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CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells

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The application of human embryonic stem (hES) cells in regenerative medicine will require rigorous quality control measures to ensure the safety of hES cell–derived grafts. During propagation *in vitro*, hES cells can acquire cytogenetic abnormalities[1–3] as well as submicroscopic genetic lesions, such as small amplifications or deletions[4]. Many of the genetic abnormalities that arise in hES cell cultures are also implicated in human cancer development. The causes of genetic instability of hES cells in culture are poorly understood, and commonly used cytogenetic methods for detection of abnormal cells are capable only of low-throughput analysis on small numbers of cells. The identification of biomarkers of genetic instability in hES cells would greatly facilitate the development of culture methods that preserve genomic integrity. Here we show that CD30, a member of the tumor necrosis factor receptor superfamily, is expressed on transformed but not normal hES cells, and that CD30 expression protects hES cells against apoptosis.

CD30 is a member of the tumor necrosis factor receptor superfamily that was originally identified as a surface marker for the malignant Reed-Sternberg cells of Hodgkin disease[5]. The receptor is found only on a few other cell types, including activated lymphocytes, decidual cells and the stem cells of human embryonal carcinoma (EC) cells[6–8], the malignant counterparts of hES cells. Because CD30 is a well-established marker of human EC cells, we anticipated that hES cells might also express this receptor, and that signaling through CD30 might play a role in regulating their growth, survival or differentiation.

To understand the role of CD30 in pluripotent stem cell growth and differentiation, we studied its expression in more detail. Northern blot analysis of mRNA from human EC cell lines showed that, in addition to the main band of 3.8 kilobases (kb) seen in the EC cell lines corresponding to the canonical transcript, a fainter band of ~2.6 kb was expressed (**Fig. 1a**). Both the 3.8-kb and the 2.6-kb bands were downregulated during differentiation of EC cells *in vitro* (**Fig. 1b**). This 2.6-kB band was similar in size to a variant CD30 transcript described previously in myeloid leukemia cell lines[9]. This transcript, originating from alternate promoter usage, encodes a truncated variant form of CD30 consisting only of its cytoplasmic domain, and this truncated protein constitutively activates signaling pathways in the absence of ligand. Reverse transcriptase–polymerase chain reaction (RT-PCR) using a forward primer specific for the variant transcript confirmed its expression in EC cells (**Fig. 1c**). Immunoblots of EC cell lysates were probed with antisera raised against a domain in the cytoplasmic portion of the protein

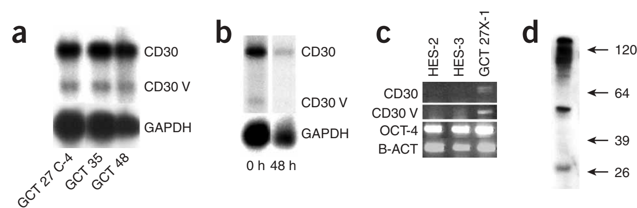

Figure 1 Expression of CD30 transcripts and protein in human EC and ES cells. (**a**) Northern blot of polyA+ mRNA from EC cell lines GCT 27C-4, GCT 35 and GCT 48, probed with full-length cDNA for CD30 or GAPDH as a loading control. (**b**) Northern blot of polyA+ mRNA from control GCT 27X-1 EC cells and from cells treated with 50 ng/ml BMP-2 for 24 or 48 h, probed with full-length cDNA for CD30 or GAPDH probes. Scanning densitometry of these Northern blots showed a 3.2-fold reduction in CD30 expression following this treatment. (**c**) RT-PCR analysis of expression of CD30 canonical and variant transcripts in HES-2 P15, HES-3 P65 and EC cell line GCT 27X-1. Oct-4 products shown as stem cell marker, GAPDH is control for RT-PCR. (**d**) Immunoblot analysis of GCT 27X-1 cell lysate using antibody to C-terminal region of CD30. Position of molecular weight markers shown to right. The 120-kDa band is the canonical form, the low-molecular-weight band is the variant form and the 57-kDa band in the middle results from known cross-reactivity of the antibody with another cellular protein.

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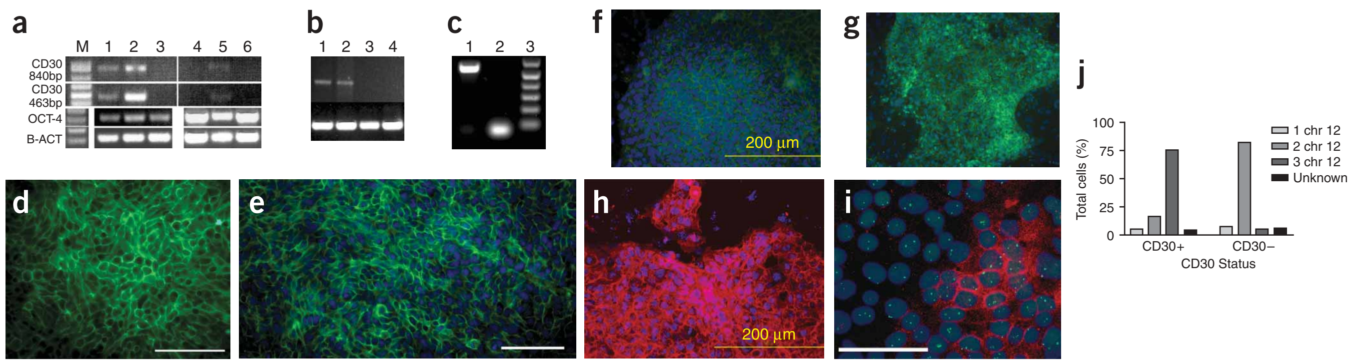

