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Tumourigenic characteristics of embryonal carcinoma cells as a model for studying tumour progression of human embryonic stem cells

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Abstract

Objectives: The objective of this study was to compare tumourigenic characteristics of human embryonic stem cells (HESCs) and embryonal carcinoma cells (ECCs) to identify a robust and simple model for studying certain aspects of cell transformation and tumourigenesis, in tumour progression of HESCs.

Materials and methods: SSEA-3 positive ECCs (NTERA-2) cells were identified and compared to HESCs (*ch*HES-20) in terms of pluripotency and differentiation capacity, growth characteristics, gene expression profiles and signalling pathways.

Results: Our results showed that NTERA-2 cells shared similarities in expression markers of pluripotency to *ch*HES-20 cells. However, NTERA-2 cells also expressed some markers of differentiation and had a tendency to differentiate towards ectodermal endpoints. We identified NTERA-2 cells with higher S-phase fraction in cell cycle distribution, anti-apoptosis markers and robust self-renewal ability, compared to *ch*HES-20 cells. Microarray analysis and real-time PCR results showed that some oncogenes were up-regulated and tumour-suppression genes were down-regulated, whereas pluripotency-related genes were up-regulated and differentiation-related genes were down-regulated, and that Wnt and Notch signalling pathways were activated during progression from ES cells to EC cells.

Conclusion: Tumourigenic characteristics of ECCs may provide a valuable insight into possible tumour progression of HESCs.

Introduction

Pluripotent stem cells have been isolated from a variety of human sources as models for investigating early human development (1, 2). Two of the most well-studied cell types include embryonic stem (ES) cells derived from the inner cell mass of blastocyst-stage embryos, and embryonal carcinoma (EC) cells, stem cells of teratocarcinomas (mixed germ cell tumours) derived from progenitors of germline cells (3). Stem cells can be defined as having extensive self-renewal capacity and the ability to differentiate into a wide variety of cell types (4). In a sense, EC cells resemble stem cells. On the other hand, EC cells have progressed from ES cells and are a malignant equivalent of ES cells (5). They thus provide a good model in which to study cell transformation and tumourigenesis, in tumour progression of ES cells.

In many respects, germ-cell tumourigenesis resembles early embryogenesis (6). Some factors and signalling pathways associated with oncogenesis inherent in stem cells, are involved in cell proliferation, differentiation and self-renewal of embryonic stem cells. Altered expression of the precise factors has now been associated with some cancers. Their exact roles remain unknown and they are considered to be a secondary effect downstream of primary causes of tumourigenicity. Thus, it is essential to determine the factors and signalling pathways that turn an oncogenic status on, or to be more precise, cancer stem cell status. Furthermore, studies of induced pluripotent stem (iPS) cells has shown that the pluripotent state can be acquired by expression of four specific genes, *OCT4*, *SOX2*, *C-Myc* and *KLF4*,

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which are frequently up-regulated in tumours. Although IPS cells are able to generate chimaeric animals, including with germline transmission, nearly 20% of the IPS-derived chimaeric offspring were found to develop tumours (7). In addition, it was shown (8) that expression of *OCT4* was no longer required for maintaining IPS cell status. Thus, while these studies have provided hope for reprogramming adult cells for therapeutic uses, they also further reiterate the necessity of identifying genes associated with oncogenesis, in pluripotential stem cells. Comparisons between expression profiles of ESCs and ECs could therefore be useful in identifying factors that distinguish pluripotency and oncogenesis.

In this study, we have examined some crucial characteristics and attributes of NTERA-2 cells. We have also compared SSEA-3 positive NTERA-2 cells and embryonic stem cells, using microarrays. Tumourigenic characteristics and gene expression profiles and signalling pathways, of EC and ES cells, were involved in tumour progression from ES cells to EC cells. We report novel insights into cell transformation and tumourigenesis of human ES cells in comparison to EC cells, with HESC.

Materials and methods

chHES-20 cell line culture

*ch*HES-20 cells were isolated by mechanical dissection of blastocysts and direct plating the inner cell mass upon human embryonic fibroblasts, in K-SR medium (patients who donated blastocysts for derivation of hESCs had provided informed consent and, the study was approved by the ethical committee of our institute). Medium contained knock-out DMEM (Gibco-BRL, St. Louis, MO, USA) supplemented with 15% serum replacement (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 1% non-essential amino acids (Gibco-BRL), 2 mM L-glutamine (Gibco BRL, Grand Island, NY, USA), 50 U/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma) and 4 ng/ml human recombinant basic fibroblast growth factor (Gibco BRL, Gaithersburg, MD, USA). *ch*HES-20 cells were routinely passaged every 6–7 days by mechanical separation.

NTERA-2 cell line culture

NTERA-2 cell line was obtained from cell culture centre of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences and the School of Basic Medicine of the Peking Union Medical College. Cryopreserved cells were thawed and plated in 60-mm tissue culture dishes (Costar, Cambridge, UK) without feeder cells. Culture medium consisted of DMEM (Gibco-BRL, St. Louis, MO, USA) supplemented with 10% FBS (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 1% non-essential amino acids (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 50 U/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma) and 4 ng/ml human recombinant basic fibroblast growth factor (Gibco BRL). Cells were cultured at 37 °C, in 5% CO_2 atmosphere and were expanded by passaging every 2–3 days using 0.05% trypsin-EDTA (Gibco BRL).

In vitro differentiation assay

*ch*HES20 cells were mechanically divided into small clumps (approximately 50–100 cells each), and NTERA-2 cells were digested using trypsin-EDTA (Gibco BRL). In the region of $0.5–1 \times 10^6$ cells were collected and injected into hind legs of 6- to 8-week old NOD/SCID mice. Ten weeks later, mice were sacrificed. Xenografts were removed and fixed in 4% paraformaldehyde (Sigma) for 24 h. After embedding in paraffin wax, tumours were sectioned and standard histopathological analysis was performed following haematoxylin and eosin (H&E) staining.

Flow cytometry

Cells were trypsinized into single cell suspensions, washed in 1× PBS, incubated in 100 μl dilute primary antibody, then treated with respective secondary antibody conjugated to FITC for 30 min at 4 °C (1:500). Approximately 100 μl cell suspension containing 1×10^6 cells was incubated with antibodies SSEA-1 (1:250), SSEA-3 (1:200), SSEA-4 (1:300), TRA-1-60 (1:400) and TRA-1-81 (1:400) along with appropriate isotype-matched controls. Results were acquired and analysed using a FACS Calibur flow cytometery (Becton Dickinson, San Jose, CA, USA). A total of 10 000 events were acquired and analysed in each case, to determine percentage differential expression of each cell surface marker.

