differences in hematopoietic reconstitution abilities among human UCBs [13,14].

The NOD/SCID mouse system was first exploited by Gan et al. to compare human hematopoietic stem cells transplanted immediately after collection with those subjected to *ex vivo* culture with stromal cells; they found a decrease in the rate of repopulating cells after transplantation of cultured hematopoietic cells [13]. We transplanted UCBs into NOD/SCID mice and analyzed the hematopoietic cells present in peripheral blood and the bone marrow. We also determined the gene expression profiles of the $CD34^+$ UCB cells in their pre-transplantation condition, and searched for an expression signature that correlated with the success of transplantation of the $CD34^+$ UCB cells.

2. Materials and methods

2.1. CD34-positive cells

$CD34^+$ UCB cells were obtained from the Stem Cell Resource Network in Japan (Banks at Miyagi, Tokyo, Kanagawa, Aichi, and Hyogo) through the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan).

2.2. Mice

Seven-week-old female NOD/SCID mice were purchased from CLEA Japan (Tokyo, Japan). The mice were used within two weeks of delivery. Four to six hours prior to cell transplantation, the mice were given a 300 cGy dose of γ-rays.

2.3. Transplantation assay

$CD34^+$ cells (3×10^5 cells) from each sample of UCB were suspended in 600 μl MEM-α containing 10% fetal bovine serum (FBS); 200 μl of the suspension (1×10^5 cells) was then injected into the tail vein of each of three NOD/SCID mice. This procedure was repeated for each of the 12 UCB samples.

2.4. Flow cytometry

Twelve weeks after transplantation, peripheral blood samples were obtained from the retro-orbital venous plexus, and bone marrow cells were obtained after sacrifice. The peripheral blood and bone marrow cells were stained with monoclonal antibodies (MoAbs) and analyzed by FACS Calibur (BD Biosciences, San Jose, CA, USA). The red blood cells in the peripheral blood samples were lysed using red blood cell lysis buffer (140 mM NaCl, 1 mM $NaHCO_3$) prior to cell staining. The following MoAbs were purchased from BD Biosciences: a fluorescein isothiocyanate (FITC)-conjugated MoAb against human CD45 (CD45-FITC), a phycoerythrin (PE)-conjugated MoAb against human CD34 (CD34-PE), an allophycocyanin (APC)-conjugated MoAb against mouse CD45 (mCD45-APC), CD19-PE, CD33-APC, Glycophorin A-FITC, and TER119-PE. Cell viability was determined after propidium iodide (SIGMA, St Louis, MO, USA) staining. Data from $1x10^4$ living cells were collected and analyzed using CellQuest Pro (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA) analysis software. The rate of chimerism (%) was calculated from the flow cytometry data as follows: rate of chimerism of human cells (%) = [% human $CD45^+$ cells/(% human $CD45^+$ cells + % mouse $CD45^+$ cells)] × 100.

2.5. Oligonucleotide microarray analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) from an aliquot of each sample of human cryopreserved $CD34^+$ UCB cells at the time of thawing. Then, 250 ng of each total RNA was subjected to reverse transcription and isothermal linear amplification using Ribo-SPIA (NuGEN, San Carlos, CA, USA) [15], using a modification of the manufacturer's recommended protocol. The linearly amplified cDNAs served as templates for the *in vitro* transcription generating hybridization target cRNAs using the Low RNA Fluorescent Linear Amplification Kit Plus (Agilent, Santa Clara, CA, USA). The amplified cRNAs were labeled with Cy3 dye and used for hybridization to the oligonucleotide microarray (Agilent human whole genome 4 × 44) following the manufacturer's protocols. Hybridization signals were scanned with the Agilent Technologies Scanner G2505C (Agilent) and were extracted from the scanned images by the use of Feature Extraction Ver. 9.5.3 (Agilent). All microarray data reported in this paper is described in accordance with MIAME guidelines and the data has been deposited in the GEO (Gene Expression Omnibus) database at the National Center for Biological Information, National Institute of Health (USA). The accession number for the dataset is GSE19835.

2.6. Normalization of gene expression profiles

Quality control and array normalization was performed in the R statistical environment (http://www.r-project.org) using the Agi4x44PreProcess package downloaded from the Bioconductor web site (http://bioconductor.org/). The data files were appropriately edited with text editing software to render the files compatible for the Agi4x44PreProcess packages. The normalization and filtering steps were based on those described in the Agi4x44PreProcess reference manual.

2.7. Statistical analyses

A heat map of differentially expressed genes was generated using Gene Cluster 3.0 software [16] and visualized with TreeView software [17]. Overexpressed genes specific for each phenotype were identified by Student's *t*-tests, and potential false-positives were removed by the Benjamini–Hochberg method. The GSEA analysis [18] was carried out using GSEA Java desktop software (version 2.04, http://www.broadinstitute.org/gsea/). The C2 curated gene set and C2-all gene set for GSEA analysis were retrieved from the Molecular Signatures Database (MSigDB: http://www.broadinstitute.org/gsea/downloads.jsp). The DAVID functional annotation system was used as described by Huang et al. [19].

2.8. Quantification of RNAs using real-time PCR

Real-time PCR was performed using the ABI 9500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) and each amplification reaction was performed in quadruplicate. For quantification of miRNAs, the cDNAs prepared for the microarray analysis were used as templates and PCR was performed with SYBR premix Ex Taq (Perfect Real Time: Takara Bio Inc., Shiga, Japan) with GAPDH as the loading control. The nucleotide sequences used for PCR amplification are given in *Supplementary* Table 1.

3. Results

3.1. Hematopoiesis reconstitution in immunodeficient mice xenografted with $CD34^+$ cells

We first compared the relative abilities of 12 samples of $CD34^+$ UCB cells from different donors to form engraftments in immunodeficient NOD/SCID mice. Each animal was injected with 1×10^5 cells; the cells from each donor were injected into three mice and the remaining cells were used for the microarray gene expression

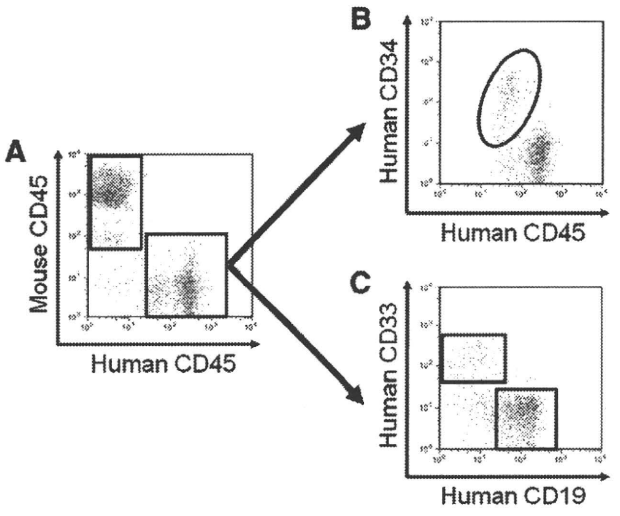

Fig. 1. Classification of human and mouse hematopoietic cells. (A) Separation of human and mouse hematopoietic cells from the bone marrow of a mouse xenograft by flow cytometry. The horizontal axis and the vertical axis indicate the signal intensities for human CD45- and mouse CD45-specific antibodies, respectively. (B) Quantification of human $CD34^+$ cells among human $CD45^+$ cells. (C) Quantification of human $CD19^+$ and $CD33^+$ hematopoietic cells among human $CD45^+$ cells. (A–C) Examples of gating are indicated.

analysis. Twelve weeks after cell transplantation, peripheral blood cells were collected from each mouse. After collection of peripheral blood, the mice were sacrificed and bone marrow cells were obtained. The presence and relative numbers of human hematopoietic cells in the peripheral blood and bone marrow were determined by flow cytometry (Fig. 1). The rate of chimerism for human hematopoietic cells was calculated as the proportion of human $CD45^+$ cells in all leukocytes in the peripheral blood and bone marrow, respectively (Fig. 1A and Section 2).

Although all of the 12 $CD34^+$ UCB samples produced human $CD45^+$ cells in at least one of the three recipient mice (>0.1% chimerism), three samples (H07041, H07056, and H07112) produced very small numbers of human $CD45^+$ cells in both the peripheral blood and bone marrow of all recipient mice; we designated these three samples as "failed UCBs" (Fig. 2). The other nine $CD34^+$ UCB samples established obvious engraftments in at least one of the recipient mice; we designated these nine samples as "successful UCBs" (Fig. 2). However, the frequency of human $CD45^+$ cells varied among the successful UCBs. For example, UCB H07088 and UCB H07133 produced rates of 6.88–35.8% and 90.0–94.3%, respectively, in the bone marrow of the mice (Fig. 2, *Supplementary* Table 2). The frequencies of $CD34^+$, $CD19^+$ and $CD33^+$ cells in the human $CD45^+$ cells were also calculated for peripheral blood and bone marrow samples that had more than 3% human $CD45^+$ cell chimerism (Fig. 1B and C, and *Supplementary* Table 2). The proportions of lymphoid and myeloid cells among the human $CD45^+$ cells in the peripheral blood and bone marrow of mice that received one of the nine successful UCBs also varied (*Supplementary* Table 2).

3.2. Gene expression profiles of the 12 human UCB samples

We sought to determine if there was any connection between the gene expression profiles of the UCBs and their abilities to achieve successful engraftment. Gene expression profiles were determined by oligonucleotide microarray analyses using total RNAs from the UCB cells. The amount of total RNA obtained from each aliquot (generally several nanograms) was insufficient to perform the assay without a further *in vitro* amplification step. After amplification of the cDNAs, complementary RNAs were used for hybridization. Probes that were positive in 75% of all samples were selected for further analyses; in total, 23,807 of the 45,015 probes tested were selected.

