCT3/4*, the *OCT3/4* promoter in the CHiPS A line was highly (55 %) methylated, while the *NANOG* promoter was demethylated. CHiPS W showed negligible methylation at the *OCT3/4* promoter and moderate methylation at the *NANOG* promoter (Fig. 2C). The results suggest a lack of retroviral silencing and therefore exogenous expression of *OCT3/4* in the CHiPS A line. Semi-quantitative RT-PCR confirmed the predominant

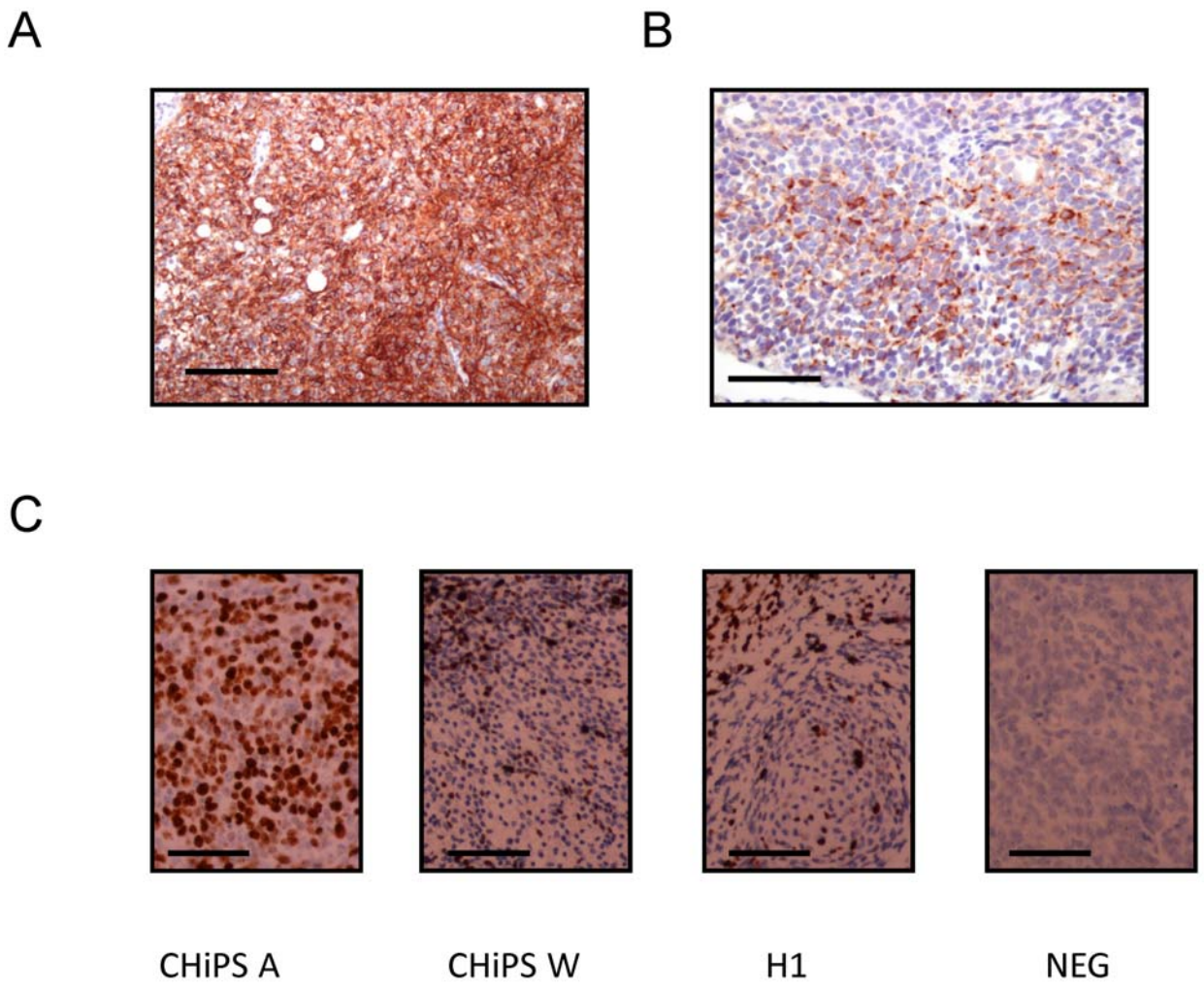

Fig. 4. Analysis of the CHiPS A-derived tumour. (**A**) Expression of CD117 (or KIT, a germ cell tumour marker). (**B**) Expression of CD99 (a primitive neuroectodermal tumours and sex cord stromal tumours marker). (**C**) Increased proliferation in CHiPS A-derived tumour, as compared to CHiPS W- and H1-derived teratomas, indicated by Ki-67 staining. NEG-negative control with CHiPS A-derived tumour. Scale bar = 100 μm.

expression of exogenous *OCT3/4* and *NANOG* in the CHiPS A line, along with endogenous genes (Fig. 2D). At the same time low levels of endogenous NANOG expression in CHiPS A line, despite complete promoter demethylation suggest other mechanisms blocking gene expression.