Figure 2 Expression of CD30 in diploid or karyotypically abnormal hES cell lines. (**a**) RT-PCR analysis of CD30 expression in HES-2, HES-3 and HES-4. Following reverse transcription, two PCR reactions were carried out for CD30, along with reactions for Oct-4 and β-actin. Lanes are as follows: lane 1, HES-2 passage 51+36, bearing partial duplication of the long arm of chromosome 1; lane 2, HES-3 passage 55+18, mosaic for trisomy 12; lane 3, HES-4 passage 41+19, diploid; lane 4, HES-2 passage 92, diploid; lane 5, HES-3 passage 78, diploid; lane 6, HES-4 passage 65, diploid. Molecular weight markers: CD30 840-bp amplicon, 650- and 850-bp markers; CD30 463-bp amplicon, 400-, 500- and 650-bp markers; Oct-4, 500- and 600-bp markers; β-actin, 200- and 300-bp markers. (**b**) RT-PCR for 840-bp amplicon of CD30 on HES-1 cells. Lanes 1–2, two samples of HES-1 P54 from Hadassah University Hospital laboratory bearing rearrangement of 1q; lanes 3–4, diploid HES-1 passages 39 and 40, respectively. (**c**) RT-PCR for variant form of CD30 in HES-2 passage 28+30 bearing duplication in 1q. Lane 1, 642-bp amplicon; lane 2, no-template control. (**d**–**h**) Staining of CD30 in HES-2 passage 51+28 with duplication in the long arm of chromosome 1 (**d**, scale bar 50 μm) or HES-3 passage 55+32 with trisomy 12 (**e**, scale bar 50 μm; preparation counterstained with Hoechst 33258), HES-3 passage 37+33 bearing (1,6) translocation (**f**, scale bar 200 μm), HES-1 passage 55 with rearrangement in 1q (**g**, same magnification as **f**) and BG01V with trisomy 12, 17 and X (**h**, scale bar 200 μm). (**i**) Triple-label staining for CD30 (red), α-centromeric probe for chromosome 12 (green) and DNA (blue) in HES-3 passage 55+20 mosaic for trisomy 12 (scale bar 50 μm). (**j**) Quantitative analysis of relationship between chromosome 12 copy number and CD30 staining in HES-3 hES cell cultures mosaic for trisomy 12.

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(C-terminal epitope). The C-terminal antisera reacted with bands of 120, 55 and 25 kilodaltons (kDa) (**Fig. 1d**); the size of the 25-kDa band is that expected of the variant form of CD30 protein, and it was not detected in blots probed with antibodies against the N-terminal ectodomain (not shown).

HES cells share many properties with pluripotent EC cells, including morphology, cell-surface antigen and gene expression profile, growth requirements and differentiation capacity. To investigate whether CD30 expression is tumor specific or a general property of human pluripotent cells, we examined expression of CD30 in colonies of hES cells using immunocytochemistry for full-length CD30 protein and RT-PCR for both the canonical and variant cytoplasmic forms of CD30. Antibodies Ber-H2 or M67, both of which react with the ectodomain of CD30, yielded background staining only on colonies of hES cell lines HES-2, HES-3 and HES-4 that were positive for the stem cell antigen GCTM-2 (Ber-H2 staining of HES-4, **Supplementary Fig. 1** online) but stained cultured human EC cells in a characteristic pattern decorating the cell border (Ber-H2 staining of GCT 27X-1, **Supplementary Fig. 1** online). Whereas RT-PCR products representing both forms of CD30 transcript were obtained from human EC cell line GCT 27X-1, none were obtained from mRNA from the hES cell lines (**Fig. 1c** and below). The presence of mRNA for the transcription factor Oct-4 and the housekeeping gene β-actin confirmed the integrity of EC and hES cell RNA.

Sequence analysis of human EC cDNA for CD30 revealed no mutations in the coding sequence of the gene (not shown), and Southern blot analysis of restriction fragment digests of tumor and normal tissue DNA from 17 patients with testicular germ cell tumors (GCTs) of diverse histology showed an identical pattern of bands in lymphocyte and tumor DNA (example, **Supplementary Fig. 2** online). Thus, no evidence for major rearrangements or deletions in the CD30 gene was found. Previous studies[10] have identified a CCAT repeat–containing microsatellite region in the promoter region of the CD30 gene; polymorphisms in the length of this repeat region can alter the activity of the promoter. We isolated DNA from three EC cell lines and from two normal human leukocyte samples, and used PCR to amplify the microsatellite region. The lengths of the PCR products varied within the range of the normal population as described elsewhere[10]; there was no evidence of marked truncation or expansion of the microsatellite in the cell lines (**Supplementary Fig. 2** online). Thus CD30 expression seems to be an epigenetic feature of malignant stem cells.