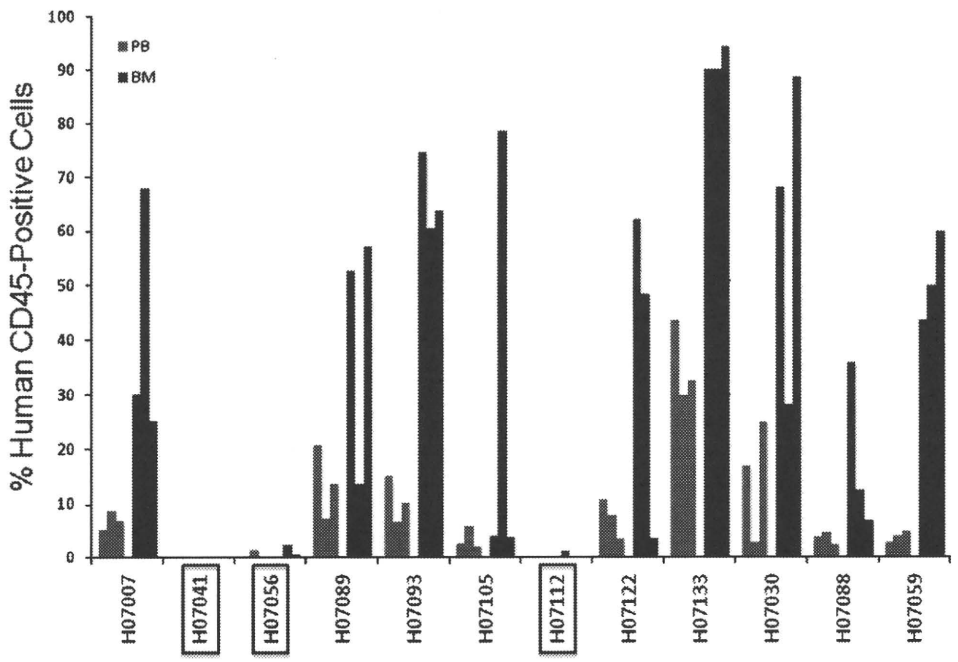

Fig. 2. Chimerism in irradiated NOD/SCID mice following injection of cryopreserved human $CD34^+$ UCB cells. The bar graphs indicate the percentages of human $CD45^+$ cells (see Fig. 1A and Section 2) from either the bone marrow (purple bar) or the peripheral blood (blue bar) of mouse xenografts. Each bar represents a single mouse. The names under the horizontal axis indicate the different human UCB samples and the boxed names indicate those that failed to engraft (see text).

Table 1
Genes that are overexpressed in successful or failed UCBs.

Gene Symbol	Accession Number	ENTREZ ID	Category	*t*-Test	Fold change
Overexpressed in successful UCBs					
DNHD1	AK074178	144132	Dynein heavy chain domain 1	0.003748741	4.754
HOXB4	NM_024015	3214	Homeobox B4	0.001103195	4.548
SMC1A	NM_006306	8243	Structural maintenance of chromosomes 1A	0.000781411	4.378
MED1	BC060758	5469	Mediator complex subunit 1	0.00311929	4.041
SNRNP48	NM_152551	154007	Small nuclear ribonucleoprotein 48 kDa (U11/U12)	4.04958E-05	3.252
ZNF12	NM_016265	7559	Zinc finger protein 12	0.000123475	3.079
CEBPB	NM_005194	1051	CCAAT/enhancer binding protein (C/EBP), beta	0.000592075	3.064
CASKIN1	NM_020764	57524	CASK interacting protein 1	7.18248E-05	3.039
CYFIP2	NM_001037332	26999	Cytoplasmic FMR1 interacting protein 2	0.000128743	2.849
IL17D	NM_138284	53342	Interleukin 17D	8.5289E-05	2.827
HIST2H2AB	NM_175065	317772	Histone cluster 2, H2ab	0.0018657	2.820
C9orf102	NM_020207	375748	Chromosome 9 open reading frame 102	0.004144549	2.788
ZNF331	NM_018555	55422	Zinc finger protein 331	0.000439891	2.775
DKK3	NM_015881	27122	Dickkopf homolog 3 (*Xenopus laevis*)	0.003874938	2.735
CAMKK2	NM_172215	10645	Calcium/calmodulin-dependent protein kinase kinase 2, beta	0.000346607	2.689
C2orf30	NM_015701	27248	Chromosome 2 open reading frame 30	0.003991442	2.652
CDC25A	NM_001789	993	Cell division cycle 25 homolog A (*S. pombe*)	0.000536286	2.634
OXSR1	NM_005109	9943	Oxidative-stress responsive 1	2.57832E-06	2.600
RIN3	NM_024832	79890	Ras and Rab interactor 3	0.000346183	2.582
EEA1	NM_003566	8411	Early endosome antigen 1	3.31698E-05	2.567
HLA-DRB5	NM_002125	3127	Major histocompatibility complex, class II, DR beta 5	0.001474265	2.548
GOT1	AL581249	2805	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	0.003025096	2.529
AGRN	NM_198576	375790	Agrin	0.004551986	2.502
PPM1D	NM_003620	8493	Protein phosphatase 1D magnesium-dependent, delta isoform	0.001089659	2.481
ZBED1	NM_004729	9189	Zinc finger, BED-type containing 1	0.001104421	2.480
ZFAND5	NM_006007	7763	Zinc finger, AN1-type domain 5	0.000927518	2.465
ETS2	NM_005239	2114	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	0.000142473	2.459
PTPMT1	BC020242	114971	Protein tyrosine phosphatase, mitochondrial 1	5.88749E-06	2.421
ARHGAP1	NM_004308	392	Rho GTPase activating protein 1	0.003940737	2.403
PRICKLE3	NM_006150	4007	Prickle homolog 3 (*Drosophila*)	0.000505149	2.371
SH3BP4	NM_014521	23677	SH3-domain binding protein 4	0.002583403	2.353
WIPF2	NM_133264	147179	WAS/WASL interacting protein family, member 2	0.003588251	2.317
OTUD1	AB188491	220213	OTU domain containing 1	0.001538639	2.299
DGKD	NM_152879	8527	Diacylglycerol kinase, delta 130 kDa	0.000501851	2.297
RNF31	NM_017999	55072	Ring finger protein 31	0.000146202	2.289
NSFL1C	NM_182483	55968	NSFL1 (p97) cofactor (p47)	0.002998104	2.288
AZIN1	NM_015878	51582	Antizyme inhibitor 1	0.002484085	2.264
ASXL1	NM_015338	171023	Additional sex combs like 1 (*Drosophila*)	0.001918932	2.255
LRRC8A	NM_019594	56262	Leucine rich repeat containing 8 family, member A	0.000265201	2.251
MANBA	NM_005908	4126	Mannosidase, beta A, lysosomal	0.001475313	2.245
PCBD1	NM_000281	5092	Pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	0.001161179	2.242
GNB1L	NM_053004	54584	Guanine nucleotide binding protein (G protein), beta polypeptide 1-like	0.000379775	2.241
NMRAL1	NM_020677	57407	NmrA-like family domain containing 1	0.000664578	2.239
EIF1AY	NM_004681	9086	Eukaryotic translation initiation factor 1A, Y-linked	0.001956033	2.234
ASAP1	NM_018482	50807	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1	0.000730755	2.231
C18orf10	NM_015476	25941	Chromosome 18 open reading frame 10	0.000138316	2.228
ZNF330	NM_014487	27309	Zinc finger protein 330	0.00275585	2.216
ATP5G3	NM_001002258	518	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	0.000183274	2.211
OGDH	NM_002541	4967	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	0.000577585	2.211
PIGY	NM_001042616	84992	Phosphatidylinositol glycan anchor biosynthesis, class Y	0.000395444	2.180
MLL5	NM_182931	55904	Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, *Drosophila*)	0.001801175	2.172