RT-PCR

Total RNA was isolated from cell pellets using TRIZ reagent (Sigma), and between 10 ng and 5 μg cDNA was synthesized from total RNA in a 20 μl reaction. RNA was reverse-transcribed in a thermocycler using viral reverse transcriptase, along with random primer according, to the manufacturer's protocol, supplied by Fermentas Company. PCR amplification of different genes was performed using Taq DNA polymerase (Promega, Madison, WI, USA). PCR products were subjected to electrophoresis on 1.5% agarose gel containing

ethidium bromide at 0.5 μg/ml. PUC Mix marker, 8 (MBI-Fermentas, St. Leon-Roth. Nukleotide) was used to estimate size of amplified bands. Supplementary Table 1 lists primers used for RT-PCR.

Analysis of real-time PCR

Total RNA was extracted from 1×10^6 *ch*HES-20 and NTERA-2 cells. Four microlitres aliquot total RNA was reverse-transcribed in a T-GRADIENT thermocycler (Biometra, Gottingen, Germany) using Revert-Aid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Specific primer pairs (as shown in list, supplementary Table 2) were designed for real time RT-PCR, which was performed using Light Cycler Fast Start Master DNA Sybr Green I kit (Roche, Penzberg, Germany) according to protocol provided. To determine specificity of amplified products, melting curve analysis was performed. No amplification of non-specific products was observed. Cycle threshold (Ct) values were obtained for the tested genes and relative gene expression level was calculated by subtracting Ct value of 28s (control gene) from Ct value of the target gene, generating the ΔCt value. Relative change in NTERA-2 cells versus *ch*HES-20 cells was obtained using the $2^{-\Delta\Delta Ct}$ method (9) and 95% confidence interval was accepted.

Cell proliferation analysis

For analysis of cell proliferation, 1×10^7 ES cells were cultured for 12 h in 20 μM 5-ethynyl–2′-deoxyuridine (EdU) medium and later were harvested and stained using Click-iT^{TM} EdU Alexa Fluor® 488 cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Fluorescence data were collected using FACScalibur apparatus (Becton Dickinson). Data were calculated as mean ± SEM of at least three separate cultures. Statistical significance was determined using Student's *t*-test with $P \leq 0.05$ considered significant.

Analysis of apoptosis

To measure early apoptosis, cells were examined after staining with Annexin V-FITC (Bender MedSystems, Vienna, Austria). Fluorescence data were collected using FACScalibur apparatus (Becton Dickinson) and further analysed using ModFit 2.0 software (Becton Dickinson). Annexin V labelled with FITC enables identification and quantification of apoptotic cells on single-cell basis, by flow cytometry. Staining cells simultaneously with Annexin V–FITC and non-vital dye propidium iodide allows (bivariate analysis) discrimination of intact cells (annexin V-FITC negative, PI negative), early apoptotic (annexin V-FITC positive, PI negative) and late apoptotic or necrotic cells (annexin V-FITC positive, PI positive). Data were calculated as mean ± SEM of at least three separate cultures. Statistical significance was determined using Student's *t*-test with $P \leq 0.05$ considered significant.

Immunomagnetic cell separation

After being harvested as described above, cells were incubated for 30 min with primary antibodies as described, followed by a washing step and incubation with corresponding magnetically labelled secondary antibody (Miltenyi Biotec, Auburn, CA, USA) for 15 min at 4 °C. Positive and negative fractions were separated using MiniMACS LC cell columns (Miltenyi Biotec) according to the manufacturer's protocols. After fluorescence labelling, samples of obtained positive and negative fractions were reanalysed by FACS to assess purity.

Microarrays and data analysis

Total RNA was extracted from SSEA-3 positive cells, sorted from *ch*HES-20 and NTERA-2 cells, using RNeasy (Qiagen, Chatsworth, CA, USA). One microgram total RNA was primed with 100 ng Oligo dT-T7 primer and reverse-transcribed using Superscript II (Invitrogen). A second strand was synthesized and the double stranded cDNA was purified with DNA Clean and Concentrator (Zymo Research, Orange, CA, USA). *In vitro* transcription reaction was performed for 9 h with T7 RNA polymerase. In the first round, RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and in the second, amplification was performed similar to the first round, but with 100 ng RNA and 500 ng random hexamers. ENZO BioArray HighYield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY USA) was used to incorporate biotin-labelled nucleotides in the second round dscDNA, then RNA was purified using RNeasy. Fragmentation was completed using the standard protocol. Prior to hybridization on GeneChip array, test3 array of housekeeping controls was analysed to determine sample suitability for GeneChip arrays. Hybridized arrays were subsequently scanned for data analysis. Detailed RNA amplification protocol is available upon request. The hybridization mixture was heated at 99 °C for 5 min, then at 45 °C for 5 min, followed by centrifugation at 13 000 ***g*** for 5 min. Gene chips were pre-hybridized in 200 ml of 1 hybridization buffer for 10 min at 45 °C with mixing at 60 rpm. in the hybridization oven. Then pre-hybridization buffer was replaced with 200 ml hybridization mixture and incubated for 16 h at 45 °C, and mixed at 60 rpm.

Hybridization mixture was removed and stored at −70 °C. Each chip was filled with 250 ml of non-stringent washing buffer (6 X SSPE, 0.01% Tween-20). Chips were scanned using an Affymetrix Scanner 3000 (Affymetrix). Gene expression signals were collected using Affymetrix GCOS V1.1.1 software. Up-regulated and down-regulated gene distributions on each chromosome were analysed using Dchip 2004 software.

Results

NTERA-2s with characteristics of ES cells

After thawing, NTERA-2 cells were seeded on plates at $7 \times 10^4/cm^2$ density; they maintained EC phenotype. As such, cells became confluent every 2–3 days and aggregated to form nested regions. The expanded NTERA-2 cells had high nucleus/cytoplasm ratio and one or two nucleoli. Results of gene expression analysis by RT-PCR showed that NTERA-2 cells expressed specific markers, such as *Oct-4, Nanog, Sox-2, TERF1, TERF2, REX, FGF4, Cripto, Thy1* and *LEFTYA*. Immunohistochemistry showed that they were SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and Oct4 positive, but SSEA-1 negative (Fig. 1a). Also, NTERA-2 cells had alkaline phosphatase and telomerase activity. Grown at low density they were found to exhibit the following markers: *CD90+, CD31+, CD34+, Brachyury+, AFP, VE-cadherin+, Flt-1+, and Nestin+*. Thus, in summary, the NTERA-2 population expressed markers associated with undifferentiated pluripotent cells (*Oct4, Nanog, SSEA-4, TRA1-60, TRA1-81, hTERT*), as well as a marker profile indicating differentiation towards all three germ layers (Fig. 1b). Flow cytometric analysis further demonstrated that considerable populations of NTERA-2 cells were immunoreactive with cell surface antigens such as SSEA-3 (89.07%), SSEA-4 (96.43%), TRA-1-60 (53.12%) and TRA-1-81 (48.48%) (Fig. 2). Their gene expression profiles indicated that the cells had similar characteristics to human ES cells.