The CD30 expression studies on hES cells described above were carried out on cultures maintained in serum-containing medium in the presence of a mouse embryonic fibroblast feeder layer, with subcultivation by mechanical dissection of colonies into small clumps of cells. Under these conditions, the hES cell lines studied displayed remarkable karyotypic stability (**Supplementary Table 1** online). We also grew hES cells using the system of Amit *et al.*[11] (medium supplemented with serum replacement and fibroblast growth factor 2 (FGF-2), and embryonic fibroblast feeder layer support), or that of Xu *et al.*[12] (conditioned medium from embryonic fibroblast and Matrigel (BD-Biosciences) pretreatment of the culture surface), and passaged the cells following dispersal by trituration after treatment with either cell dissociation buffer or collagenase. Under these conditions karyotypically abnormal cells appeared in some cultures of HES-1, HES-2 and HES-3, and overgrew the diploid population (**Supplementary Table 1** online). The lesions included duplications of part of the long arm of chromosome 1, trisomy 12, a lesion observed previously by others in hES cell cultures[1,2] (overrepresentation of 12p is also highly characteristic of human germ cell tumors of the testis[13], and EC cell lines retain the lesion during prolonged passage *in vitro*[14]), a balanced translocation between chromosomes 1 and 6, and a complex lesion on the long arm of chromosome 1 (46, XX, der(1)), involving duplication of q2 and inverted duplication of q3–4.

Abnormal variants of cell lines HES-1, HES-2 and HES-3 showed a faster population doubling time compared with diploid cells (∼30 h versus 72 h under these growth conditions, data not shown). To assess differentiation and growth requirements *in vitro*, we compared the effect of withdrawal of FGF-2 on the proportion of stem cell

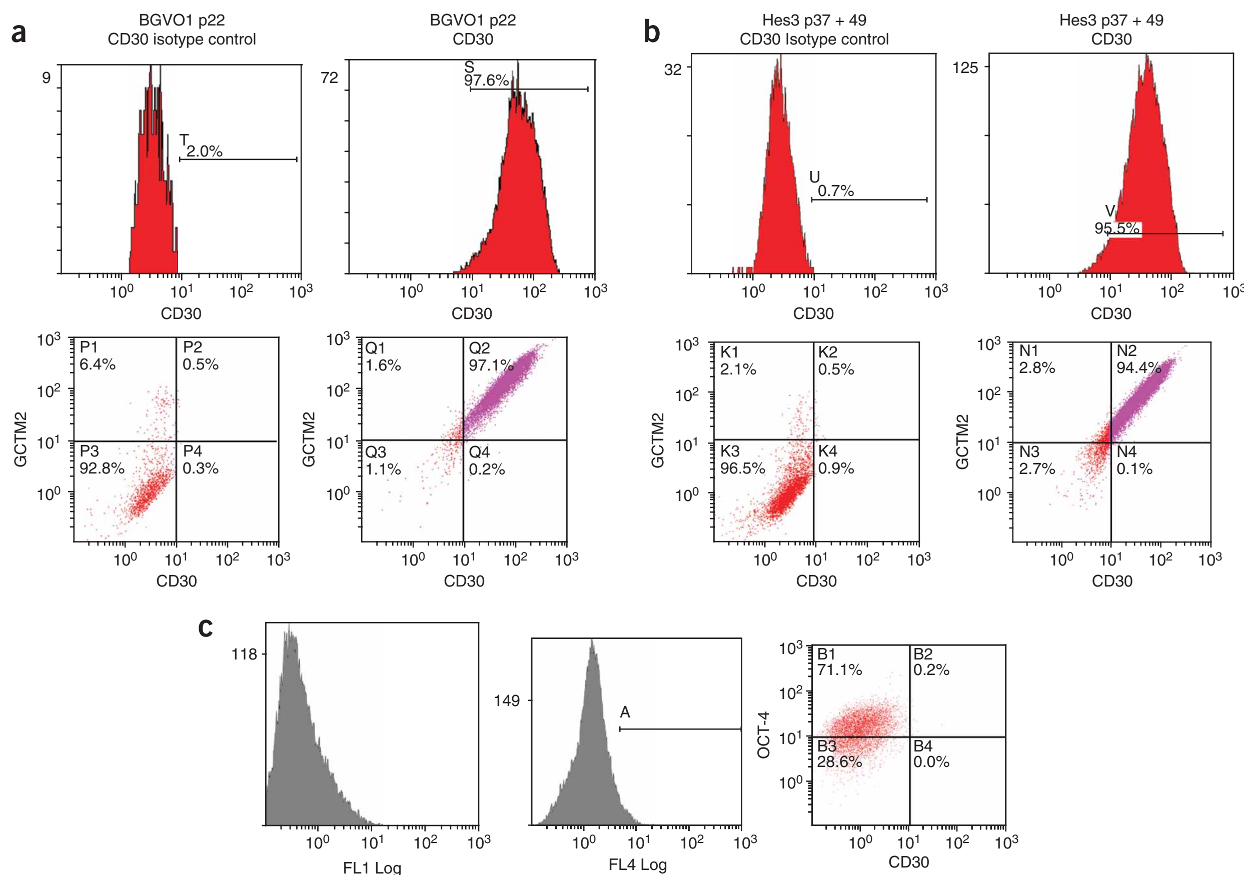

Figure 3 Flow-cytometric analysis of CD30 expression in abnormal and diploid hES cell lines. (**a**) BG01V; (**b**) HES-3 passage 37+49 (46XX, t(1;6)(p22;q15)); (**c**) HES-2 passage 31+11 (46XX). In **a** and **b**, top panels show actual profiles for isotype control and CD30, and bottom panels show dot plots for double labeling with GCTM-2. In **c**, dot plot for double label of Oct-4 and CD30 shown at top, with raw profiles for isotype control and CD30 below.