PAIP1	NM_006451	10605	Poly(A) binding protein interacting protein 1	0.001278086	2.168
SCYL2	NM_017988	55681	SCY1-like 2 (*S. cerevisiae*)	3.57747E-05	2.160
MCTS1	AK096956	28985	Malignant T cell amplified sequence 1	0.000323927	2.137
TRNAU1AP	NM_017846	54952	tRNA selenocysteine 1 associated protein 1	0.001160239	2.137
MAP1A	NM_002373	4130	Microtubule-associated protein 1A	0.001014758	2.131
TNIP1	NM_006058	10318	TNFAIP3 interacting protein 1	0.000254844	2.130
PPP3CC	NM_005605	5533	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	0.0004302	2.111
CDC45L	NM_003504	8318	CDC45 cell division cycle 45-like (*S. cerevisiae*)	0.004108453	2.107
KIAA0841	AB020648	23354	KIAA0841	0.00307293	2.095
MTERFD1	NM_015942	51001	MTERF domain containing 1	0.002752437	2.094
INTS12	NM_020395	57117	Integrator complex subunit 12	0.001394525	2.081
EZH2	NM_004456	2146	Enhancer of zeste homolog 2 (*Drosophila*)	0.000585999	2.078
AGPAT3	NM_020132	56894	1-Acylglycerol-3-phosphate O-acyltransferase 3	0.003953771	2.077
PDE6B	NM_000283	5158	Phosphodiesterase 6B, cGMP-specific, rod, beta	0.002411658	2.061
POMGNT1	NM_017739	55624	Protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase	0.001779407	2.054
ACOX1	NM_004035	51	Acyl-coenzyme A oxidase 1, palmitoyl	0.002677004	2.054
BCORL1	AK021694	63035	BCL6 co-repressor-like 1	0.000131308	2.042
ARMC8	AL096748	25852	Armadillo repeat containing 8	0.003651856	2.033
ARMC1	NM_018120	55156	Armadillo repeat containing 1	0.001292391	2.021
SPOCK2	NM_014767	9806	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	0.003554045	2.017
Overexpressed in failed UBCs					
SSBP3	NM_001009955	23648	Single stranded DNA binding protein 3	0.003603576	4.842
ALKBH6	NM_198867	84964	alkB, alkylation repair homolog 6 (*E. coli*)	0.001413295	3.318
DHX57	NM_198963	90957	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	0.00170115	2.968
NIP30	NM_024946	80011	NEFA-interacting nuclear protein NIP30	0.000183566	2.725
TMEM134	NM_025124	80194	Transmembrane protein 134	0.003386533	2.516
RNPC3	AK057799	55599	RNA-binding region (RNP1, RRM) containing 3	5.00896E-05	2.505
ELK4	NM_001973	2005	ELK4, ETS-domain protein (SRF accessory protein 1)	0.001518504	2.471
TMEM216	NM_016499	51259	Transmembrane protein 216	0.000180326	2.432
GPR150	NM_199243	285601	G protein-coupled receptor 150	0.000487991	2.370
ZFP112	NM_013380	7771	Zinc finger protein 112 homolog (mouse)	4.54855E-05	2.359
ATM	NM_000051	472	Ataxia telangiectasia mutated	0.000542207	2.300
BCAT1	NM_005504	586	Branched chain aminotransferase 1, cytosolic	0.000193566	2.289
PHF2	NM_005392	5253	PHD finger protein 2	0.000820897	2.227
IRX3	NM_024336	79191	Iroquois homeobox 3	0.002209041	2.220
ZNF490	NM_020714	57474	Zinc finger protein 490	0.00040782	2.153
LOC442211	XR_019545	442211	Similar to Vacuolar ATP synthase 16 kDa proteolipid subunit	0.003343215	2.147
PECAM1	NM_000442	5175	Platelet/endothelial cell adhesion molecule	0.000314337	2.131
STON2	NM_033104	85439	Stonin 2	0.000675103	2.096
GSTM2	NM_000848	2946	Glutathione S-transferase mu 2 (muscle)	0.001806696	2.049
GTF2H1	NM_005316	2965	General transcription factor IIH, polypeptide 1, 62 kDa	0.003923314	2.034
DCLRE1A	NM_014881	9937	DNA cross-link repair 1A (PSO2 homolog, *S. cerevisiae*)	0.001829853	2.032
AGAP1	NM_001037131	116987	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	0.000611158	2.027
HCN4	NM_005477	10021	Hyperpolarization activated cyclic nucleotide-gated potassium channel 4	0.001048363	2.002

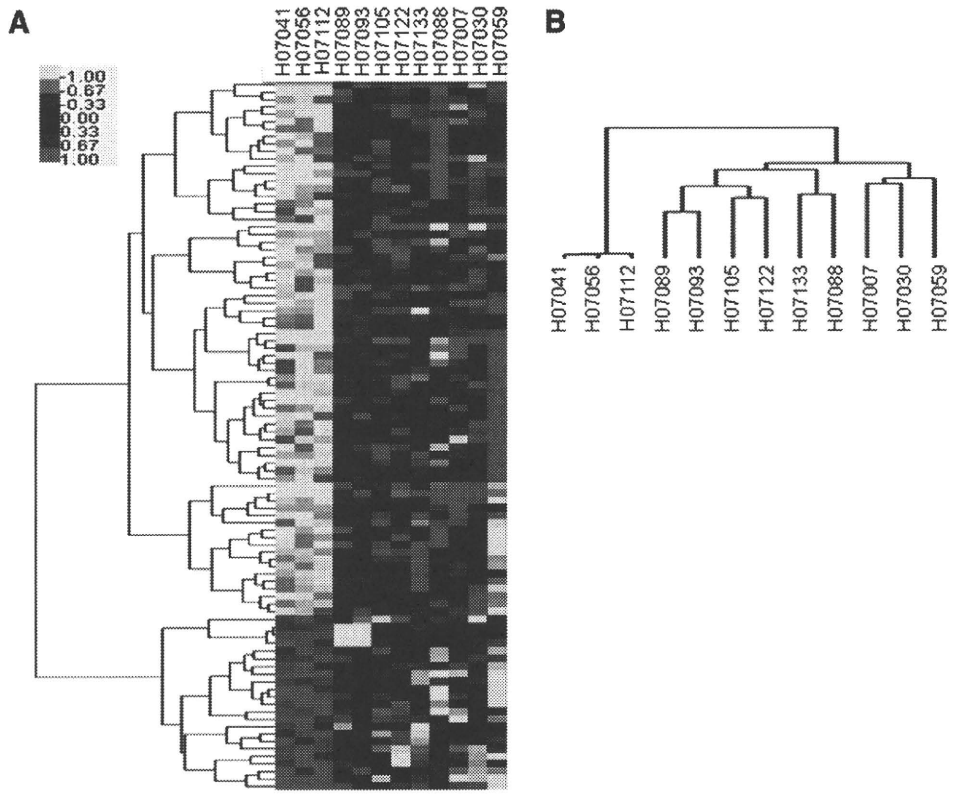

Fig. 3. Differentially expressed genes in successful and failed UCB samples. (A) Gene expression heat map of human UCB samples showing differential expression in successful and failed UCB samples. The map shows genes whose expression showed a larger than 2-fold increase or decrease and were statistically significant ($p < 0.005$) (see Table 1). Genes that were expressed at above or below the average level of the twelve samples are indicated in red and green, respectively. The color bar is the logarithmic indicator of the fold difference of each gene expression from the average, i.e., 1.0 means a larger than 2-fold increase and −1.0 means a less than 0.5-fold decrease. (B) Dendrogram of human UCB gene expression profiles constructed using the differentially expressed genes.

Initially, we employed a hierarchical clustering analysis to profile gene expression in the human $CD34^+$ UCB cells. However, this analysis did not separate successful and failed UCBs (data not shown). We concluded that our collection of UCBs might not be large enough to extract biologically meaningful signatures from gene expression profiles through an unsupervised approach.

In order to identify candidate genes responsible for success or failure of engraftment, we compiled a list of genes showing differential expression between successful and failed UCBs. A combination of relative level (fold change) of expression and statistical significance (Student's *t*-test) was used to distinguish these genes. Genes that showed a larger than 2-fold increase or decrease in gene expression between successful and failed UCBs, and also showed a statistically significant difference ($p < 0.005$) are listed in Table 1.

In total, 71 genes were found to be upregulated in successful UCBs and 23 in failed UCBs (Table 1). Many of the genes showing upregulation in successful UCBs are important for cell growth and differentiation in hematopoietic cells, such as *HOXB4, ETS2, CDC45L*, and *SMC1A* (Table 1).

In the expression heat map for the genes listed in Table 1, genes that expressed above or below the average level of the twelve UCB samples are indicated in red or green, respectively (Fig. 3A). The dendrogram was obtained by cluster analyses based on the differentially expressed genes (Fig. 3B). The graph indicates that failed UCBs were clearly separate from successful UCBs and consisted of a single cluster (Fig. 3B).

3.3. GSEA and DAVID analyses confirm upregulation of cell growth related genes in successful UCBs

Following identification of differentially expressed genes, we performed GSEA analyses in order to obtain biologically relevant insights. Four gene sets were selected as specifically enriched in

Table 2
Gene sets significantly overrepresented in successful and failed UCBs.

Gene set name	# of genes	ES	NES	NOM p-val	FDR q-val	FWER p-val
Enriched in successful UCBs						
SCHUMACHER MYC UP	53	0.561	2.085	0.000	0.026	0.021
ZHAN MM CD138 PR VS REST	23	0.648	2.007	0.000	0.048	0.077
P21_ANY_DN	22	0.611	1.859	0.000	0.193	0.473
UVB_NHEK2_DN	74	0.477	1.869	0.000	0.228	0.442
Enriched in failed UCBs						
DAC_PANC50_UP	26	−0.597	−1.976	0.000	0.127	0.167

successful UCBs (Table 2). Three of the four sets of genes have an unambiguous role in cell growth activity: the SCHUMACHER_MYC_UP gene set consists of downstream genes of the *MYC* oncogene in B-cells [20]; the ZHAN_MM_CD138_PR_VS_REST gene set is overexpressed in multiple myelomas with poor prognosis [21]; the P21_ANY_DN gene set includes genes that are downregulated by the tumor suppressor p21 [22]. However, it is currently not clear whether the UVB_NHEK2_DN gene set is active in cell growth [23]. The results of the GSEA analysis suggest that successful UCBs may be more committed to cell growth than the failed UCBs. All gene sets that were significantly enriched in either successful or failed UCBs are listed in *Supplementary* Table 3.

The DAVID annotation service was also employed for the functional analysis of differentially regulated genes in successful and failed UCBs. For this analysis, we chose a 1.5-fold increase or decrease in gene expression with $p < 0.005$ as the criteria for gene selection from successful and failed UCBs expression profiles. These criteria resulted in the selection of 577 and 327 genes, respectively, from the gene expression profiles of successful and failed UCBs (*Supplementary* Table 4). The GO terms for the biological processes of the significantly enriched genes (fold enrichment >1.5, FDR <5%) for both groups are given in Table 3. From examination of Table 3, it is clear that the successful UCBs had high expression of cell cycle related genes, such as those in "GO:0007049 cell cycle", whereas developmental and morphogenesis-related genes were upregulated in failed UCBs.