Differentiation potential of CHiPS A

The CHiPS A line, despite its ES-like morphology, failed to differentiate *in vitro* (Fig. 3A). During differentiation via embryoid bodies (EBs), in contrast to the CHiPS W line and the control hESC H1 line, EBs of CHiPS A dissociated over time (Fig. 3A). We also tried to direct differentiation of CHiPS A under adherent conditions using 10 μM all-trans retinoic acid or to induce differentiation on a Matrigel-coated dish by bFGF withdrawal. However, we were unable to obtain mature EBs and cells ultimately dissociated over time (data not shown). To investigate the differentiation commitment to the three embryonic germ layers, we analysed the acquired expression of *FOXA2* (endoderm), *MSI1* (ectoderm), and *Brachyury* (mesoderm) marker genes within the EBs of CHiPS A and CHiPS W from day 0 to day 8 (Fig. 3B). We observed the expected change in all the markers analysed in the CHiPS W and hESC H1 lines, indicating proper differentiation commitment. However, none of the markers increased with time in the CHiPS A line (Fig. 3B). We decided to verify whether this disability regarding *in vitro* differentiation extended into a defect in an *in vivo* teratoma formation assay. The histological examination showed that the CHiPS A cells formed invasive (multiple infiltrations into the surrounding tissue, lack of defined borders) germ cell-like tumours when injected into NOD-SCID mice (Fig. 3C). In the same assay, the CHiPS W cells formed benign teratomas, as expected (Fig. 3C). To confirm the germ cell-like nature of the CHiPS A tumour, we also verified the expression of known seminoma markers *DAZL, GDF3, KIT,* and *STELLAR,* as well as the pluripotency markers *NANOG, OCT3/4,* and *SOX2* (Ezeh *et al.*, 2005; Gopalan *et al.*, 2009) (Fig. 3D). We observed strong endogenous