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marker–positive cells 15 d after subculture of HES-3 bearing trisomy 12 to the effect of factor withdrawal on diploid HES-3 cells passaged only twice in the serum-free system. Withdrawal of FGF-2 induced differentiation in diploid ES cells under these conditions but had no effect on the abnormal cells in this assay (**Supplementary Fig. 3** online). Alterations in differentiation capability of the abnormal cell lines were also apparent in transplantation assays. When injected into immunodeficient mice, the abnormal cell lines did not form malignant ECs, unlike EC cells (**Supplementary Fig. 4** online), and still showed differentiation into various types of tissue. However, compared with teratomas formed by diploid ES cells (**Supplementary Fig. 4** online), teratomas formed by the abnormal cells (**Supplementary Fig. 4** online) contained a much greater proportion of primitive, undifferentiated cells. Seven mice were injected, each at two sites, with cells from two of these karyotypically abnormal lines, and 14 tumors obtained showed similar histology with a preponderance of poorly differentiated cells.

The karyotypically abnormal ES cell sublines expressed CD30 transcripts (**Fig. 2a,b**). The expression of CD30 was not an inevitable consequence of growth in the absence of serum; diploid cultures of cell line HES-4 grown for as many as 20 passages in serum-free medium were negative for CD30 transcripts or surface staining (**Fig. 2a**, lane 3, immunostaining not shown). Karyotypically abnormal cells also expressed transcripts encoding the variant form of CD30, similar to EC cells (HES-2, **Fig. 2c**). Expression of this transcript was downregulated following treatment of abnormal cell line BG01V with 50 ng/ml bone morphogenetic protein 4 (BMP-4) for 4 d (**Supplementary Fig. 5** online). All abnormal lines derived from HES-1, HES-2 and HES-3 showed typical surface staining for CD30, as did an independently derived line containing extra copies of 12, 17 and X (ref. 3; **Fig. 2d–h**). That the abnormal HES-3 subline was mosaic for trisomy 12 when the abnormality was first detected permitted us to evaluate the relationship between karyotype and CD30 expression within a mixed population of cells grown under identical conditions. Combined indirect immunofluorescence analysis for CD30 and fluorescence *in situ* hybridization with an α-centromeric probe for chromosome 12 (**Fig. 2i**) showed a clear concordance between CD30 expression and the presence of an extra copy of chromosome 12 (quantitative analysis, **Fig. 2j**).

Flow-cytometric analysis provided confirmation of the immunostaining data; diploid cell line HES-2 showed a very low proportion of $CD30^+$ cells, but an aneuploid variant of HES-3 with a (1;6) translocation, and BG01V both showed a very high proportion of $CD30^+$ cells (**Fig. 3**).

Cell line HES-2 reproducibly gives rise to aneuploid variants when cultured in serum replacement medium. We examined the early stages of emergence of populations of $CD30^+$ subpopulations of cell line HES-2 to determine the relationship between CD30 expression and the cells' susceptibility to spontaneous or induced apoptosis. Combined terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and CD30 labeling of cells followed by flow cytometry showed that $CD30^+$ cells that gradually emerged during cultivation in