Differentiation of chHES20 and NTERA-2 cells in vitro

Both NTERA-2 cells and *ch*HES-20 cells gave rise to embryonic bodies (EBs) in suspension culture for up to 7 days (Fig. 3a, b). After re-plating d7 EBs on to dishes, various types of cells developed over 15 days. A sample of cells was removed at 0, 5, 10 and 15 days of differentiation and gene expression of markers indicative of endoderm (*AFP*), mesoderm (*KDR*) and ectoderm (*PAX6*) were assayed by real-time PCR . Expression of Nanog decreased significantly by day 5, specially in *ch*HES-20 cells (Fig. 3c). Expression of *AFP* increased significantly after 5 days differentiation of *ch*HES-20 cells, but did not increase obviously after day 5 of NTERA-2 cells (Fig. 3d). The ectodermal marker, *Pax6*,

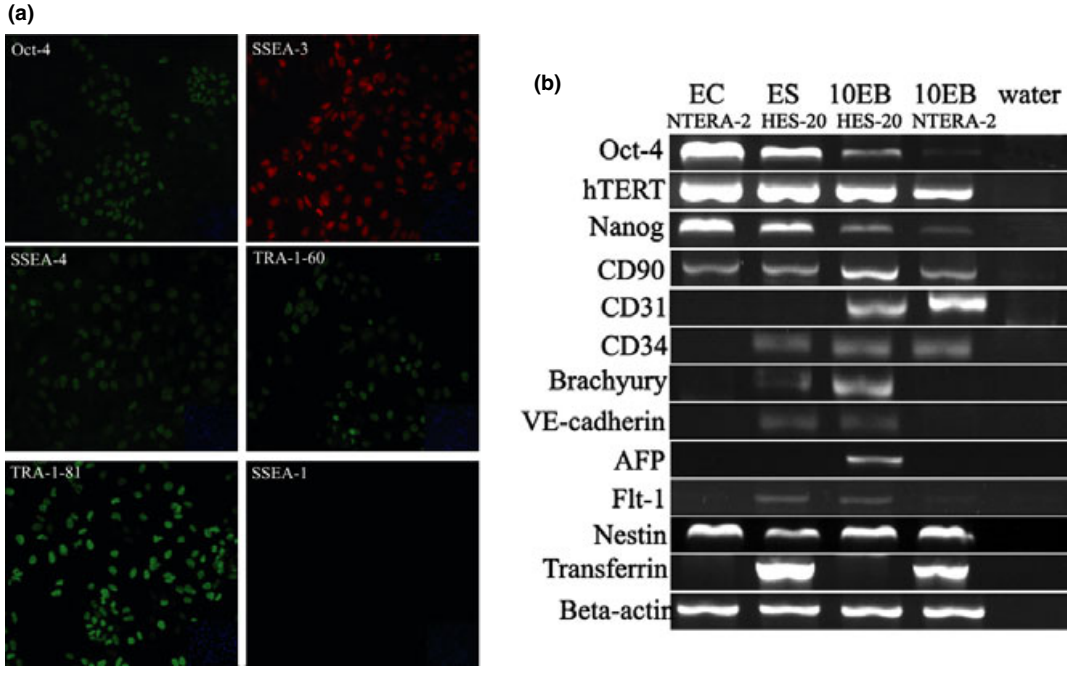

Figure 1. Assessment of the undifferentiated state of NTERA-2 cells by immunofluorescence analysis. (a) NTERA-2 cells were Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 positive and SSEA-1 negative. (b) Comprehensive expression of markers related to pluripotency and differentiation, including *Oct-4, hTERT, Nanog, CD90, CD31, CD34, Brachyury, VE-cadherin, AFP, Flt-1, Nestin and Transferrin*.