3.4. *HOXB4* and other cell cycle related genes are upregulated in successful UCBs

Next, we sought to confirm the conclusion from the microarray analysis that cell growth related genes were overexpressed in successful UCBs. We performed real-time RT-PCR analyses (qPCR) of four genes of interest: *CDC45L*, *C/EBP-β*, *ETS2*, and *HOXB4*. Due to the limited amount of cDNA available, only samples from six UCB samples (H07007, H07041, H07056, H07089, H07093, and H07015) could be used for qPCR. Of these, H07041 and H07056 are failed UCBs. We found that the amount of normalized qPCR product from the four mRNAs showed a good correlation with the signal intensity of the corresponding microarray probes (Fig. 4).

4. Discussion

The present study indicated that $CD34^+$ cells from 12 different human UCBs showed various abilities to reconstitute hematopoiesis in sublethally irradiated NOD/SCID mice. Gene expression profiling of these UCBs suggested that those that were successful at engraftment had increased expression of genes associated with cell growth compared to failed UCBs. To date, this is the first report to describe a relationship between the engraftment ability of mouse xenografts and gene expression profiles in human $CD34^+$ UCB cells. Indeed, our results suggest that the gene expression profile of human $CD34^+$ UCB cells reflect their potential for successful establishment of hematopoiesis in UCB transplantation.

There are several reports describing the gene expression profiles of human UCBs [24–27]. However, these studies provided no information on the relationship between gene expression profiles in human $CD34^+$ UCB cells and their relative abilities for bone marrow engraftment in mouse xenografts. We found that different cryopreserved human $CD34^+$ UCB cells varied in the extent of engraftment they yielded in mouse xenografts. This variation raises the question of what factors determine successful engraftment by cryopreserved human $CD34^+$ UCB cells? It is well known that the relative numbers of hematopoietic stem cells is a critical quality factor for UCBs [5,8,10]. The study by Cairo et al. further showed that the colony formation activity of human UCBs was correlated with ethnicity, sex, and the delivery methods of the donors [11]. It is an open question as to why these factors should be correlated with the number of stem cells in UCBs.

Several transcription factors are candidate mediators of cell growth for human $CD34^+$ UCB cells, for example, *HOXB4* and *ETS2*, which were upregulated more than 2-fold in successful UCBs compared to failed UCBs (Table 1). *HOXB4* is a major factor for the growth and maintenance of 'stemness' in embryonic stem cells [28]. Several groups have reported that introduction of *HOXB4* into UCB cells contributed to the *ex vivo* expansion of cell numbers [29,30]. *ETS2* is an oncogene that plays critical roles in cell growth signal transduction in various tissues [31–33]. Our results here are compatible with the known characteristics of these transcription factors.

C/EBP-β is another transcription factor upregulated in successful UCBs. The *C/EBP-β* is a critical factor for cell differentiation and expansion of the number of progenitor cells committed to the B-cell lineage; it also promotes tumor growth in several types of malignancies [34]. In contrast, the tumor suppressor *ATM* was included in the set of upregulated genes in the failed UCBs. *ATM* is activated by DNA damage and can induce cell cycle arrest [35].

Our experimental approach demonstrates the practicality of molecular assessment of the quality of human $CD34^+$ UCB cells. At present, it is not clear whether any candidate cell surface

Table 3
GO terms for biological processes enriched in successful and failed UCBs (>1.5-fold, FDR <5%).

GO category	Term	Count	%	*p*-Value	Fold enrichment	FDR (%)
Enriched in successful UCBs						
GO:0006512	Ubiquitin cycle	38	6.60	0.00	2.51	0.00
GO:0043687	Post-translational protein modification	71	12.33	0.00	1.59	0.19
GO:0006888	ER to Golgi vesicle-mediated transport	9	1.56	0.00	4.78	1.03
GO:0006281	DNA repair	19	3.30	0.00	2.42	1.80
GO:0007049	Cell cycle	44	7.64	0.00	1.67	1.86
GO:0046907	Intracellular transport	37	6.42	0.00	1.76	2.13
GO:0065003	Macromolecular complex assembly	31	5.38	0.00	1.88	2.17
GO:0015031	Protein transport	36	6.25	0.00	1.76	2.70
GO:0048193	Golgi vesicle transport	11	1.91	0.00	3.40	2.78
GO:0022607	Cellular component assembly	32	5.56	0.00	1.81	3.37
GO:0000074	Regulation of progression through cell cycle	29	5.03	0.00	1.87	3.43
GO:0051726	Regulation of cell cycle	29	5.03	0.00	1.86	3.71
Enriched in failed UCBs						
GO:0019222	Regulation of metabolic process	60	20.34	0.00	1.54	0.66
GO:0009653	Anatomical structure morphogenesis	29	9.83	0.00	1.96	1.47
GO:0031323	Regulation of cellular metabolic process	57	19.32	0.00	1.52	1.53
GO:0050793	Regulation of developmental process	11	3.73	0.00	3.25	4.04

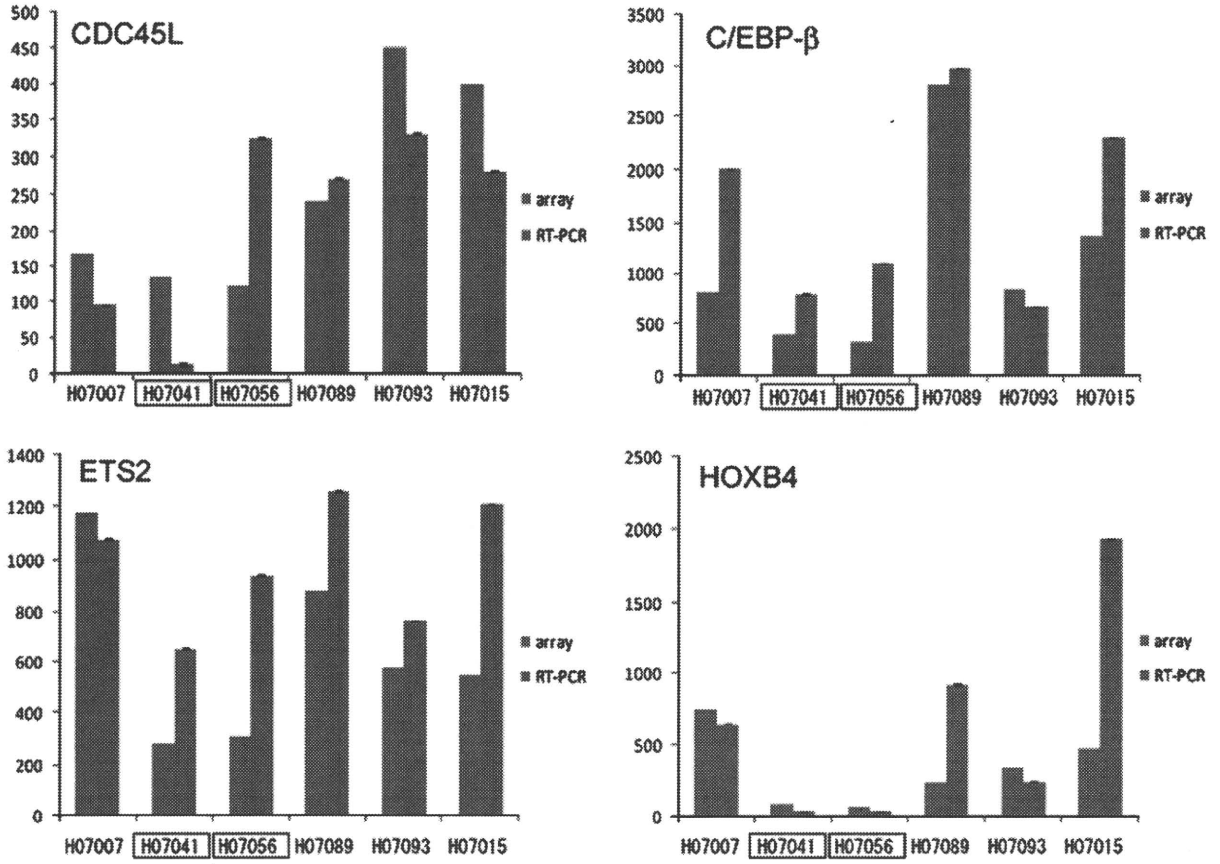

Fig. 4. A quantitative RT-PCR analysis was used to evaluate overexpressed genes in the successful UCB samples: the results for *CDC45L*, *C/EBP-β*, *ETS2*, and *HOXB4* are shown. The vertical axis indicates the relative expression ratio of each gene normalized against GAPDH. The names under the horizontal axis indicate the different human UCB samples and the boxed names indicate those that failed to engraft (see text).

markers for successful UCBs are included among the identified upregulated genes (Table 2). Nevertheless, it will be valuable to establish robust molecular markers for potentially successful $CD34^+$ UCB cells using functional gene expression profiling.

5. Conclusions

The quality of cryopreserved human $CD34^+$ UCB cells was variable and their respective gene expression profiles might reflect these qualitative differences and provide clinically relevant and versatile surrogate markers for human $CD34^+$ UCB cell quality. In addition, the results in this study suggest that cell growth is an important trait for the successful engraftment of human $CD34^+$ UCB cells.

Support and financial disclosure declaration

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.010.