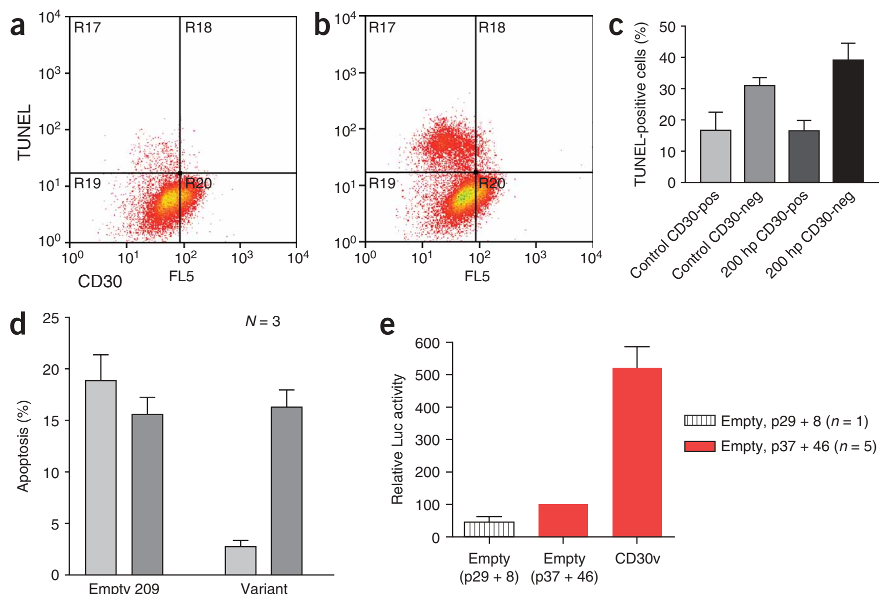

Figure 4 Combined analysis of CD30 expression and apoptosis in HES-2 serum replacement cultures by flow cytometry and inhibition of apoptosis by expression of CD30 variant. (**a**,**b**) Typical flow cytometry profiles for spontaneous apoptosis and H_2O_2-induced apoptosis, respectively. Gates for isotype control stainings are indicated within the panels. (**c**) Quantitation of data from three experiments as shown in **a** and **b**. Bars show percentage of TUNEL-positive cells for (left to right) control CD30-positive cells, control CD30-negative cells, CD30-positive cells treated with 200 μM hydrogen peroxide, CD30-negative cells treated with 200 μM hydrogen peroxide. Values are mean ± s.e.m. of three separate experiments. (**d**) Inhibition of spontaneous apoptosis in diploid HES-2 cells following transfection of cDNA encoding variant CD30. Level of apoptosis in transfected cells (identified by EGFP expression, light gray bars) is compared to that in nontransfected cells (dark gray bars) in cultures transfected with bicistronic vector containing EGFP only (empty vector) or EGFP and CD30 variant (variant). Values are mean ± s.e.m. of three separate experiments. (**e**) Activation of NF-κB signaling by CD30 in hES cells. Basal reporter activity was higher in aneuploid HES-3 cells bearing (1,6) translocation, compared with diploid hES cells, and cotransfection with the CD30 variant greatly increased reporter activity. Values are mean ± s.e.m. of three separate experiments.

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serum replacement had lower levels of spontaneous apoptosis and were less sensitive to apoptosis induced by H_2O_2 (**Fig. 4a–c**). To determine whether expression of CD30 could account directly for the decreased sensitivity of hES cells to apoptosis, we introduced cDNA encoding the variant form of CD30 under the control of a constitutive promoter into diploid hES cells in a bicistronic construct that also included green fluorescent protein (GFP). We evaluated cell survival using TUNEL labeling, and compared CD30 variant transfected cells with cells transfected with vector-encoded GFP cDNA only. Expression of CD30 variant was clearly associated with a decreased level of apoptosis in this short-term assay (**Fig. 4d**).

We also considered whether CD30 signaling activates the NF-κB pathway in hES cells. Abnormal cell line HES-3 with a (1;6) translocation showed a higher basal level of reporter activity when transfected with a construct incorporating NF-κB binding sites driving luciferase expression, compared with diploid cells (**Fig. 4e**). The reporter activity was greatly enhanced when the cells were cotransfected with the CD30 variant construct along with the luciferase plasmid. Incubation with an inhibitor of NF-κB transcriptional activity showed 95% inhibition of reporter activation, confirming the specificity of the assay (data not shown).

It was first observed many years ago[15] that CD30 might prove a useful marker for human EC in diagnostic histopathology, and subsequent observations have provided ample confirmation of this finding[7,16]. Nonetheless, the biological function of CD30 in human EC stem cells has remained obscure. The finding that diploid hES cells lack detectable CD30 transcripts or CD30 protein on their surfaces, combined with evidence in the mouse that CD30 is not required for embryonic and fetal development[17], suggest that CD30 expression is not characteristic of pluripotent cells in the embryo or *in vitro*, but is instead peculiar to transformed human pluripotent cells. This ectopic expression of CD30 in human EC is apparently unrelated to changes in its coding sequence, or to gross rearrangements or deletions in the gene in tumor DNA, but could involve changes in DNA methylation patterns, or other epigenetic alterations.

The emergence of karyotypically abnormal sublines of HES-1, HES-2 and HES-3 and other cell lines under conditions of serum-free growth[1,2], and the development of submicroscopic DNA lesions under these culture conditions[4], contrast with the genetic stability of these and other ES cell lines under the original culture system[4,18–20] used to derive the cell lines, but it is clearly not an inevitable consequence of propagation in the absence of serum and/or a feeder layer. One important difference between our original culture method and the newer techniques is the use of cell dissociation buffer or enzymes to disperse cells in the serum-free systems: whereas the original technique rarely if ever affects the transfer of single ES cells, other dissociation techniques are more likely to result in passage of single cells or very small clumps of cells. The cloning efficiency of single human pluripotent stem cells is very low in any existing culture system. Even pluripotent EC cells show <0.5% cloning efficiency in the presence of a feeder cell layer[21]; the cloning efficiency of our ES cell lines is even lower (M.F.P., unpublished data), and others[11] have made similar observations. Such low cloning efficiency probably results directly from the process of dissociating hES cell or EC colonies to single cells, as well as cultivation at low density per se, because even the presence of a feeder cell layer does not ensure efficient clonal growth. Thus serial passage as single cells probably presents a strong selective barrier to survival of pluripotent stem cells.

As we have shown, CD30 expression provides a significant survival advantage for pluripotent stem cells that express it. Evidence that CD30 might function in an autocrine, cell-autonomous fashion to provide a selective advantage for transformed human pluripotent cells was obtained in the present study, which showed that a variant form of CD30 was expressed in EC cells and karyotypically abnormal hES cells. This variant receptor was previously shown to transactivate gene expression through NF-κB and to induce differentiation in HL-60 cells in a ligand-independent fashion[9]. Our results confirm activation of the NF-κB signaling pathway by the variant form of CD30 in hES cells, and they also show that the basal level of this pathway is higher in aneuploid cells expressing CD30 than in their normal counterparts. The sensitivity to spontaneous or induced apoptosis of hES cells transfected with a bicistronic expression vector containing CD30

variant cDNA and enhanced GFP (EGFP) was reduced, compared with cells expressing EGFP alone, as measured by TUNEL assay. The survival effect was apparent under conditions that drive death or differentiation of human pluripotent stem cells, namely dissociation to single cells and subcultivation in the absence of a feeder cell layer[21]. Such effects of CD30 overexpression are consistent with its postulated action in promoting the survival of other types of transformed cells, for example, lymphomas[22].