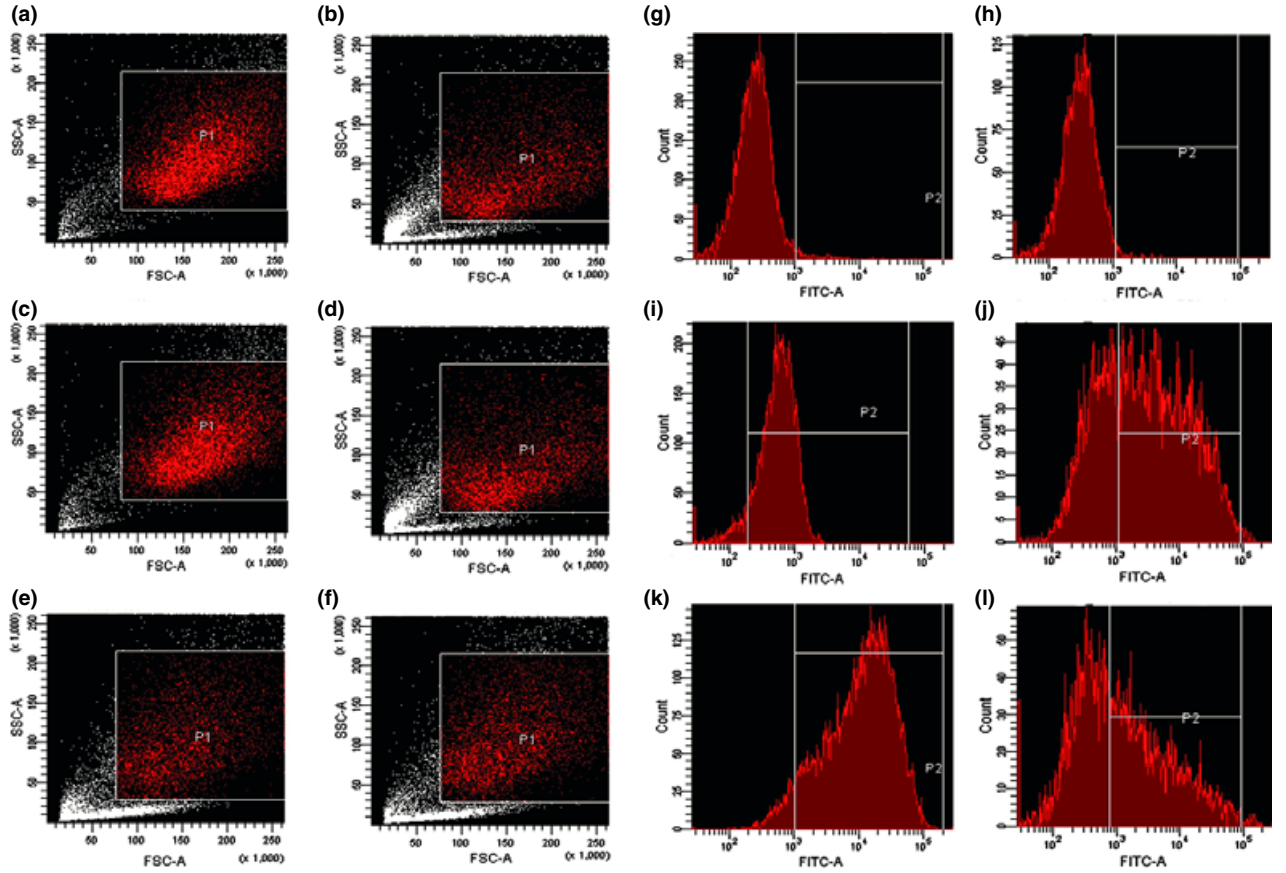

Figure 2. Immunophenotyping of NTERA-2 cells by flow cytometry. Flow cytometric analysis showing that populations of NTERA-2 cells were immunoreactive to cell surface antigens. Mouse IgG was used as isotype control (Figure a and g). SSES-1 staining was negative (Figure b and h). SSEA-3 (89.07%) (Figure c and i), TRA-1-60 (53.12%) (Figure d and j), SSEA-4 (96.43%) (Figure e and k) and TRA-1-81 (48.48%) (Figure f and l).

increased significantly by 5 days differentiation of *ch*HES20 and NTERA-2 cells, but more obviously in *ch*ES20 cells (Fig. 3e). Likewise, expression of mesodermal marker, *KDR*, increased in *ch*HES20 cells, but its expression in NTERA-2 cells increased only slightly after 5 days (Fig. 3f) and change in absolute value of *ch*HES-20 and NTERA-2 cells showed the same tendency (see Supplementary Fig. 1). Our results illustrate two important points. First, compared to *ch*HES-20 cells, NTERA-2 cells were more difficult to differentiate into all three germ layer cells under the same induced differentiation conditions. Secondly, compared to mesoderm and endoderm differentiation, NTERA-2 cells were easy to differentiate into ectoderm under spontaneous differentiation conditions.

NTERA-2 cells formed malignant tumours in vivo

1×10^6 SSEA-3 positive NTERA-2 (Fig. 4a) and *ch*HES-20 cells (Fig. 4b) were injected into hind legs of SCID mice and histopathological examination of tumours formed was carried out 10 weeks later. Cells of NTERA-2 xenografts and morphology of tissues were disordered and typical characteristics of malignant tumours were observed. The cells had infiltrated local muscles at the explant site, but no metastases was found in distant organs (Fig. 4c). Xenografts from *ch*HES-20 cells however, differentiation into cells of the three germ layers with clear tissue morphology, including primitive nerve tissue with rosette structure, immature mesenchymal tissue and nested regions consisting of primary cells (Fig. 4d). These results indicated that tumourigenicity of NTERA-2 cells was different from that of more typical ES cells.

Lower ratio of apoptosis and higher ratio of cell proliferation in NTERA-2 cells

Comparison of levels of spontaneous apoptosis and cell proliferation between NTERA-2 cells and *ch*HES20

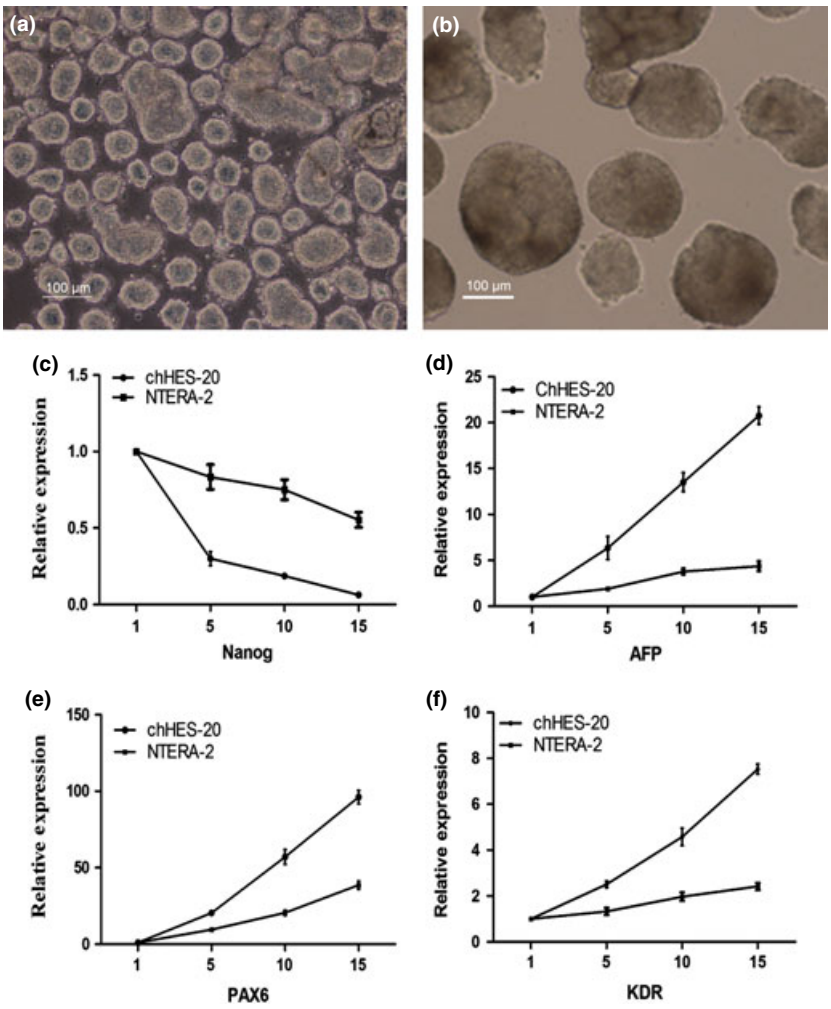

Figure 3. Differentiation *in vitro* of NTERA-2 cells and *ch*HES-20 cells. NTERA-2 cells (a) and *ch*HES-20 cells (b) gave rise to embryonic bodies. (c) *Nanog*, a marker of undifferentiated ES cells, (d) *AFP*, an endoderm marker, (e) *PAX6*, an ectoderm marker, and (f) *KDR*, a mesoderm marker were assayed at each time point over a period of fifteen days. Expression levels of the markers are shown relative to normal *ch*HES-20 cells.

cells was carried out. For *ch*HES-20 cells, mean level of spontaneous apoptosis was 10.7 ± 0.45% (Fig. 5a), but for NTEAR-2 cells, mean level of apoptosis was 2.47 ± 0.24% (Fig. 5b,c). The Edu incorporation assay shows proportion of Edu positive NTERA-2 cells was 63 ± 0.67%, but for *ch*HES20 cells, it was 46 ± 1.3% (Fig. 5d), indicating more DNA synthesis in NTERA-2 cells. Together, lower levels of apoptosis and higher levels of cell proliferation indicated that NTERA-2 cells had more capacity for proliferation compared to *ch*HES20 cells.