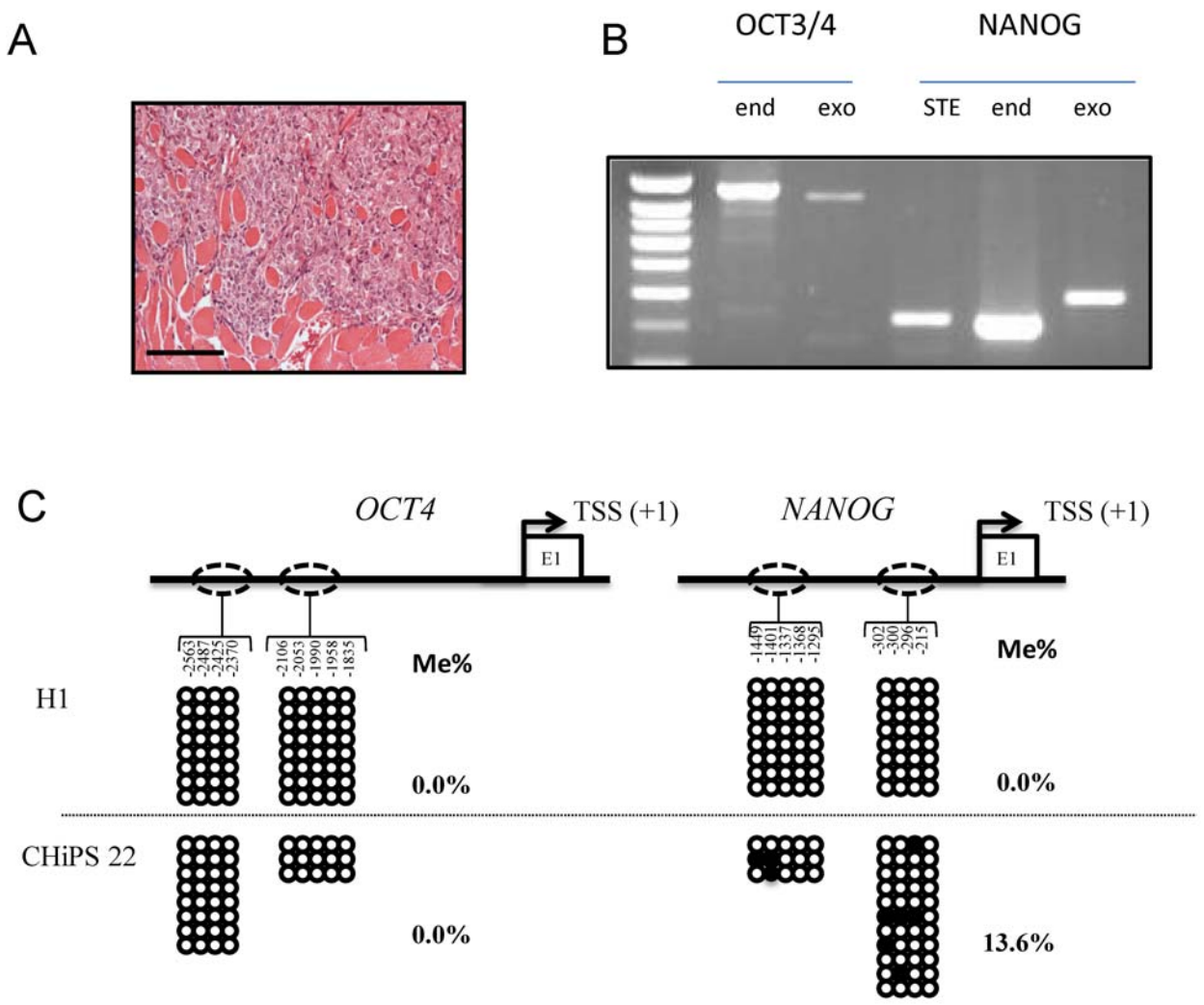

Fig. 5. Characterisation of CHiPS 22 line. (**A**) Haematoxylin and eosin staining of germ cell-like tumour derived from CHiPS 22 cells. Scale bar = 100 μm. (**B**) Expression of endogenous and exogenous OCT3/4 and NANOG in CHiPS 22 line by RT-PCR. Exogenous NANOG is expressed from two constructs, indicated by NANOG exo and STE (Stemcca). (**C**) Bisulphite genomic sequencing of OCT4 and NANOG promoters in CHiPS 22 and H1 lines. Each row represents an individual sequencing reaction for a given amplicon. Open and filled circles equal unmethylated and methylated CpGs dinucleotides, respectively. Percentage of methylation for each cell line is shown on the right.

and exogenous NANOG expression as well as GDF3 and STELLAR. DAZL and KIT were weakly expressed (Fig. 3D). However, KIT gave a very strong signal in the immunohistochemical assessment of the CHIPS A-derived tumor (Fig. 4A) as well as CD99, a known marker of primitive neuroectodermal and sex cord stromal tumours (Fig. 4B). In line with commonly increased proliferation in tumours (Soini *et al.*, 1998), the CHiPS A-derived tumours showed a higher proliferative index compared to the CHiPS W- and H1-derived teratomas (Fig. 4C).

To investigate whether priming the cells with NANOG could have activated the carcinogenic program during reprogramming, regardless of the choice of other reprogramming genes, we repeated human fibroblast reprogramming, first with *NANOG* and then with the set of four Yamanaka reprogramming factors (*OCT3/4, SOX2, MYC, KLF4*) expressed from one polycistronic vector (Sommer et al., 2009). The resulting iPSC line, the CHiPS 22, also failed to differentiate *in vitro*. Importantly, in the teratoma formation assay, the CHiPS 22 cells gave rise to germ cell-like tumours, as did the CHiPS A cells (Fig. 5A). Semi-quantitative RT-PCR analysis of pluripotency gene expression showed that similarly to the CHiPS A line, transgenes for *OCT3/4* and *NANOG* were not silenced, however, the relative quantities of the endogenous *versus* exogenous transcripts resembled those of the CHiPS W (Fig. 5B). Similar to the CHiPS W and by contrast to the CHiPS A, the OCT3/4 promoter of the CHiPS 22 was found hypomethylated (Fig. 5C). Therefore, we conclude that priming cells with NANOG before complete reprogramming might result in a germ cell-like tumour phenotype *in vivo,* regardless of the reprogramming factors used.