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Check your cultures! A list of cross-contaminated or misidentified cell lines

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Mini Review

Continuous cell lines consist of cultured cells derived from a specific donor and tissue of origin that have acquired the ability to proliferate indefinitely. These cell lines are well-recognized models for the study of health and disease, particularly for cancer. However, there are cautions to be aware of when using continuous cell lines, including the possibility of contamination, in which a foreign cell line or microorganism is introduced without the handler's knowledge. Cross-contamination, in which the contaminant is another cell line, was first recognized in the 1950s but, disturbingly, remains a serious issue today. Many cell lines become cross-contaminated early, so that subsequent experimental work has been performed only on the contaminant, masquerading under a different name. What can be done in response—how can a researcher know if their own cell lines are cross-contaminated? Two practical responses are suggested here. First, it is important to check the literature, looking for previous work on cross-contamination. Some reports may be difficult to find and to make these more accessible, we have compiled a list of known cross-contaminated cell lines. The list currently contains 360 cell lines, drawn from 68 references. Most contaminants arise within the same species, with HeLa still the most frequently encountered (29%, 106/360) among human cell lines, but interspecies contaminants account for a small but substantial minority of cases (9%, 33/360). Second, even if there are no previous publications on cross-contamination for that cell line, it is essential to check the sample itself by performing authentication testing.

Key words: authentication, cell culture, cell lines, cross-contamination, DNA profiling, misidentification

Additional Supporting Information may be found in the online version of this article.

Novelty and Impact: This manuscript reviews the literature relating to cross-contamination of cell lines. Its novelty comes from the inclusion of a list of known cross-contaminated cell lines (over 300 lines named), allowing researchers to check their own cell lines with reference to the article. Recent developments in this field, including methods of authentication testing, are also discussed.

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Cell Lines as Model Systems

Continuous cell lines represent a readily accessible and easily studied resource for research into health and disease. These cell lines have acquired the ability to proliferate indefinitely if grown in the appropriate culture conditions; usually this is a rare event, since the majority of cells even in tumor tissue will cease proliferation after a limited number of cell divisions.[1] However, once established, a continuous cell line can be repeatedly passaged, reliably recovers from cryopreservation and retains many of the properties of its cell type or tissue of origin.[2,3] These advantages make continuous cell lines effective, and widely used, model systems for normal cellular processes and for a variety of disease states.

Cell lines are particularly attractive models for studying malignant disease. The genetic changes in tumor-derived cell lines closely resemble those of the tumors of origin.[4] Moreover, the genetic changes required to establish continuous cell lines from normal cells recapitulate many of the genetic changes occurring in cancer.[5,6] These genetic changes are required to overcome replicative senescence, in which normal cells continue to be metabolically active but are restricted from further division.[1] Cells able to overcome senescence continue

proliferating until their telomeres become so short that the chromosomes undergo fusion-breakage-bridge cycles and the ensuing genomic instability results in culture crisis. Occasionally (at a rate of ~1 in 10^7 cells), an immortalized cell will emerge from crisis and begin to divide again, yielding a continuous cell line.[1] The changes seen throughout this process have many parallels within cancer development, both for malignancy in general and when considering specific tumor types.[7,8]

Despite these advantages, numerous cautions have emerged from the literature regarding appropriate use of cell lines as model systems.[9,10] Even where cultures have been transformed through the introduction of specific genes, cell lines that have passed through replicative senescence and crisis are aneuploid, heteroploid and genotypically and phenotypically unstable, resulting in considerable heterogeneity within the culture.[10] This instability will cause changes in the characteristics of the cell line but a further consequence may result: alterations in a cell line can be accepted by the user as intrinsic to that culture when there is actually extrinsic contamination present.

Cell Line Cross-contamination and Misidentification

Cell lines become contaminated when a foreign cell line or microorganism is introduced without the handler's knowledge. Although we do not wish to minimize the problem of microbial contamination, we will focus on cell line cross-contamination in this article. Cross-contamination may arise due to several causes, including poor technique (spread *via* aerosols or accidental contact), use of unplugged pipets, sharing media and reagents among cell lines and use of mitotically inactivated feeder layers or conditioned medium, which may carry contaminating cells if not properly eliminated, for example, by freeze-thaw and filtration.[11] In addition, a cell line can be replaced by another as a result of misidentification by confusing cultures during handling, mislabeling or poor freezer inventory control. Simple errors during labeling of culture flasks, truncation of the cell line name or typographic errors in a published manuscript, can result in significant confusion for years after the event when another researcher attempts to use the same cell line for ongoing experimental work.[12]

Cross-contamination may occur "early," in which case the original cell line has probably never existed independently, or "late," where the tested sample has been overgrown but other stocks of the original may still exist.[13] Unfortunately, cell lines generally become cross-contaminated early, while still within the originating laboratory.[14] This is not surprising: cultures can remain in crisis for a prolonged period of time before emergence of an immortalized population and this is a time when a single cell, if introduced from a separate cell line, would rapidly take over the culture.

There are now a number of studies pointing out the severity of this problem and the need to take urgent action to minimize cross-contamination and its consequences.[9,15–17] Ten years ago, the German Collection of Microorganisms and Cell Cultures (DSMZ) published data from its identification testing of cancer cell lines submitted by various laboratories for deposit at the cell bank.[14] They found that 18% of 252 submitted cell lines were cross-contaminated with more than half of cases arising within only 6 laboratories. Subsequent work by the DSMZ, extending the number of cell lines tested (Fig. 1), shows that of 598 leukemia–lymphoma cell lines (the group provided with the most complete genetic data), 187 (31%) were contaminated with Mycoplasma and/or a second cell line with 38 (6%) of cell lines contaminated with both. These data suggest that poor practice within some laboratories results in contamination of multiple cell lines with multiple contaminants, which can then be disseminated more widely if these cultures are used by others.

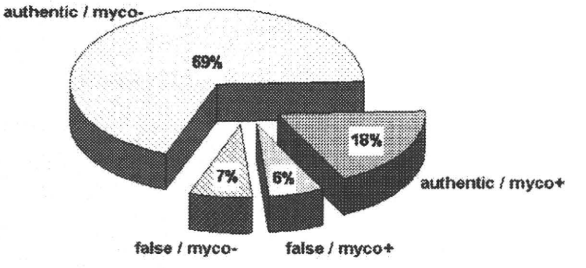


Figure 1. Rates of contamination for leukemia–lymphoma cell lines. Percentages of cross-contaminated and Mycoplasma-contaminated cell lines from a dataset of 598 leukemia and lymphoma cell lines analyzed by the German cell line bank DSMZ. "False/authentic" refers to the presence or absence of cross-contamination; "myco+/myco−" refers to the presence or absence of Mycoplasma contamination. Cell lines fall into the following categories: authentic/myco− (n = 411, 69%); authentic/myco+ (n = 108, 18%); false/myco− (n = 41, 7%) and false/myco+ (n = 38, 6%). (Courtesy of Hans Drexler, DSMZ.)

Other studies have pointed out that testing of cell lines is often infrequent, resulting in the failure to detect contaminated samples. John Ryan of Corning Life Sciences conducted surveys of seminar attendees in 1990, asking about Mycoplasma contamination; 50% were not currently performing testing and only 18% said they tested their cultures regularly. Almost 1 in 4 respondents (23%) had experienced Mycoplasma contamination, but with such a low level of testing, it is likely that the real figure was much higher.[18] Other data on cross-contamination were published in 2004 by researchers at the University of California, Berkeley, where Walter Nelson-Rees worked on this problem in the 1970s, focusing on the HeLa cell line.[19] Of 483 respondents to a questionnaire on cell line usage, 35% were using cell lines obtained from another laboratory rather than a cell line repository, but almost half of all respondents performed no testing for cross-contamination.[20]

A practical example of the consequences of cell line contamination can be found in a recent study published by Berglind *et al.*[21] The authors analyzed data within the UMD_p53 (2007) database, which includes information on the p53 status of 1,211 cell lines. Discrepancies were found in p53 status for 23% (88/384) of cell lines where data have been published by 2

independent laboratories. It is likely that many of these discrepancies arose due to work with cross-contaminated samples; the authors noted that many groups rely on previously published reports of a cell line's p53 status,[21] resulting in further confusion when interpreting results from these cell lines.

Cell banks have the expertise to detect such cross-contamination, and have been proactive in publishing reports of cross-contaminated cell lines,[22,23] in publishing test results online[24] and in developing new detection methods.[25–27] Unfortunately, however, cell banks have also reported reluctance from many researchers to deposit cell lines for distribution.[28] Such repositories specialize in the detection of cross-contamination and it is unlikely that most laboratories have comparable resources in this regard. In addition, many researchers obtain cell lines from one another, rather than approaching the originator or purchasing the cell line from a cell bank performing quality control testing. This may be faster or cheaper than obtaining cultures from a reputable source but the practice makes contamination more prevalent and harder to detect.

Practical Responses

Having defined the problems, it is time to focus on what can be done. Several cancer-related journals, including the International Journal of Cancer, have recently responded to these issues by changing their policies to require evidence of authentication with all submitted manuscripts using continuous cell lines.[29,30] Their response underscores the need for laboratories to come to grips with cell line cross-contamination and misidentification. Every researcher involved in cell culture will have cell lines currently in culture, stored in liquid nitrogen or may be commencing work on a new cell line. Put practically, how can you know if your cell lines are cross-contaminated?

There are 2 important answers to this question:

1. Check the literature, for example, by searching the PubMed database using the cell line name and "cross-contamination."
2. Check your cultured cells. Unless a cell line has come directly from a repository or other laboratory performing identification testing, it should be tested on arrival, and all cultures should be periodically tested while in use, before cryopreservation and when thawed from liquid nitrogen.[31] A variety of methods are available for authentication; for human cell lines, short tandem repeat (STR) profiling is the current international reference standard and is recommended as an easy and economical way to confirm cell line identity by comparison to donor tissue or to other samples of the cell line held by laboratories worldwide.[26]

Checking the Literature: A List of Cross-Contaminated Cell Lines

A 2004 survey of abstracts within the PubMed database would suggest that inappropriate usage of cross-contaminated cell lines is increasing,[20] despite many years of publication on this issue. It is possible that many researchers simply cannot find existing references to cross-contamination so, to make this already published work more accessible, we have surveyed the literature and other online resources for references to cell line contamination. The resulting list of cross-contaminated cell lines is included as Electronic Supporting Information.