Whole expression characteristics of NTERA-2 cells

To examine potential differences in gene expression, SSEA-3 positive cells from *ch*HES20 and NTERA-2 cells were sorted, for analysis of transcription profiles (GSM172582 and GSM172579). MAS 5.0 software detection algorithm (Affymetrix) was used to determine presence or absence of each gene represented on the array. Any gene with detection class of 'present' was considered present for that array. Venn diagrams were constructed to illustrate presence or absence of gene expression in cells of the two lines. From this analysis, we found that 2091 genes were uniquely expressed in *ch*HES-20 cells, 887 genes were uniquely expressed in NTERA-2 cells (Fig. 6a) and 12396, 11192 genes were expressed in *ch*HES-20 and NTERA-2 cells. Among the 887 unique genes in NTERA-2 cells, most were involved with tumourigenesis (Table 1). Levels of gene expression were significantly different between the two cell lines, with a higher number of genes expressed in *ch*HES-20 cells (Fig. 6b).

To discover whether expression of genes in NTERA-2 cells was associated with non-random genomic amplification in certain 'hot spot' areas, we created a map of chromosomal locations of these differentially expressed genes. All down-regulated genes in NTERA-2 cells were distributed randomly across all chromosomes (data not shown), but most of up-regulated ones

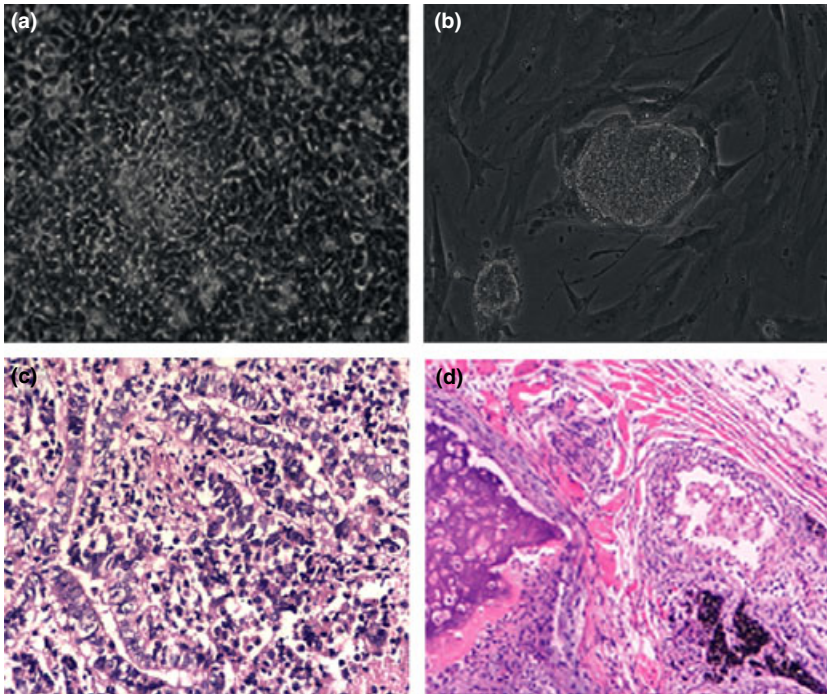

Figure 4. Teratoma formations of NTERA-2 cells and *ch*HES-20 cells. (a) morphology of NTERA-2 cells and (b) *ch*HES-20 cells on feeders, (c) Xenografts from NTERA-2 cells displayed typical characteristics of malignant tumours. (d) Xenografts from *ch*HES-20 cells showed possible differentiation into all three germ layers, including pigment retinal epithelium, epidermal tissues and intestines, muscle, adipose tissue, cartilage.

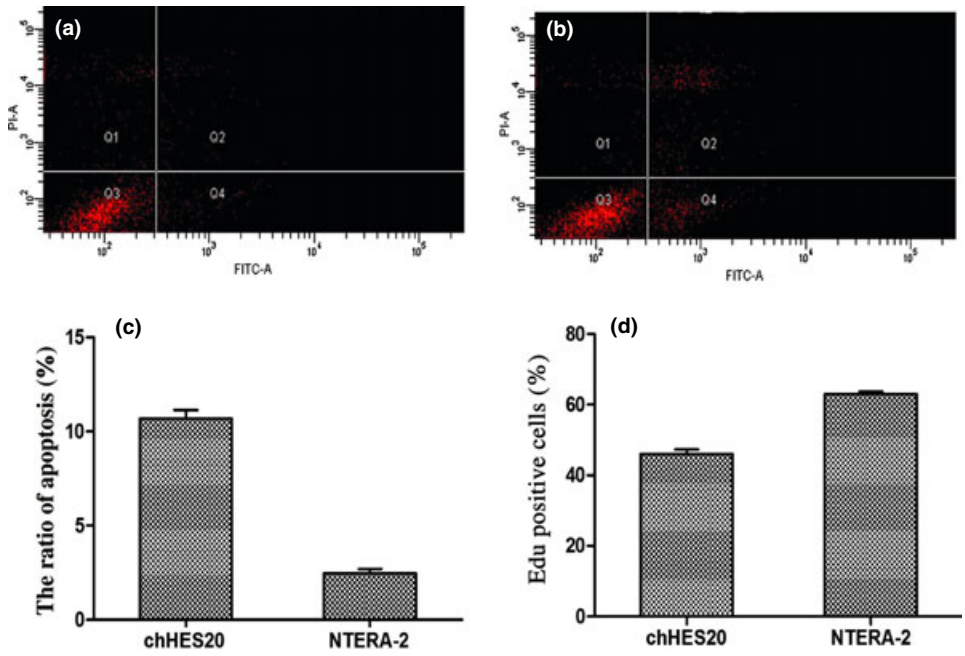

Figure 5. Lower ratio of apoptosis and higher ratio of cell proliferation in NTERA-2 cells compared to *ch*HES-20 cells. (a) level of apoptosis in NTERA-2 cells. (b) level of apoptosis in *ch*HES-20 cells. (c) comparison of apoptotic level between NTERA-2 cells and *ch*HES20 cells. (d) higher level of cell proliferation in NTERA-2 cells compared to *ch*HES-20 cells. Data were calculated as mean ± SEM of at least three separate experiments.