Molecular characterisation

It has been previously demonstrated that genomic abnormalities in hESC might lead to neoplastic progression (Werbowetski-Ogilvie *et al*., 2009). To investigate the

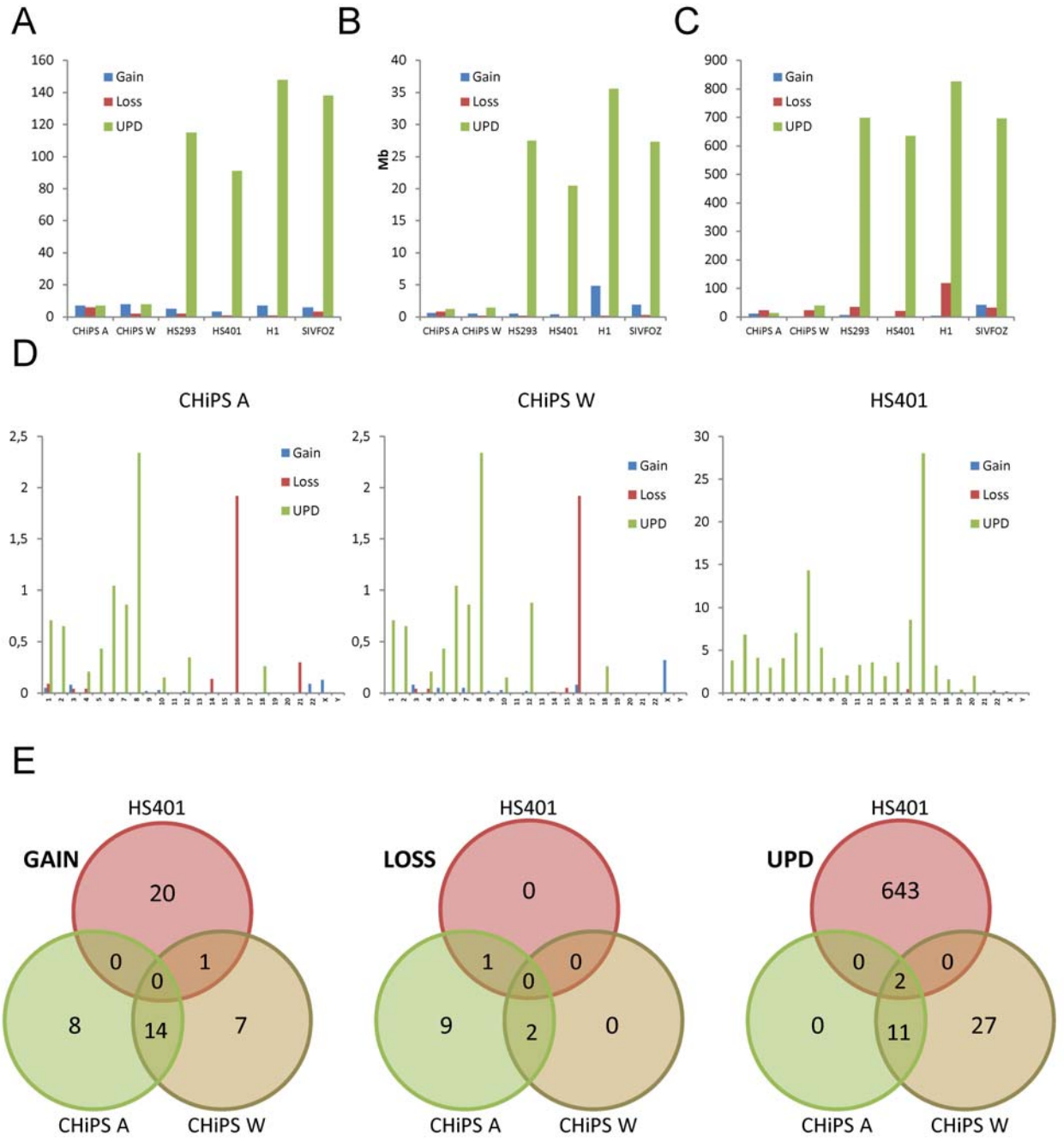

Fig. 6. Chromosomal variations in CHiPS A and CHiPS W lines. (**A**) Total number of variations, (**B**) genomic coverage of the variations and (**C**) total number of mutated genes. H1, HS293, HS401, SVF02 – human embryonic stem cell lines, HFF – human feeder fibroblasts. (**D**) Per chromosome coverage of genomic variations and (**E**) the number of genes in either deleted, duplicated or UPDs (uniparental disomies) regions in CHiPS A, CHiPS W and ES401 lines.

origin of the oncogenic potential of the CHiPS A line, array comparative genomic hybridisation (aCGH) was performed to evaluate the presence of genomic signatures. We compared the CHiPS A and CHiPS W lines with four hESC lines. This revealed some chromosomal variations, which were not detected previously with G banding. Surprisingly, the total observed abnormalities were minor, compared to the ESC lines (Fig. 6A,B). Detailed analysis of each chromosome showed that the gains and losses were mainly concentrated in chromosomes 1, 3, 14, 16, 21, 22, and X for CHiPS A and 3, 16, and X for CHiPS W (Fig. 6D). There were also multiple uniparental disomies affecting chromosomes 1, 2, 4, 5, 6, 7, 8, 9, 10, 12, and 18 in both lines in the same manner, suggesting a parental origin. Surprisingly, thorough examination revealed only a few genes present in the affected regions (Fig. 6C). Of these, 26 differed between the CHiPS A and the CHiPS W lines, 10 in the deleted regions and 16 in duplicated regions (Fig. 6E). Those genes were CHiPS A-specific, since they were also absent, apart from the olfactory receptor OR11H12, from the mutated regions of the control hESC line (HS401) (Table 2). Unexpectedly, the genes did not