To generate this list, the authors examined the PubMed database, references within other articles relating to this topic and the websites of 5 cell banks: the American Type Culture Collection (ATCC), DSMZ, European Collection of Cell Cultures (ECACC), Japanese Collection of Research Bioresources and the RIKEN Bioresource Center Cell Bank. A Wikipedia list of contaminated cell lines was also accessed (http://en.wikipedia.org/wiki/List_of_contaminated_cell_lines). Cross-contaminated cell lines are listed by name along with their species and cell type (both claimed and actual), the name of the contaminating cell line where identified, the reference in which this was reported and the PubMed ID number where available. Notes are also included for some cell lines. The list is made available in Excel spreadsheet or PDF format for easy accessibility.

The cell lines listed within this database are divided into 2 tables. Supporting Information Table 1 contains those cell lines where cross-contamination occurred as an early event, and thus where there is no original material remaining. Supporting Information Table 2 contains those cell lines where it is thought cross-contamination occurred as a late event and where original stocks may still exist. A full list of references is also given.

The current list of cross-contaminated cell lines (version 6.4) contains 360 cell lines, 346 in Supporting Information Table 1 and 14 in Supporting Information Table 2, drawn from 68 references. Cell lines affected are primarily human, although cultures from at least 8 other species are included, and come from a wide spectrum of tissue types. The cell or tumor type is given within the list where known; extensive work has been done by some cell banks and laboratories in this area to characterize the actual cell type or tumor type.[22,32] In some cases, this work has shown that a cell line carries the correct name but its cell or tumor type has been incorrectly identified, for example, the cell line RPMI-6666 was initially thought to have come from Hodgkin lymphoma but is now known to be an EBV-positive B-lymphoblastoid cell line.[22]

Common features for cross-contaminating cell lines within the current list are summarized in Table 1. It can be seen that most cross-contamination events have arisen from within the same species but a substantial minority (9%, 33/360) involved cross-contamination from a second species. For the intraspecies contaminants, all of those detected were human but it is likely that this relates to the difficulty of detecting intraspecies contaminants for nonhuman species. The commonest contaminant remains the HeLa cell line

Table 1. Cross-contaminating cell lines

Type of contaminant	Number of cell lines affected
Intraspecies	
Human	324
Nonhuman	0
Interspecies	33
Correct name—incorrect cell type (misidentified)[1]	3
Total	360
Contaminating cell line—12 most frequent	**Number of cell lines affected**
HeLa (human cervical adenocarcinoma)	106
T-24 (human bladder carcinoma)	18
HT-29 (human colon carcinoma)	12
CCRF-CEM (human acute lymphoblastic leukemia)	9
K-562 (human chronic myeloid leukemia)	9
U-937 (human lymphoma)	8
OCI/AML2 (human acute myeloid leukemia)	8
Hcu-10 (human esophageal carcinoma)[2]	7
M14 (human melanoma)	7
HL-60 (human acute myeloid leukemia)	6
PC3 (human prostate carcinoma)	6
SW-480, SW620 (human colon carcinoma)[3]	6

[1]For additional misidentified cell lines see Drexler *et al.*[22] [2]Hcu-10 carries the same genetic identity as Hcu-18, Hcu-22, Hcu-27, Hcu-33, Hcu-37 and Hcu-39; it is unclear which is the correct identity (see Electronic Supporting Information for reference). [3]SW480 and SW620 come from the same donor and therefore carry the same genetic identity (see Electronic Supporting Information for reference).

(29%, 106/360), followed by T-24 (5%, 18/360) and HT-29 (3%, 12/360).

It is important for such a list to be continually updated and feedback is welcome for this purpose. An earlier version of the database was released online by ECACC[31]; 6 cell banks have now agreed to make the database available online and to update this information where necessary. Current website addresses for access to the list of cross-contaminated cell lines are given in Table 2. In future, it is envisaged that the current list of misidentified cell lines will be included in a new initiative improving access to authentication data. The Standard Development Organization at the ATCC is in the process of producing an international standard for human cell line identification based on STR profiling (ATCC SDO Workgroup ASN-0002, manuscript submitted). Strict criteria for STR profiles derived from cancer cell lines are being developed. One consequence of this initiative is that funding is being sought for a quality controlled and curated cell line database with free access into which the database described here will be incorporated.

Table 2. Websites for ongoing access to the list of cross-contaminated cell lines

Cell bank	Website address
ATCC	http://www.atcc.org/
CellBank Australia	http://www.cellbankaustralia.com/
DSMZ	http://www.dsmz.de/
ECACC	http://www.hpacultures.org.uk/collections/ecacc.jsp
JCRB	http://cellbank.nibio.go.jp/
RIKEN Bioresource Center Cell Bank	http://www.brc.riken.go.jp/lab/cell/english/guide.shtml

Checking Your Cultures: Authentication of Cell Lines

Even if a search of the literature shows no indication that a cell line is contaminated, it is still essential to test the sample that you are working with. Authentication testing should be considered in a positive light, as an essential part of good cell culture practice[33] and as an assurance for researchers, funding bodies and journals that the cell line used is a valid experimental model.[17]

There are a number of methods for testing cell line identity. When the issue of cross-contamination was first identified, HeLa contaminants were detected through a combination of isoenzyme and chromosomal analysis.[19,34] Both techniques continue to be used but there are also many newer molecular approaches. Commonly used authentication methods are summarized in Table 3; what factors should be considered when choosing between these methods?

The expertise of the laboratory holding the cell line is an important factor. For example, laboratories with experience in cytogenetics would have the skills to identify species through karyotype analysis and cell lines through the presence or absence of appropriate markers.[35] Although this is an older approach, it still allows clear identification of cell lines, and many cell banks have published karyotypic information on their cell lines to allow comparison to well-characterized stocks. It should be noted that tumor-derived cell lines can be surprisingly difficult to harvest for cytogenetic analysis[35] and are typically heteroploid making interpretation difficult: the experience of the operator is important for success.

The species of cell lines held within the laboratory is also important. Although some authentication methods can be used on more than 1 species, molecular methods such as STR profiling are only successful for a single species; other species will simply fail to amplify.[26] This may not be an issue for laboratories working only with human samples but clearly is a significant factor for groups working with rodent cell lines. In this regard, multilocus DNA fingerprint analysis has a clear advantage, since probes are able to hybridize to a wide variety of species.[25] Unfortunately, although successful within a single laboratory, it can be challenging to compare DNA fingerprints across several experimental runs, and it is difficult to exchange data among laboratories or for cell

Table 3. Commonly used methods for authenticating cell lines

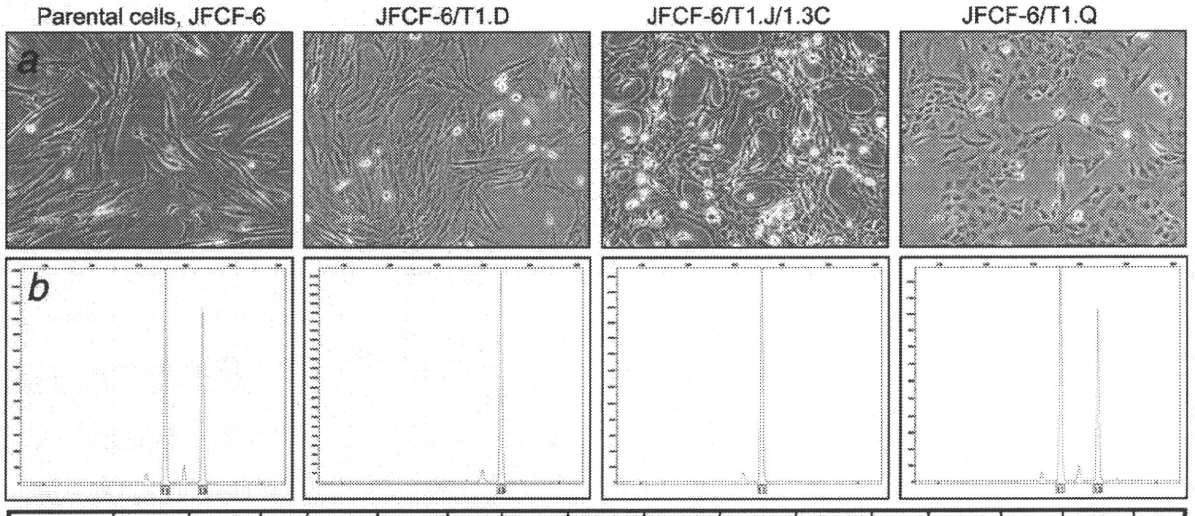
Name	Description	Purpose	References
Chromosomal analysis/karyotyping	Involves preparation of a metaphase spread with chromosome banding and painting to identify chromosome number and markers	Separates species, plus individual cell lines if detailed analysis performed	Ref. 35
Isoenzyme analysis	Biochemical method separating isoenzymes by electrophoresis; isoenzyme mobility may vary within or across species. Kits available include the Authentikit gel electrophoresis system	Separates species, sometimes individuals	Refs. 36,37
Multilocus DNA fingerprint analysis	Molecular method detecting variation in length within minisatellite DNA containing variable numbers of tandem repeat sequences. Analysis is by Southern blot hybridization using probes 33.6 and 33.15, M13 phage DNA, or oligonucleotide sequence	Separates individual cell lines across multiple species	Refs. 25,38
Short tandem repeat (STR) profiling	Molecular method detecting variation in length within microsatellite DNA containing variable numbers of short tandem repeat sequences. Analysis is by PCR with comparison to set size standards; usually available in a kit format allowing amplification of up to 16 loci	Separates individual cell lines within a single species	Refs. 26,39
Polymerase chain reaction (PCR) fragment analysis	Molecular method involving amplification of specific genes or gene families, aiming to detect variations in exon/intron sequence, transcript splicing, or the presence of pseudogenes. Genes examined include the aldolase gene family and the beta-globin gene	Separates species only	Refs. 40,41
Sequencing of "DNA barcode" regions	Involves sequencing of a DNA fragment from the mitochondrial gene cytochrome *c* oxidase subunit I, with comparison to sequence obtained from online databases. This "DNA barcode" has been shown in practice to distinguish a broad range of animal species	Separates species only	Refs. 27,42

banks to publish such fingerprints online. It is advisable to always compare the test sample to a known sample within the same experiment, ideally using DNA from the blood or tissue of the original donor.