were clustered in chromosomes 1 and 12 (Fig. 7a). We then monitored chromosomal distribution mapping of highly expressed genes in NTERA-2 cells by normalizing length of each chromosome and number of genes in each. We found that there was increased frequency of up-regulated genes on chromosomes 1 and 12 com-

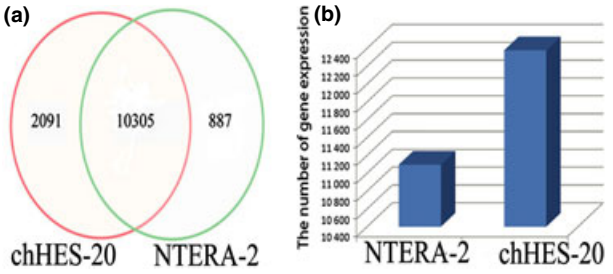

Figure 6. Whole gene expression of NTERA-2 cells compared to *ch*HES-20 cells. (a) Overlaps between genes enriched in NTERA-2 cells and *ch*HES-20 cells. Region of overlap between two circles indicates number of genes and EST probe sets expressed in both cell lines. (b) Numbers of gene expression in Affymetrix chips.

Table 1. Function analysis of unique genes in NTERA-2 cells

Term	Count	Percentage
Oncogene	18	0.99
Mesoderm development	64	3.52
Synaptic transmission	35	1.92
Developmental processes	196	10.8
Induction of apoptosis	22	1.21
Pre-mRNA processing	32	1.76
MAPKKK cascade	24	1.32
Neurotransmitter release	15	0.82
Receptor protein tyrosine kinase signalling pathway	25	1.37
Neurogenesis	60	3.30
Protein phosphorylation	65	3.57
Phospholipid metabolism	19	1.04
Oncogenesis	45	2.47
Mitosis	38	2.09
Cell cycle	90	4.94
Neuronal activities	57	3.13
mRNA polyadenylation	6	0.33
Skeletal development	16	0.88

pared to all other chromosomes (Fig. 7b,c and Table 2).

Up-regulation of pluripotency-related genes and down-regulation of differentiation-related genes

To assess stem-cell characteristics of NTERA-2 cells, we focused on expression profiles of selected genes that are known to regulate self-renewal, pluripotency and differentiation in both ES cells and adult stem cells. Of 10 genes previously shown to be important in pluripotency and self-renewal regulation (10), we found that *POU5F1*, *Nanog*, *LDB2*, *GABRB3*, *FGF4*, *FGF13*, *DNMT3B*, *LDB2* and *CD9*, were up-regulated in NTERA-2 cells, while expression of differentiation-related genes FN1, *MCFD2*, *MSI12*, *NEDD4L*, *PT2*, *PAX6*, *OTX2*, *MCFD2*, *CALB1*, *L1CAM* of 21ectoderm development-related genes, *KDR*, *FLT1*, *HLA-B*, *PITX2*, *THBS1*, *THBS2* of 28 mesoderm development-related genes, and *AFP*, *CER1*, *GATA6* of 7 endoderm development-related genes down-regulated in NTERA-2 cells, whereas expression level remained unchanged for the rest. These results suggest that, compared to *ch*HES20 cells, NTERA-2 cells are more capable of undergoing self-renewal, but less capable of differentiating.

Down-regulation of tumour-suppressor genes and up-regulation of oncogenes

Thirteen tumour suppressor genes, including *PTPRG*, *PTCH*, *SMAD4*, *PTEN*, *RERE*, *RPL10A*, *TIMP1*, *CDH1*, *APC*, *TP53*, *BRCA1*, *MSH2* and *NME1*, were expressed in both samples. *SMAD4*, *PTEN*, *RERE*, *CDH1*, *APC*, *TP53* and *BRCA1* had lower expression in NTERA-2 cells. *PTPRG*, *PTCH*, *RPL10A*, and *TIMP1* were up-regulated but *NME1* and *MSH2* showed no significant difference in their expression levels. All 10 oncogenes studied, including *FGFR1*, *MDM2*, *BCL2*, *LMO2*, *ERBB2*, *TPM3*, *NTRK1*, *MET*, *CDK4*, and *LMO1*, were expressed in NTERA-2 cells and *ch*HES-20 cells and *FGFR1* and *CDK4* expressions were up-regulated. Our results show that most oncogenes were involved in oncogenesis and embryogenesis, but dysregulation of tumour-suppressor genes may be the main reason for tumourigenesis of NTERA-2 cells.

Sequential activation of signalling pathways favour cell transformation and tumour progression of human ES cells

EC cells that progressed from ES were their malignant equivalent (5). To explore signalling events during the procedure, we evaluated interactive roles of a candidate set of signalling molecules at the stage of change of human ES cells to NTERA-2 cells. Growth factor genes, *HDGF*, *PDGFA*, *TGFBR3*, *EGF*, *FGF4*, *FGF19*, *IGFBPL1*, *PDGFA* and *IGFBP7*, growth factor receptor genes *EGFR*, *FGFR2*, *NGFRAP1*, *FGFR3*, *FGFR1* and cell surface receptor-linked signal transduction genes *FZD2*, *STC1*, *FGFR1*, *IFITM1*, *PDGFA* were all up-regulated. Our results show that the proposed set of growth factors and their receptors becomes activated in malignant ES cells *in vitro*.