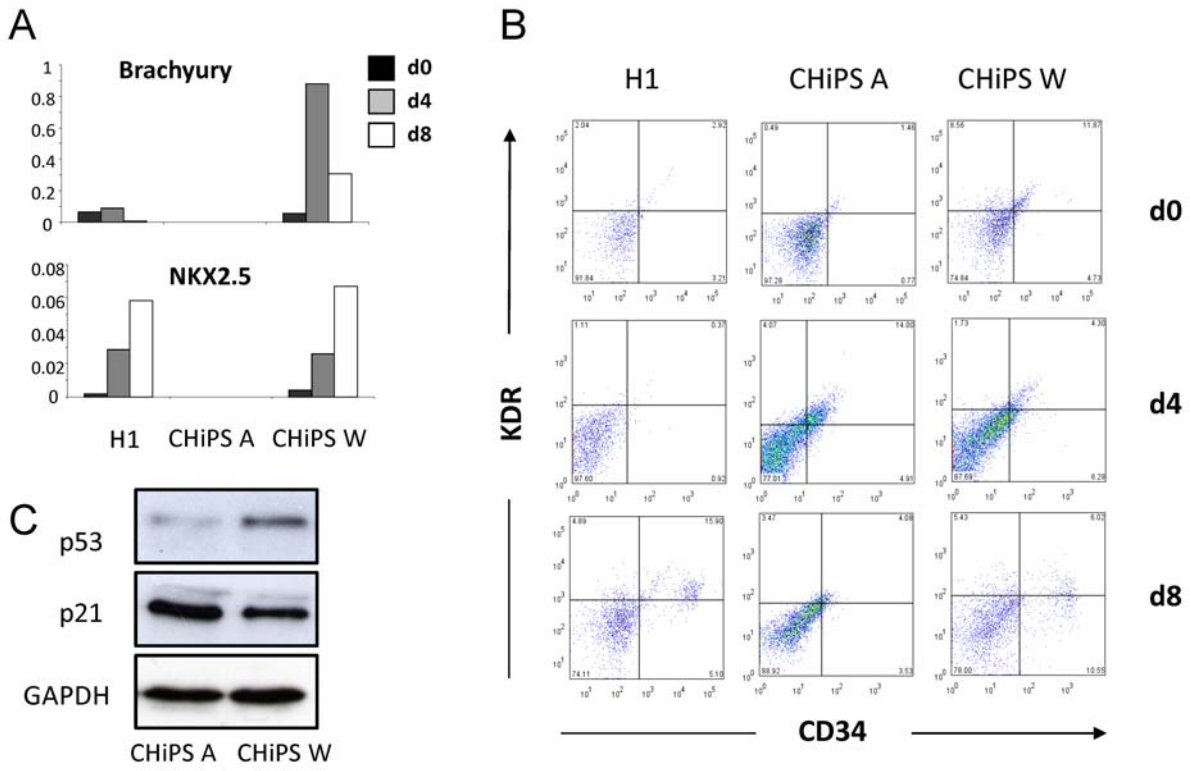

Fig. 7. Analysis of cardiac and haematopoietic differentiation, as well as p53 and p21 expression in CHiPS A and CHiPS W lines. (**A**) Differential quantitative expression of cardiac markers Brachyury and NKX2.5 at day 0, 4 and 8 of cardiac differentiation in CHiPS A, CHiPS W and H1 lines. (**B**) FACS analysis of the expression of the haematopoietic markers KDR and CD34 at day 0, 4 and 8 of haematopoietic differentiation. (**C**) Protein levels of p53 and p21 in CHiPS A and CHiPS W assessed by western blot, GAPDH – loading control.

include any tumour suppressor genes or oncogenes, nor did they encompass any known pluripotency genes (Table 2). The gene ontology analysis performed for three classes (Gene Ontology Molecular Function (GOMF), Biological Processes (GOBP) and Kegg pathways) showed multiple differences between the two lines, as summarised in Table 3. Of particular interest were potential defects in the cardiac and haematopoietic differentiation pathways in the CHiPS A line, as also revealed in the comparative pathway analysis between the CHiPS A and HS401 lines (Table 4).