The obvious advantage of STR profiling lies in the use of control samples to generate a numerical code for each sample, which precisely identifies that cell line and which can be readily shared and published online. It is primarily for this reason that STR profiling is recommended as an international reference standard for human cell lines[26] and accepted within the legal system for human identity testing.[39] STR profiling is based on the presence of STRs within the human genome that exist at variable lengths throughout the population. Each of the repeat regions to be analyzed (usually tetra or pentanucleotide repeats in noncoding sequence) is amplified by PCR using primers carrying fluorescent tags and electrophoresed in a sequencing gel; the precise length of each allele is determined and compared with size standards and controls. This allows identification software to assign a number to each allele at that locus (see, *e.g.*, Fig. 2). The combination of multiple loci—classically 13, as used in the FBI Laboratory's Combined DNA Index System (CODIS)—gives sufficient data to uniquely identify that individual.

STR profiles for individual cell lines and panels have now been reported by many laboratories (*e.g.*, Ref. 44) and are published online by several cell banks. However, there are some cautions to be aware of when using this approach. It is accepted within the forensic field that tumor samples are not as genetically stable as other tissue sources for STR profiling, because of loss of heterozygosity and microsatellite instability.[45,46] This is even more evident in tumor-derived cell lines, where evolution or genetic drift continues to occur with passage.[47] When searching an online database of STR profiles from cell lines, the user needs to look for close matches and not just identical matches; most studies would agree that 80% similarity is an appropriate threshold for declaring a match when comparing cell line profiles.[26,44] There may also be a significant start-up cost if testing in-house; in addition to an STR kit, access to methods for DNA extraction, precise quantitation, fragment analysis and software for STR profile identification is required.

The fact that STR profiling is only suitable for distinguishing cell lines of a single species has led to the need to re-examine authentication of nonhuman cell lines. Laboratory rodent samples will always be difficult to identify precisely due to inbreeding; laboratories working with rat or mouse cultures may wish to examine strain identity rather than authentication of individual cell lines, particularly if they have expertise in single nucleotide polymorphism (SNP) or single sequence length polymorphism (SSLP) analysis,



c

Cell Line	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA
Parental	10,13	29,31	11	10,13	14,16	9.3	8,12	11,13	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.D	13	29,31	11	10,13	14	9.3	8	13	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.J/1.3C	10,13	29,31	11	10,13	14,16	9.3	8,12	11	17,20	14,15	16,17	11	12,19	X,Y	9,11	21
JFCF-6/ T1.Q	10,13	29,31	11	10,13	14,16	9.3	8,12	11,13	17,20	14,15	16,17	11	19	X,Y	9,11	21

Figure 2. Example of STR profile generation and interpretation. An example of STR profiling is given for the JFCF-6 cell fibroblast strain and 3 of its immortalized derivatives, JFCF-6/T1.D, JFCF-6/T1.J/1.3C and JFCF-6/T1.Q.[43] Derivatives were established after transfection with SV40 early region DNA and were handled by CellBank Australia through its Culture and Return service. DNA from each culture was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Mulgrave, Australia), which includes primers for 16 STR loci. Amplified sequence was analyzed using an ABI PRISM 3100 Genetic Analyzer and data files were assessed using GeneMapper ID software (Applied Biosystems). (*a*) Photographs taken of each culture, comparing parental cells to the morphology of each derived cell line (scale bar = 100 μm). Each derivative has a markedly different morphology, showing the need for authentication testing to confirm that derivatives correspond to the parental strain. (*b*) Examples of STR peak amplification for the D16S539 locus of each culture. Amplification varies at this locus due to genetic drift during establishment of the 3 JFCF-6–derived cell lines. The peaks shown correspond to specific allele sizes known to exist at this locus and confirmed using size standards and controls supplied with the kit (data not shown). (*c*). STR profiles for JFCF-6 and derived cell lines; the locus shown in B, D16S539, is highlighted in grey. Despite the differences seen due to genetic drift, the profiles for derived lines closely match the parental cell strain and all of these cultures are correctly identified.

which can be used for strain identification.[48,49] SNP analysis can also be used to identify individual samples[50] and has been used for cell line authentication,[51] making it a method of great promise for application to human and nonhuman samples alike. Laboratories working on specific cell types may be able to use expressed markers for identification, as 1 laboratory has done recently, publishing a technique for identification of hybridomas based on sequencing of light-chain variable regions.[52]

A simple method has recently emerged to help detect interspecies contamination. The term DNA barcoding here refers to amplifying a specific 648 bp fragment of the mitochondrial gene, cytochrome *C* oxidase subunit I (COI), using primers developed by Folmer *et al.*[53] Sequence divergences within this fragment allow species discrimination across almost all animal phyla.[42] Although debate is ongoing as to whether DNA barcoding is sufficient for assignment of species in taxonomic terms,[54] it is clear that the technique can readily identify the species of an unknown specimen if compared with previously sequenced reference material in online databases.[55] DNA barcoding has been tested for species identification of cell lines[27] and its use would reduce the incidence of interspecies cell line

contamination, found here to cause almost 1 in 10 of all published cross-contamination events.

Whatever the authentication method used, it should be clearly recorded within the researcher's experimental notes, and the result should be linked if possible to the laboratory's liquid nitrogen records, so that quality control for frozen vials is clearly evident. When publishing experimental work, the Material and Methods section should include the correct and full name of the cell line used, its origin (with appropriate references), the source of the cultures used and details of authentication testing.

Conclusions

Cell line contamination is a serious issue that detracts from the use of cell lines as model systems to help us understand a broad range of diseases, including cancer. Responding practically by checking each cell line before it is used, searching for previous references and authenticating the sample itself is worthwhile and will reduce the risk and subsequent consequences of contamination long-term.

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CELL LINE

Establishment of induced pluripotent stem cells from human neonatal tissues

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Abstract

Following the success in establishing human induced pluripotent stem (iPS) cells, research into various applications of the cells derived from human iPS cells has begun in earnest. The use of iPS cell-derived cells in clinical therapies is one of the most exciting of the possible applications. However, the risk of tumorigenicity is the biggest potential obstacle to use iPS cell derivatives in the clinic. It should be noted that the human cells used to generate iPS cell lines may have acquired genetic mutations and these might influence the tumorigenicity of the cells. In particular, the cells of older people have a higher risk of genetic mutations than those of younger people. Here, we show that iPS cells could be derived from short-term cultures of neonatal tissues. The established human iPS cells expressed various markers of undifferentiated cells and formed teratoma in immunodeficient mice. The human iPS cells derived from neonatal tissues may represent a clinical material possessing less tumorigenicity.

Key words: clinical application, neonatal tissue, induced pluripotent stem cells.

INTRODUCTION

The development of a method to generate human induced pluripotent stem (iPS) cell lines[1–4] has stimulated a considerable number of studies into the potential applications of these cell lines. The use of iPS cell-derived cells in clinical therapies is one of the most exciting of the possible applications. However, the risk of tumorigenicity is the biggest potential obstacle to use iPS cell derivatives in the clinic. Initially, the method for producing iPS cell lines involved the integration of exogenous genes into the host genome. Recent modifications to the methodology have obviated the need for retention of exogenous genes to generate iPS cell lines.[5–7] These methodological changes may reduce the problem of potential tumorigenicity.

However, even when iPS cells are established with methods that avoid the integration of exogenous genes, the risk of tumorigenicity of iPS cell derivatives remains similar to that of embryonic stem (ES) cell derivatives. One potentially problematic aspect of iPS cells compared with ES cells is that they are established from somatic cells that may have already acquired genetic mutations. In addition, the cells of older people have a higher risk of genetic mutations than those from younger people. This potential problem has to be taken into account prior to the use of iPS cell derivatives in the clinic.

Although neonatal tissues such as umbilical cord, fetal membrane, and placenta are readily available,[8] they are usually discarded if they are not required for immediate use. Provided the mother of a neonate agrees to allow the neonatal tissues to be used in basic research

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and/or clinical applications, these tissues could providea useful resource without the complicating factor of critical ethical concerns. Here, we show that iPS cells can be derived from short-term cultures of neonatal tissues.

MATERIALS AND METHODS

Cell sources of iPS cells

Fibroblast-like cells derived from human umbilical cord (HUC-F2, HUC-Fm and HUC-5 derived from three different neonates) and from human fetal membrane (HFM-1 derived from a neonate) were obtained from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and were cultured in minimum essential medium-α (MEM-α; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA).

iPS cell generation

pMXs retroviral vectors were obtained from addgene (Cambridge, MA, USA) and used to express Oct3/4, Sox2, Klf4, and c-Myc. To produce the recombinant pseudo-type retrovirus, plasmid DNA was transfected into 293T cells along with the *gag-pol* expression plasmid (pCAGGS *gag-pol*) and the vesicular stomatitis virus G glycoprotein (VSV-G) *env* expression plasmid (pMD/G VSV-G) by FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA), and supernatant from the transfected cells was collected to infect the cells from neonatal tissues.