Activation of Wnt and Notch signalling pathways

Microarray results showed that Wnt and Notch signalling pathways were preferentially activated in NTERA-2 cells compared to *ch*HES20 cells. In all pathways analy-

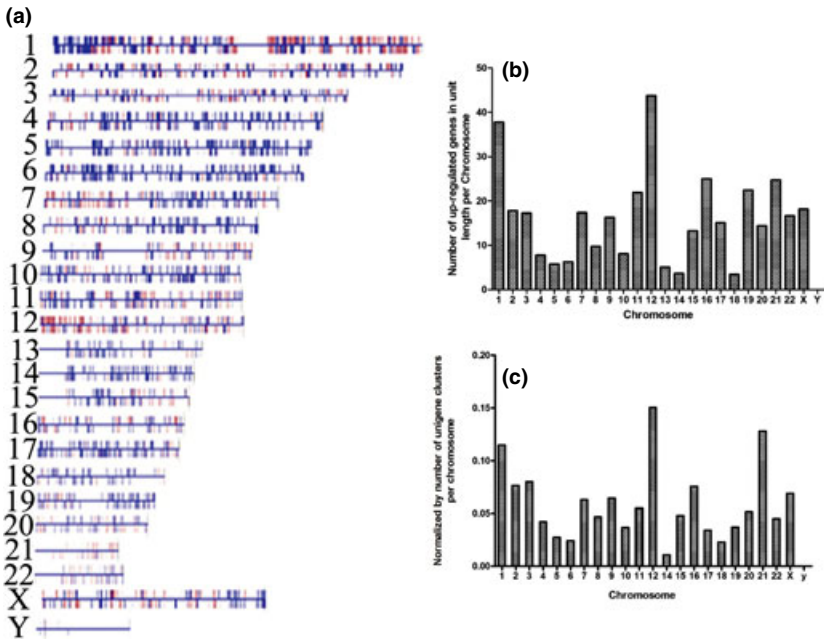

Figure 7. Chromosomal distribution of up- and down-regulated genes normalized by gene numbers and chromosome sizes. (a) Up-regulated genes (red) relatively clustered on chromosomes 1 and 12. Down-regulated genes (blue) were distributed randomly across all chromosomes. (b) Distribution of up-regulated genes per chromosome normalized by size of chromosomes on each chromosome. Chromosomal size was acquired from Ensemble 7. (c) distribution per chromosome normalized by number of UniGene clusters on each chromosome. Number of UniGene clusters (Build 34, version 3) was from NCBI.8.

Table 2. Chromosomal distribution of the over-expression genes in karyotypically aberrant *ch*HES-20 cells by normalizing with the length of each chromosome and the number of the UniGene clusters

chr	The number of up-regulated genes	The relative length of the chromosome	The normalized number of gene expression by chromosome size	The number of genes in per chromosome	The normalized ratios of gene expression by number of Unigene clusters per chromosome
1	124	8.44	37.80	2776	0.1149
2	31	8.02	17.83	1866	0.0766
3	40	6.83	17.27	1473	0.0801
4	28	6.30	7.78	1164	0.0421
5	22	6.08	5.76	1281	0.0273
6	26	5.90	6.27	1528	0.0242
7	27	5.36	17.35	1474	0.0631
8	27	4.93	9.74	1025	0.0468
9	25	4.80	16.25	1207	0.0646
10	25	4.95	8.08	1094	0.0366
11	25	4.61	21.91	1841	0.0549
12	31	4.66	43.77	1355	0.1505
13	7	3.74	5.08	556	0.0341
14	10	3.56	3.65	1220	0.0107
15	19	3.46	13.29	961	0.0479
16	31	3.36	25	1108	0.0758
17	45	3.25	15.08	1442	0.0340
18	7	2.93	3.41	438	0.0228
19	18	2.67	22.47	1624	0.0369
20	15	2.56	14.45	717	0.0516
21	9	1.90	24.74	367	0.1281
22	10	2.04	16.67	756	0.0450
X	27	5.12	18.16	1344	0.0691

sed, genes related to Wnt signalling, which is involved in self-renewal of HESCs and tumourigenesis, had significantly differential expression (11–13). Of the differentially expressed genes related to Wnt signalling, inhibitor GSK-3β was found to be down-regulated, while CTNNB1 and its downstream CCND1 were up-regu-

Table 3. The expression changes of Wnt signalling pathway-related genes in microarray

Gene symbol	Accession	Chromosomal location	Change	Change value (Log Ratio)
CCND2	AI635187	chr12p13	I	4.9
DVL3	NM_004423	chr3q27	I	1.6
LDLR	NM_000527	chr19p13.3	I	2
CSNK1E	NM_001894	chr22q13.1	I	1.2
RAC1	BG292367	chr7p22	I	1.6
CCND1	BC000076	chr11q13	I	2.9
PRKCI	L18964	chr3q26.3	I	1.1
FZD2	L37882	chr17q21.1	I	1.5
CTNNB1	NM_001904	chr3p21	I	1.6
WNT5B	NM_030775	chr12p13.3	I	4.8
RHOA	AF498970	chr3p21.3	D	−0.4
RAC1	AJ012502	chr7p22	D	0.1
PRKCZ	NM_002744	chr1p36.33-p36.2	D	−0.1
MYC	NM_002467	chr8q24.12-q24.13	D	−1.2
PPP2R5E	NM_006246	chr14q23.1	D	−0.1
APC	AI375486	chr5q21-q22	D	−0.7
FZD7	NM_003507	chr2q33	D	−1.9
GSK-3β	NM_002093	chr3q13.3	D	−1.4
FZD6	NM_003506	chr8q22.3-q23.1	D	−0.2
PRKCI	L18964	chr3q26.3	D	−0.1
PRKCB1	M13975	chr16p11.2	D	0.2
PRKCQ	L01087	chr10p15	D	0.3
PAFAH1B1	L13387	chr17p13.3	D	−2.5
PPP2R5C	AW772123	chr14q32	D	−1.2
PRKCA	AF035594	chr17q22-q23.2	D	−1.9
FZD3	NM_017412	chr8p21	D	−1.8
FRAT1	NM_005479	chr10q24.1	D	0.2
C2orf31	NM_030804	chr2q34	D	−1.4
CSNK1E	T51255	chr22q13.1	D	−0.7

Change, the change in tendency of NTERA-2 cells compared with chHES20 cells; I, increase; D, decrease.

lated in NTERA-2 cells. *DVL3*, *FZD2*, *LDLR*, *WNT5A*, *RAC1* and *WNT5B* were up-regulated (Table 3). Of all genes related to Notch signalling pathway, *NOTCH2*, *JAK-STAT* cascade, *STAT5B*, *STAT3 CDKN1A* and *CFLAR* were down-regulated. Genes related to cell cycle and transcription regulation, such as those for cyclin D1, CDKN1B, HES1 and SATA6, were up-regulated. Differential expression of these genes was further validated by real-time PCR (Table 4 and Fig. 8). These results suggested that activated Wnt and Notch signalling pathways may be the main reason for malignant transformation from HESCs to EC cells, and this appears to be similar to results observed in other stem-cell tumourigenesis studies (14,15).

Discussion

CSCs have been found in many solid tumours (16) and reports have shown that they originate from normal stem cells. Molecular mechanisms causing, and resulting from, transformation from normal stem cells to malignant stem cells has recently attracted more interest.