To evaluate the prognostic values of aCGH analysis, we investigated whether the cells were capable of cardiac and directed haematopoietic differentiation *in vitro*, via embryoid body formation. In line with the aCGH data, quantitative analysis of the expression of the *TBX1*/*Brachyury*, a major regulator of cardiac mesoderm formation, and *NKX2-5*, a cardiopoietic marker, revealed their absence from the CHiPS A line during *in vitro* cardiac differentiation (Fig. 7A). Further differentiation led to beating cardiomyocytes in case of the CHiPS W (Chicha *et al*., 2011), but not in the CHiPS A, which failed to differentiate and dissociated over time. It has been previously shown that the CHiPS W line is capable of undergoing haematopoietic differentiation (Chicha *et al*., 2011). FACS analysis of differentiating embryoid bodies from the CHiPS W and the hESC H1 line showed an increase in the CD34+/KDR+ cell population with differentiation time; however, these vascular endothelial surface antigen markers were absent from the CHiPS A line (Fig. 7B).

The TP53 (p53) tumour suppressor is an established factor connecting tumourigenesis and reprogramming to pluripotency, (reviewed in Tapia and Scholer, 2010). Therefore, we analysed p53 and p21 protein levels in the CHiPS A and CHiPS W cell lines. In the normal, pluripotent CHiPS W cell line, the proportion of p53 to p21 was higher than in the tumourigenic CHiPS A cell line (Fig. 7C).

Discussion

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) offer great therapeutic promise. However, their potential tumourigenicity must be addressed before they can be taken into the clinic. In 1997, Bonnet and Dick proposed the new concept of cancer stem cells (CSCs) (Bonnet and Dick, 1997), a small selected population within a tumour, characterised by stem-like genomic and epigenetic signatures, including for instance OCT3/4 and NANOG expression. It is still unclear where CSCs originate, but reprogramming and carcinogenesis share many common features. For instance, deactivation of the antitumour p53-p21 barrier increases the yield of iPSC generation (Hong *et al.*, 2009). For the therapeutic use of stem cells in the future, it is of crucial importance to discriminate between normal and cancer stem cells; however, proper cellular models are still missing.

Table 2. List of the genes in the affected regions characteristic for a given line.

CHiPS A but not CHiPS W	CHiPS W but not CHiPS A	CHiPS A but not HS401
WDR63	ZDHHC11	WDR63
MCOLN3	BRD9	MCOLN3
SYDE2	FAM115A	SYDE2
GJA5	OR2F2	GJA5
GJA8	OR2F1	GJA8
GPR89B	OR2Q1P	GPR89B
NR5A2	PAOX	NR5A2
FAM58B	PCDH11X	FAM58B
GATA2		GATA2
OR11H12		C3orf27
NF1L6		RPN1
NF1L4		TMED10P2
TMPRSS15		MIR720
PPIAL3		UGT2B28
EFCAB6		RBPJP6
SULT4A1		ATP5A1P6
PNPLA5		MTG1
HMGN2L9		CYP2E1
		SPRN
		STX2
		RAN
		NF1L6
		NF1L4
		TMPRSS15
		PPIAL3
		EFCAB6
		SULT4A1
		PNPLA5
		HMGN2L9
		ZNF81
		ZNF182
		ZNF630
		SPACA5

In the present work, we generated two reprogrammed cell lines from human dermal fibroblasts. The first line, CHiPS W, had a differentiation potential comparable to a normal human ESC line while the other, CHiPS A, failed to undergo *in vitro* and *in vivo* differentiation and induced germ cell-like tumours, confirmed by marker expression analysis. We sought to characterise genomic variations at the genome-wide level and to associate these with the behaviour of the cell lines used in this study. G-banding was used to assess variations at the macroscopic level and, subsequently, Affymetrix SNP6.0 arrays were run to detect copy number variations (CNVs) at kb resolution. On this scale, no variations were detected that could be associated with tumour suppressor or oncogenes. Although a number of common structural variants, like InDels or short inversions, escape the methods used in this study, CNVs represent the predominant form of variation, and have the highest *de novo* mutation rate in the genome. Our results suggest that the role of genomic variations in determining the tumourigenic phenotype might not be a major one, which implies epigenetic-level mechanisms leading to tumourigenic characteristics. However, considering multiple reports of iPSCs genomic instability (Gore *et al.*, 2011; Hussein *et al.*, 2011; Laurent *et al.*, 2011; Mayshar *et al.*, 2010; Pasi *et al.*, 2011), it remains an open question whether the genomes of the derived cell lines remain stable over time.