To produce the recombinant pseudo-type lentivirus, human Oct3/4 and c-Myc cDNAs were obtained as Human Fetus Marathon-Ready cDNA and Human Bone Marrow Marathon-Ready cDNA, respectively, from Clontech (Mountain View, CA, USA) and human Sox2 and Klf4 cDNAs were obtained from German Science Center for Genomic Research. The cDNAs were inserted into the pENTR/D-TOPO entry vector plasmid (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. The cDNAs in pENTR/D-TOPO were then transferred to the pCSII-EF-MCS-IRES2-Venus lentiviral vector plasmid using the Gateway LR clonase (Invitrogen). The VSV-G-pseudotyped lentiviral vectors were produced by transient transfection of three plasmids, the packaging plasmid (pCAG-HIVgp), the VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev), and the lentiviral vector plasmid into 293T cells. Human neonatal tissue cells were transduced with lentiviral vectors and nearly 100% of transduction efficiency was confirmed by fluorescence microscopy for Venus expression. Six to ten days after transduction, the cells were harvested by trypsinization and 5–10 × 10^4 cells were replated on mouse embryonic fibroblast (MEF) feeder cells in a 100 mm dish.

Viral infection and iPS cell generation were performed essentially as has been described previously.[1] Generated iPS cells were maintained essentially as has been described previously.[9]

Feeder cells

The iPS cells were established and maintained on MEF feeder cells. MEFs were obtained from 14 day embryos of ICR mice as previously described.[10] Pregnant ICR mice were obtained from Charles River Japan (Tsukuba, Ibaraki, Japan). MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 10% FBS (Sigma) and were treated with Mitomycin C (10 μg/mL) for 1.5–2.0 h prior to their use as feeder cells.

Alkaline phosphatase staining and immunocytochemistry

Alkaline phosphatase staining was performed using an alkaline phosphatase substrate kit 4 (Vector Laboratories, Burlingame, CA, USA). Immunocytochemistry was performed essentially as described previously.[9] Primary antibodies used were SSEA-4 (Millipore, Billerica, MA, USA), Tra-1-60 (Millipore), Tra-1-81 (Millipore), Oct3/4 (Santacruz, Santa Cruz, CA, USA), and Nanog (Reprocell, Yokohama, Kanagawa, Japan). Secondary antibodies used were Alexa Fluor 546 anti-mouse IgG (Molecular Probes, Eugene, OR, USA) to detect SSEA-4, TRA-1-60, TRA-1-81 and Oct3/4, and Alexa Fluor® 546 anti-rabbit IgG (Molecular Probes) to detect Nanog.

Karyotype analysis

Karyotype analysis was performed essentially as described previously.[9]

Teratoma formation assay

The teratoma formation assay was performed as described previously,[1] except that the cells were transplanted into the sub-capsular space of the testis.

Short tandem repeat polymorphism analysis

Short tandem repeat (STR) polymorphism analysis was carried out on genomic DNA using a PowerPlex1.2 kit (Promega, Madison, WI, USA), which is polymerase chain reaction (PCR) based.[11]

RESULTS

Generation of iPS cells from human neonatal tissue cells

Approximately 2 weeks after initiating the generation of iPS cells, some granulated colonies appeared that were dissimilar to ES cells in morphology. We observed distinct types of colonies that were flat and resembled ES cell colonies 3–5 weeks after initiation of culture. Eventually, all of the human cell types tested provided ES-like colonies in their cultures. We picked some representative colonies from each culture and placed these in fresh cultures. All of the colonies continued to proliferate when grown on MEFs in primate ES cell medium containing basic fibroblast growth factor (FGF). Of note, iPS cells established with lentiviral vector containing Venus did not express Venus at all, i.e. the expression of exogenous genes was silenced in the established iPS cells.[1]

The human iPS cell lines generated in this study are described in Table 1. All of the cell lines were morphologically similar to human ES cells, with the exception of

Table 1 List of information regarding established human iPS cell lines

Cell name	Source cells	Introduced genes	Vector origin	Origin	Karyotype	Teratoma
HiPS-RIKEN-1A	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XX	Observed
HiPS-RIKEN-1B	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XX	ND
HiPS-RIKEN-1C	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1D	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1E	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-1F	HUC-F2	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2A	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-2B	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2C	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2D	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2E	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-2F	HUC-Fm	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-12A	HUC-5	OcT3/4, Sox2, Klf4	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-12B	HUC-5	OcT3/4, Sox2, Klf4	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-13A	HUC-5	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-13B	HUC-5	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-11A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	46, XY	Observed
HiPS-RIKEN-11B	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Neonate	ND	ND
HiPS-RIKEN-3A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	46, XY	Observed
HiPS-RIKEN-3C	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-3D	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-3E	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Retrovirus	Mother	ND	ND
HiPS-RIKEN-4A	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	46, XY, t(6;9)(p22;q32)	Observed
HiPS-RIKEN-4B	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4C	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4D	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4E	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4F	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4G	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND
HiPS-RIKEN-4H	HFM-1	Oct3/4, Sox2, Klf4, c-Myc	Lentivirus	Mother	ND	ND

HUC-F2, HUC-Fm, and HUC-5 are fibroblast-like cells derived from human umbilical cord. HFM-1 are fibroblast-like cells derived from human amniotic membrane. The cellular origins of human induced pluripotent stem (iPS) cells were confirmed using short tandem repeat polymorphism analysis (see text and Materials and methods). Transplants of iPS cells into immunodeficient mice that generated teratomas with all three types of germ layer are indicated by "observed". ND, not done.

HiPS-RIKEN-3C (data not shown). Although HiPS-RIKEN-3C continued to proliferate for more than one month, this line did not appear to be comprised of ES-like cells (data not shown). The nature of these apparently non-ES-like cells in HiPS-RIKEN-3C remains to be determined.

Expression of gene markers of the undifferentiated state

Alkaline phosphatase activity and the expression of stage specific embryonic antigen-4 (SSEA-4), tumor-related antigen-1–60 (TRA-1-60), TRA-1-81, Oct3/4, and Nanog were detected in all of the iPS cell lines (Fig. 1), with the exception of HiPS-RIKEN-3C.

Teratoma formation

To evaluate the *in vivo* pluripotency of these new iPS cells, we transplanted the cells into the sub-capsular space of the testis of immunodeficient mice. Tumor formation was screened at about 8 weeks after transplantation. Histological examination of the tumors showed the presence of ectoderm-, mesoderm-, and endoderm-derived tissues (Fig. 2).

Authentication of the origin of the new iPS cell lines

To confirm that the newly generated iPS cell lines were derived from neonatal tissue cells, we compared the results of an STR polymorphism analysis of the original neonatal tissue cells and the new iPS cell lines (data not shown). All of the iPS cell lines were confirmed to be derived from the source cells (Table 1). Interestingly, we also found that the amniotic membrane cells, HFM-1, were a mixture of cells from two individuals. Since we also possessed umbilical cord cells from the neonate, we were able to confirm that the neonate provided some of the cells. It is highly likely that the other contributor was

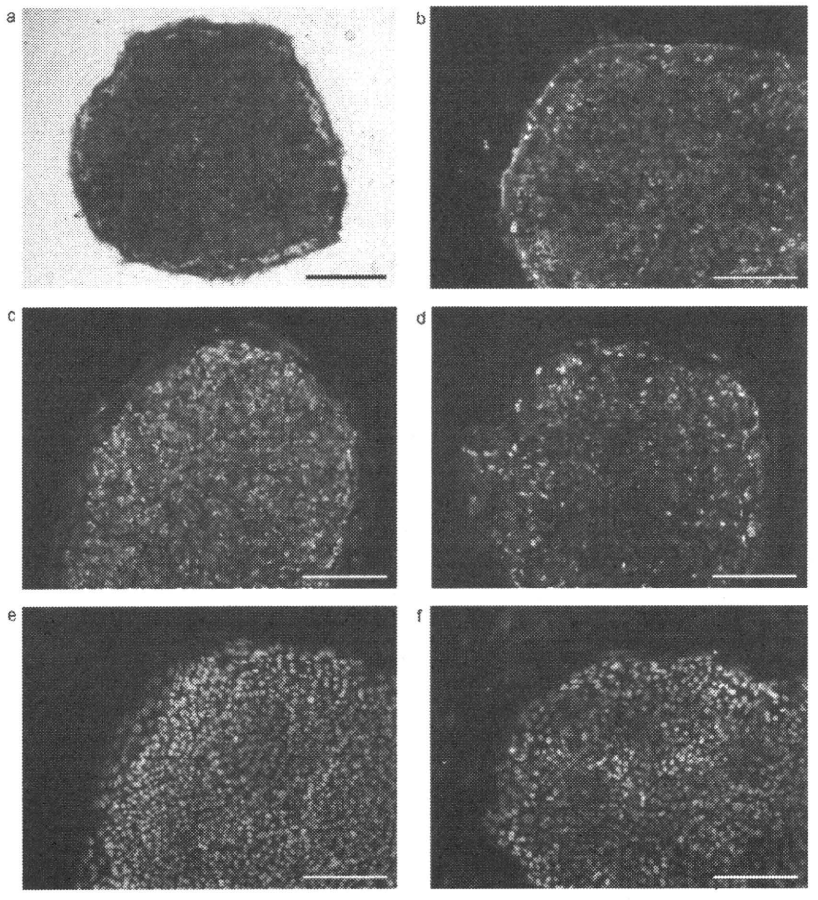

Figure 1 Expression of marker genes for the undifferentiated embryonic stem (ES) cell-like state in cells of the clone HiPS-RIKEN-1A. (a) alkaline phosphatase. (b) SSEA-4. (c) Tra-1-60. (d) Tra-1-81. (e) Oct3/4. (f) Nanog.