While this manuscript was in preparation, Enver *et al.*[23] reported a comparison of the biological properties and transcription profile of an aneuploid hES cell line with that of its diploid parent. These studies showed that the abnormal line had a higher cloning efficiency and a faster population doubling time. Several differences in gene expression between the two cell lines were observed, most notably an increase in transcripts for the Notch ligand *DLK1*.

Additional extensive multicenter studies will be required to determine whether CD30 expression always accompanies the development of karyotypic abnormalities in hES cells, whether expression is activated by other genetic lesions not evident at the level of a G-banded karyotype and whether expression of this surface antigen is necessarily associated with altered biological properties in the abnormal cells. Such studies could be carried out under the auspices of the International Stem Cell Initiative[24]. It is entirely possible that CD30 expression might be an adaptive response of the cell that predisposes to genetic damage, because if expression were an epigenetic adaptive response of hES cells to stress, it might permit survival of cells under suboptimal conditions at the expense of greater tolerance for accumulation of DNA damage. The absence of CD30 expression in diploid ES cells, the very limited range of its expression in other cell types, its nearly universal presence on the surface of human EC cells *in vivo* or after extensive passage *in vitro*, and its appearance on five abnormal but nonmalignant ES cell sublines, each bearing a distinct single chromosomal abnormality, all indicate that the presence of this antigen on the cell surface will prove a useful marker of early as well as late stages of transformation of human pluripotent stem cells. CD30 antigen expression provides a simple means for monitoring ES cell cultures for abnormal variant cells, even if they are present only at low frequency in a population, through the use of flow cytometry, and for quantitative assessment of the effects of modifications in ES cell culture methodology on the emergence of such abnormal cell populations. Available immunotoxin conjugates[25] could be used to purge ES cell cultures of abnormal cells bearing CD30. CD30 thus represents a prototype biomarker for monitoring and maintaining the genetic health of hES cell cultures.

METHODS

Cell lines and indirect immunofluorescence. EC cell lines GCT 27C-4, GCT 27X-1, GCT 35 and GCT 48, derived from nonseminomatous human testicular GCT, were grown as described elsewhere[26]. Induction of differentiation of GCT 27X-1 was achieved by treatment with BMP-2 (ref. 27) or by subculture as single cells in the absence of a feeder cell layer[21]. ES cell lines HES-2, HES-3, HES-4, HES-5 and HES-6 were grown under standard conditions as described; spontaneous differentiation of these cell lines was induced by cultivation for prolonged periods at high cell density[18]. All of these cell lines express surface markers characteristic of primate ES cells, express transcripts for Oct-4, FoxD3, TDGF-1 and GDF-3, and form teratomas in immunodeprived mice. BG01V cells were induced to differentiate[28] by treatment with 50 ng/ml BMP-4.

Cell lines HES-2, HES-3 and HES-4 were all transferred at several passage levels to the culture system described by Amit *et al.*[11] using either collagenase or cell dissociation buffer to disaggregate colonies of ES cells for subculture and then maintained to different passage levels in this system. The culture medium was supplemented with 20% KnockOut Serum Replacement (Invitrogen) and 4 ng/ml FGF-2, and feeder cells were used at a density of $2 \times 10^4/cm^2$. Cell line HES-2 was also grown in the culture system of Xu *et al.*[12] on Matrigel (BD-Biosciences)-treated surface in medium supplemented with KnockOut Serum Replacement, 4 ng/ml FGF-2 and 50% mouse embryo fibroblast conditioned medium. Cells were harvested using cell dissociation buffer to disaggregate the colonies for subculture. Cell line BG01V was maintained in the system described by Amit *et al.*[11]

The passage level of cells grown under standard conditions is annotated herein as P_x, where x is the number of passages from primary culture of the inner cell mass. The passage level of cells grown in serum-free medium is annotated as P_{x+y}, where x represents the passage level at which the cells were transferred into the serum-free system and y represents the number of passages under serum-free conditions.

Colonies of hES cells consisting predominantly of cells expressing stem cell markers were fixed in ethanol and stained with monoclonal antibodies Ber-H2 (DAKO) and M67 (Immunex Corporation) against CD30, followed by anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (DAKO), and for double-label stains, control mouse serum, monoclonal antibody GCTM-2, biotinylated rabbit anti-mouse IgM (DAKO), and Texas Red–streptavidin (Amersham Biosciences). EC cells were used as a positive control for the immunostaining. In some experiments, CD30 staining was visualized using goat anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen).

Karyotype analysis. Standard G-banding karyotype analysis was carried out at several passage levels as listed in **Supplementary Table 1** online. A total of 20 metaphases were evaluated.