The p53 tumour suppressor plays a pivotal role in cancer prevention (reviewed in Molchadsky *et al.*, 2010) but also, as recently demonstrated, in reprogramming somatic cells to pluripotency (Hong *et al*., 2009; Kawamura *et al.*, 2009; Utikal *et al.*, 2009). Recent microRNA profiling analysis of iPSCs and cancer cell lines also demonstrated differences in the status of the p53 network in these two cellular populations (Neveu *et al.*, 2010). Morigucchi *et al*. have proposed that a higher ratio of p53 to p21 might be responsible for shifting the balance from stemness to tumourigenicity in iPSCs (Moriguchi *et al.*,

Table 3. Process affected in CHiPS A as compared to CHiPS W.

embryonic heart tube development
positive regulation of phagocytosis
cell-cell junction assembly
embryonic placenta development
lens development in camera-type eye
blood vessel development
pituitary gland development
positive regulation of angiogenesis
cell fate determination
embryonic development
positive regulation of specific transcription from RNA polymerase II promoter
regulation of endocytosis
cell junction organization
cell maturation
embryonic limb morphogenesis
appendage morphogenesis
limb development
RNA polymerase II transcription factor activity enhancer binding
channel activity
scavenger receptor activity
steroid hormone receptor activity
sulfotransferase activity
sequence-specific DNA binding
Maturity onset diabetes of the young

2010). Our results do not support the model proposed by Moriguchi, as the ratio of p53 to p21 was lower in the tumourigenic CHiPS A line than in the CHiPS W line. The lack of differentiation within the CHiPS A-derived teratomas resembles tumours derived with p53-deficient iPSCs, using the four Yamanaka factors (Hong *et al*., 2009). Accordingly, we observed lower p53 protein expression in our tumourigenic line CHiPS A, compared to the CHiPS W. This is in accordance with the crucial role of p53 in limiting tumourigenicity. At the same time, the p53/p21 ratio in the CHiPS 22 line, contrary to the CHiPS A, was similar to the CHiPS W, suggesting that other mechanisms are involved (data not shown).

At this stage of investigation, we can only speculate on the possible molecular mechanism leading to the observed phenotype. Reprogramming of somatic cells into the CHiPS A and CHiPS W lines was achieved by initial pre-transduction with NANOG. A recent report by Tiemann *et al.* shows the importance of the stoichiometry of RGs in the reprogramming process (Tiemann *et al.*, 2011). It remains to be determined if the pre-selection with puromycin in the CHiPS W line significantly changed the final stoichiometry of the RGs, allowing better reprogramming into a stable pluripotent state as compared to the CHiPS A and CHiPS22 lines. Considering the Tiemann *et al.* data, it is plausible that a distinct stoichiometry of RGs controls not only full reprogramming, but also the balance between the pluripotent and tumourigenic phenotypes.

Although NANOG is not considered to be cancer inducing, it might have pre-induced the cell to additional

Table 4. Process affected in CHiPS A as compared to HS401.

cell separation during cytokinesis
microvillus assembly
embryonic heart tube development
protein amino acid N-linked glycosylation via asparagine
positive regulation of phagocytosis
drug metabolic process
cell-cell junction assembly
digestive system development
gut morphogenesis
mitotic spindle organization
acrosome reaction
embryonic placenta development
lens development in camera-type eye
pituitary gland development
plasma membrane fusion
positive regulation of angiogenesis
protein export from nucleus
blood vessel development
xenobiotic metabolic process
RNA export from nucleus
cell fate determination
androgen receptor signaling pathway
positive regulation of specific transcription from RNA polymerase II promoter
cytokinesis
regulation of endocytosis
dolichyl-diphosphooligosaccharide-protein glycotransferase activity
calcium-dependent protein binding
SNAP receptor activity
SNARE binding
glucuronosyltransferase activity transferring glycosyl groups
transferase activity
androgen receptor binding
oxidoreductase activity acting on paired donors with incorporation or reduction of molecular oxygen
oxidoreductase activity acting on paired donors with incorporation or reduction of molecular oxygen
RNA polymerase II transcription factor activity enhancer binding
oxygen binding
transcription regulator activity
scavenger receptor activity
steroid hormone receptor activity
Metabolism of xenobiotics by cytochrome P450
Drug metabolism - cytochrome P450
Pentose and glucuronate interconversions
Ascorbate and aldarate metabolism
Maturity onset diabetes of the young
Linoleic acid metabolism
SNARE interactions in vesicular transport
Porphyrin and chlorophyll metabolism
N-Glycan biosynthesis
Androgen and estrogen metabolism
Starch and sucrose metabolism