Table 4. The ΔCt values of genes obtained from the real-time RT-PCR

Genes	*ch*HES-20		NTERA-2	
	Mean	±SD	Mean	±SD
Pluripotency-related gene				
Nanog	9.70	1.44	9.01	0.57
POU5F1	5.32	0.90	4.47	0.85
Sox2	9.60	1.95	8.28	0.78
Oncogenes				
FGFR1	11.95	0.53	9.46	0.36
CDK4	11.22	1.18	8.10	0.47
Wnt signalling pathway-related genes				
GSK-3Beta	12.77	0.16	14.83	0.32
Beta-Catenin	25.05	1.08	22.37	0.48
Cyclin D1	11.77	1.22	9.73	0.34
FZD2	12.32	0.82	9.49	0.41
WNT5A	11.78	0.36	9.23	0.57
WNT5B	11.29	0.37	9.78	0.38
Tumour-suppression genes				
Tp53	10.97	1.07	12.31	0.35
PTEN,	9.60	0.36	11.21	0.38
APC	10.21	0.72	11.35	0.31

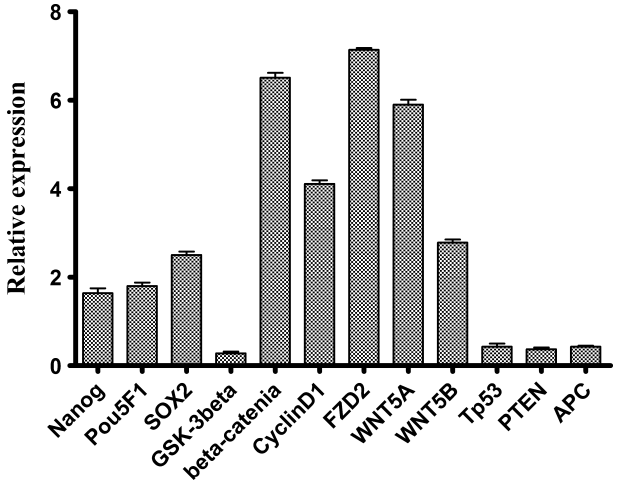

Figure 8. Relative expression level of genes between NTERA-2 cells and *ch*HES-20 cells.

NTERA-2 cells are derived from teratocarcinomas, a subset of tumours that result from neoplastic transformation of primordial germ cells (PGC). NTERA-2 cells are a type of stem cell derived from teratocarcinomas and are generally considered to be the malignant counterpart of ES cells (5). Teratocarcinomas provided not only PGCs in embryonic stage but also development of human ES cells in a phase of suboptimal culture conditions. In this study, we selected embryonal carcinoma cells (NTERA-2 cell line) as a model for studying cell transformation and tumourigenesis of human ES cells, in suboptimal culture conditions.

A primary characteristic of pluripotent stem cells is their ability to maintain their 'sternness' for unlimited periods of time, *in vitro*. In our study, we found a set of important pluripotent markers and cell-surface antigens specific to human ES cells (17–19), and these results are in accordance with our recent report on derivation and characterization of a human ES cell line (20). At the same time, NTEAR-2 cells also expressed some differentiation markers, showing that they were not totally equivalent to ES cells. Higher levels of S-phase cells and lower levels of apoptosis were found with some disparities and limitations in differentiation between NTERA-2 cells and *ch*HES-20 cells. This indicates that differentiation was impaired and embryonic traits are aberrantly maintained (21) – these regarded as first steps in malignant transformation (22). Expression profiles of genes related to pluripotency, oncogenes and tumour suppressor genes, have proved this point at gene level.

Identifying differential gene expression profiles in NTERA-2 cells responsible for tumourigenesis remains a challenge. Some oncogenes and tumour-suppressor genes are involved in embryogenesis and tumourigenesis. At the same time, it is also important to discover the genes with tumourigenic characteristics in ES cells, for further reprogramming safe iPS cells. In our experiments, most tumour-suppressor genes were down-regulated. The balance between oncogene and tumour suppressor genes was disordered. Down-regulation of tumour-suppressors withdrew inhibition of oncogenes, and activated oncogenes pushed progression from ES cells to malignant stem cells. These results provide insights concerning which tumour-suppressor genes could be new promising candidates for ES state maintenance, and IPS cell formation.

Another interesting issue is the mechanism of differential expression of so many genes in NTERA-2 cells compared to *ch*HES20 cells. Over-representation of transcripts may be a result of true functional up-regulation, but also may reflect gene-dosage effects caused by structural amplification (for example, duplication of parts of chromosomal regions), especially if over-represented genes appear in clusters. Analysis of up-regulated genes in our samples revealed that chromosome distribution was not random. Three overexpressed chromosomal regions were 1, 12 and X. It is well known that NTERA-2 cells are highly aneuploid , with some chromosome duplication (23). Therefore, we think that expression patterns observed in NTERA-2 cells are probably combined results of genomic amplification and increased transcriptional activation. At the same time, these results caution us to pay close attention to changes in gene expression level in chromosomes 1, 12 and X when we monitor genetic changes in ES cells in future.

Many molecular pathways that underlie tumour progression represent aberrations of normal processes that control embryogenesis. Some genetic regulatory programmes that control normal embryo development are inappropriately reactivated during tumourigenesis. In our study, results of microarray and real-time PCR showed that the Wnt and Notch signalling pathways were both preferentially activated among pathways investigated. This has suggested that both signalling pathways contributed to transformation from ES cells to EC cells. Earlier studies of EC and culture-adapted human ES cells have shown that adapted human ES cells have a tendency to progress towards malignant stem cells, such as CSCs during long-term culture, and that Wnt signalling is involved in this process (24). Our results have confirmed these findings. Expression of genes encoding components of these systems included *Wnt5A*, *Wnt5B*, *RAC1* and the malignant phenotype of Frizzled receptors Fzd2 (25). Up-regulation of these genes may also induce tumourigenesis through Wnt-beta-catenin-T-cell factor pathway fate decision and proliferation, and is strongly implicated in carcinogenesis in ES cells as reported in cancer cells (26).

Using both molecular and immunological techniques, we have generated a foundation of attributes describing NTERA-2 cells. Furthermore, tumour characteristics of early tumourigenesis employing human EC cells as a model has been explored. Despite their independent advantages and disadvantages, it is likely that both human EC and human ES cell systems will coexist to benefit monitoring of safety of HESCs during cell culture.

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Supporting Information

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

Fig. S1 Expression absolute values in different cell lines and in different time points. (a) Nanog; (b) AFP; (c) PAX6; (d) KDR.

Table S1. Primers of RT-PCR

Table S2. Primers for Real-time RT-PCR

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