RNA analysis. PolyA$^+$ RNA was isolated from EC cell lines using detergent lysis followed by oligodT Sepharose chromatography; methods for RNA isolation and Northern blot analysis are described in detail elsewhere[29]. The probe for CD30 was a full-length human cDNA probe (Immunex Corporation). RT-PCR was carried out on polyA$^+$ mRNA isolated from hES cells on oligodT magnetic beads as described elsewhere[18] or on total RNA extracted into Trizol and precipitated in isopropanol. PolyA$^+$ mRNA from ES cell lines or GCT 27X-1 or total RNA was subjected to RT-PCR to detect full-length and variant CD30 transcripts. The canonical form of CD30 mRNA was amplified using the forward primer 5′-CTGTGTCCCCTACCCAATCT-3′ (start, nucleotide position 1119, GenBank accession number M83554) and the reverse primer 5′-CACTGAGAGCATGACATCGC-3′ (start, nucleotide position 1959, GenBank accession number M83554) to yield an 840–base pair (bp) amplicon, or the forward primer 5′-AGCTAGAGCTTGTGGATTCCAG-3′ (start, nucleotide position 1517, GenBank accession number M83554) and the reverse primer 5′-GTCTTCTTTCCCTTCCTCTTCC-3′ (start, nucleotide position 1980, GenBank accession number M83554) to yield a 463-bp amplicon. The variant form was amplified using the forward primer 5′-CAGCAAGGCAAAGAGTGTGG-3′ (start, nucleotide position 15, GenBank accession number D86042) and the reverse primer 5′-AGCCAAGCTTTCACTTTCCAGAGGCAGCTGT-3′ (start, nucleotide position 687, GenBank accession number D86042) to yield a 672-bp amplicon. Amplifications were carried out over 45 cycles with an annealing temperature of 65 °C. Amplification of Oct-4 and β-actin transcripts was carried out as described[18]. The identities of all PCR products were verified by automated DNA sequence analysis.

Expression of canonical and variant forms of CD30 at the protein level. Cell lysates were prepared from human EC cell line GCT 27X-1 by extraction of cells into 150 mM NaCl, 1% Nonidet P-40 (NP-40) and 50 mM Tris, pH 8.0, followed by removal of nuclei and insoluble material by low-speed centrifugation. The lysates were separated on 10% SDS polyacrylamide gels and transferred to Hybond-P (Amersham Biosciences) membranes. The blots were probed with goat antisera against a C-terminal epitope in CD30 (Santa Cruz Biotechnology) or with Ber-H2. Detection was carried out using anti-goat immunoglobulins conjugated to horseradish peroxidase, with visualization of bands using the ECL plus chemiluminescence system (Amersham Biosciences).

DNA from tumors, patient lymphocytes and cultured cell lines. The freshly obtained tumor samples included in this study were collected in close collaboration with urologists and pathologists in the southwestern part of the Netherlands after informed consent. All tumors were obtained before

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chemotherapy and/or irradiation. Directly after surgical removal, representative parts of the tumor and adjacent normal tissue (when available) were snap-frozen, and other pieces were fixed overnight in 10% buffered formalin and embedded in paraffin. The size of the testis and the tumor was measured in three dimensions. The tumors were diagnosed according to the World Health Organization classification for testicular tumors. Nonseminomas containing both a seminoma and a nonseminoma component were classified as combined tumors, according to the British classification, instead of as nonseminomas according to the World Health Organization classification system.

High-molecular-weight DNA was isolated from the snap-frozen tissue samples (test DNA) and from peripheral blood (reference DNA), or from cell lines using standard procedures. After restriction enzyme digestion, Southern blot analysis was carried out using routine procedures; the membrane was probed with a full-length human CD30 cDNA, provided by the Immunex Corporation.

Genomic PCR for a microsatellite regulatory region in the 5′ regulatory region of the CD30 gene was carried out on DNA from several cell lines and normal human subjects; the conditions for the PCR reaction were as described[10].

Analysis of differentiation of diploid and karyotypically abnormal hES cell lines. To analyze differentiation *in vitro*, diploid HES-3 cells or HES-3 cells trisomic for chromosome 12 were grown in the culture system of Amit *et al.*[11]; control cultures of either cell subline were grown in medium supplemented with 4 ng/ml FGF-2 and test cultures were grown in the absence of the factor. At 15 d after subculture, cells were harvested and analyzed by flow cytometry for expression of the GCTM-2 stem cell antigen as described[30]. To study differentiation *in vivo*, we inoculated SCID mice with diploid HES-3 or HES-4 cultures, or karyotypically abnormal sublines of HES-2 and HES-3, beneath the testis capsule[18], and analyzed the resulting lesions by routine histological examination. All animal experimental protocols adhered to the guidelines of the National Health and Medical Research Council, and were approved by the Monash Medical Center Animal Ethics Committee.

Analysis of CD30 expression and chromosome 12 copy number. HES-3 cells (passage 55+20) mosaic for trisomy 12 were seeded and cultured for 1 d in medium supplemented with knockout serum replacer and FGF-2. Cells were treated with 75 mM $CaCl_2$ for 5 min at 37 °C and fixed in methanol–acetic acid (3:1) for 5 min at 4 °C. Slides were next air dried and stored at –20 °C. Slides were incubated in denaturation solution (70% formamide, 2 × SSC, pH 7.4) for 5 min at 73 °C and dehydrated by subsequent 1-min incubations in 70%, 85% and 100% ethanol. The green fluorescent chromosome 12 probe was prepared according to the manufacturer's instructions (Vysis), denatured at 73 °C for 5 min and kept at 50 °C. Slides were warmed on a 50 °C heated stage and 10 µl of prepared probe applied for 4 h at 42 °C. Next, slides were washed in 0.4 × SSC–0.3% NP-40 (pH 7.0) for 2 min at 73 °C and then in 2 × SCC–0.1% NP-40 (pH 7.2) for 1 min. Slides were next washed once in PBS and incubated for 1 h at 37 °C in a Ber-H2 antibody to CD30 (DAKO) in PBS, washed three times in PBS, and incubated in a 1:50 dilution of sheep anti-mouse Alexa Fluor 546 (Molecular Probes) in PBS for 1 h at 21 °C. Slides were next washed three times in PBS and counterstained with 4′-6-diamidino-2-phenylindole (DAPI; 0.1 µg/ml) for 10 min at 21 °C, washed once in PBS and mounted with VECTASHIELD (Vector Laboratories). The specimens were examined with a DMR Leica fluorescence microscope fitted with a mercury lamp using the ultraviolet, fluorescein and Texas Red channels: images were captured with a Leica DC200 camera using Leica DC200 software. The captured images were superimposed and the number of green spots per blue nucleus of cells that were $CD30^+$ and CD^- were scored. A total of 600 cells from randomly selected fields were counted.