cancer pathway-activating changes. Considering that CHiPS 22, another iPSC line with similar characteristics, could be obtained using a different set of reprogramming factors but with the same pre-induction with NANOG, the molecular mechanism behind the process is probably NANOG-related. While it is known that NANOG expression suppresses differentiation in murine ESCs (Chambers *et al.*, 2003), a recent loss-of-function analysis in cancer cell lines has demonstrated a role of NANOG in tumour development (Jeter *et al.*, 2009). In addition, Lindgren *et al.* have shown that failed repression of *Nanog* in *Pten* null mouse ESCs leads to tumour initiation (Lindgren *et al.*, 2011). In germ cell tumours and derived cell lines, methylation of CpGs in the *NANOG* promoter has been correlated with the differentiation state (Nettersheim *et al.*, 2011). Consequently, the lack of silencing of endogenous and/or exogenous *NANOG* expression during the CHiPS A and the CHiPS 22 differentiation *in vitro* and in the tumours might be directly responsible for the observed phenotype. Whether pre-induction of fibroblasts with NANOG carried by excisable factors or other transient means would result in the same cellular phenotype remains to be determined.

We believe that the resulting phenotype is an outcome of complex and interrelated epigenetic changes. The role of NANOG seems to be crucial; however, CHiPS A and CHiPS 22 differ despite their similar phenotypes. For instance, the CHiPS A line lacks *SOX2* expression. Nevertheless the re-introduction of *SOX2* using a lentiviral vector did not rescue the phenotype and the new SOX2+ / CHiPS A line still failed to differentiate (data not shown). Furthermore, the tumourigenic CHiPS 22 line expresses SOX2, which altogether suggests that the molecular mechanism is not SOX2-dependent. Similarly, the CHiPS A line, in contrast to the CHiPS 22, overexpresses *OCT3/4* and, as previously demonstrated, ectopic expression of *OCT3/4* might block progenitor differentiation and cause carcinogenesis (Hochedlinger *et al.*, 2005). Likewise, human cancers often display altered expression of *MYC*, a proto-oncogene used here during the reprogramming of the CHiPS 22 line. It has also been demonstrated during reprogramming that the *MYC* oncogene leads to increased tumour transformation (Okita *et al.*, 2007). However, the CHiPS A was reprogrammed with the Thomson set of reprogramming genes, and its *MYC* expression was barely stimulated (data not shown), indicating that MYC is not directly responsible for the resulting carcinogenic behaviour.

Conclusion and Summary

The present data highlight five major points: priming with *NANOG* before reprogramming of human somatic cells can generate cells with the characteristics of iPS cells; despite their expression of pluripotency markers, such cell lines may fail to differentiate; *in vitro* differentiation failure might translate into germ cell-like tumour induction in the *in vivo* differentiation assay; the tumourigenic iPSC line might be genetically stable which implicates the involvement of epigenetic mechanisms in the activation of the carcinogenic program; one of the plausible mechanisms of carcinogenic program induction may involve the p21/p53 ratio.

Our results are the first describing iPSC lines that behave similarly to pluripotent cell lines *in vitro* but are unable to differentiate. These cells do however induce germ cell-like tumours *in vivo*. The tumourigenic character of the reprogrammed lines might result from epigenetic events that occurred during priming the cells with NANOG. It is therefore important to test derived iPSCs, not only at the molecular and cellular level, but also using an *in vivo* teratoma formation assay, which is essential in the assessment of the carcinogenic potential of the reprogrammed pluripotent stem cells. At the same time, we believe that our results might open new vistas in the area of stemness *versus* carcinogenesis, while the CHiPS A, CHiPS 22 and CHiPS W lines might serve as a model for comparing cellular signalling leading to normal and malignant conditions.

Acknowledgements

We would like to thank Sandrine Vianin for help with iPSC line derivation and technical support and Lasta Kocjancic-Curty for technical support. We are also grateful to Dr. Marie Cohen, Geneva University Hospital and Prof. Thanos Halazonetis, University of Geneva, for kindly sharing p53 and p21 antibodies, as well as Prof. Olivier Irion, Geneva University Hospitals for material support. This work was supported by the Swiss National Foundation (grant 310000-119938/1), the Roche Research Foundation, the Ernst & Lucie Schmidheiny Foundation, the Geneva University Hospitals, Genico, and the Swedish Research Council.

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Discussion with Reviewers

Reviewer I: Do the authors think that more epigenetic studies should be done?

Authors: Considering the increasing amount of data connecting tumourigenicity and epigenetic mechanisms it would be very interesting to investigate not only the genomics but also epigenomics of the generated lines. This would be particularly relevant in the context of NANOG but also the genes already known to participate in the processes of reprogramming. We believe that this kind of investigation if applied on the parental fibroblasts together with the "normal" and "tumourigenic" lines that we have generated, could provide interesting data on the mechanisms leading to malignancy.