Analysis of CD30 expression by flow cytometry. hES cell lines HES-2 (46 XX, passage 31+11), HES-3 (46XX, t(1;6)(p22;q15)) or BG01V (49XXY,+12+17) were harvested and stained for either GCTM-2 or Oct-4 as described earlier[29] along with CD30 (Ber-H2). Detection was carried out with Alexa Fluor 647 goat anti-mouse IgG2b (Oct-4), Alexa Fluor 647 goat anti-mouse IgM (GCTM-2) and Alexa Fluor goat anti-mouse IgG1 (CD30). Appropriate isotype controls were included in all analyses.

Assessment of spontaneous and induced apoptosis as a function of CD30 expression. hES cell line HES-2 was grown in serum replacement medium containing FGF-2 for eight passages on a mouse feeder cell layer and was either not treated or incubated with 200 µM H_2O_2 for 24 h. The culture medium was collected and centrifuged at 3,220*g* for 4 min to collect the apoptotic bodies. The cells were then harvested with Cell Dissociation Solution (Sigma) and made into a single-cell suspension by careful trituration before combining the cells with the resuspended apoptotic bodies. The cell suspension was subsequently fixed in 2% paraformaldehyde for 1 h at 21 °C and TUNEL labeled according to the manufacturer's protocol (Roche Diagnostics GmbH). The TUNEL-labeled cell suspension was then incubated with a 1:30 dilution of CD30 antibody (DAKO) for 1 h at 37 °C, washed with PBS and incubated with 1:500 dilution of anti-mouse Alexa Fluor 647 (Molecular Probes) for 1 h at 37 °C. Finally the cell suspension was washed in PBS and analyzed by flow cytometry (TMC MoFlo Cytomation Co.).

Assessment of the effect of CD30 expression on apoptosis in hES cells. Diploid cultures of HES-2 were expanded under serum-free conditions for five passages in the presence of FGF-2 on mouse embryonic fibroblast feeder layers and determined to be $CD30^-$. Cells were harvested with Cell Dissociation Solution and seeded at 50% confluence in eight-well chamber slides (Nalgene-Nunc International) coated with Matrigel (BD-Biosciences) in the presence of mouse embryonic fibroblast–conditioned medium, as described by Xu *et al.*[12]. The next day the cells were transiently transfected with 2 µg of DNA per well of pBOS-EGFP (empty vector control), pBOS-CD30-EGFP or pBOS-CD30 variant-EGFP using Fugene (Roche) at a Fugene-to-DNA ratio of 3:2 according to the manufacturer's protocol. On the second day the transfection efficiency was judged by the number of cells showing GFP fluorescence (~15–20% of all cells). The cell cultures were next fixed with 1% paraformaldehyde, and apoptosis was quantified using a commercially available TUNEL assay that labels the DNA of apoptotic cells with fluorescein isothiocyanate (Chemicon). Subsequently the fixed cell preparations were incubated with a 1:30 dilution of CD30 antibody (DAKO) overnight at 37 °C, washed three times for 15 min each with PBS and incubated with a 1:200 dilution of anti-mouse Alexa Fluor 546 (H+L) (Molecular Probes) for 1 h at 37 °C. After another three washes in PBS, nuclei were stained with DAPI (1 µg/ml) for 10 min, washed in PBS and mounted with VECTASHIELD. The slides were subsequently observed with a BX-51 Olympus fluorescent microscope and images captured with an Olympus DP-70 digital camera. Transfected cells were identified as cells that showed GFP fluorescence (and red fluorescent CD30 staining in the case of full-length CD30), and the proportion of TUNEL-positive (apoptotic) cells within this population was quantified by scoring a minimum of 500 transfected cells in random fields.

Activation of NF-κB signaling in hES cells by CD30 variant expression. HES-3 cells propagated on serum-free medium supplemented with FGF-2 in the presence of a feeder cell layer were harvested with Cell Dissociation Buffer and seeded at a density of 60% confluence onto 96-well plates coated with Matrigel. The next day the cells were overlaid with 100 µl mouse embryo fibroblast conditioned medium. The cells were subsequently transfected with 100 ng of plasmid DNA consisting of 33 ng PCS-2 lacZ with the cytomegalovirus 1E94 promoter driving β-galactosidase, 33 ng NF-κB-luciferase reporter (5× κB-luciferase construct from Stratagene) and 33 ng of either BOS-empty vector or 33 ng of BOS-CD30 variant, using 15 µl Fugene-6 per well. The next day the medium was changed again and cells grown for an additional 2 d. Then cells were lysed using 100 µl of passive lysis buffer (Promega) according to the manufacturer's instructions. To assess β-galactosidase activity (for normalization of well-to-well differences in transfection efficiency) 40 µl of lysate was used; another 40 µl was used for measurement of luciferase activity using the Promega luciferase assay system. Each condition was performed in quintuplicate on each plate, and three separate experiments were performed. The NF-κB-luciferase reporter activities were divided by the β-galactosidase activities and normalized relative to the empty vector control.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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