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Karyotypically abnormal human ESCs are sensitive to HDAC inhibitors and show altered regulation of genes linked to cancers and neurological diseases

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Abstract Genomic abnormalities may accumulate in human embryonic stem cells (hESCs) during in vitro maintenance. Characterization of the mechanisms enabling survival and expansion of abnormal hESCs is important due to consequences of genetic changes for the therapeutic utilization of stem cells. Furthermore, these cells provide an excellent model to study transformation in vitro. We report here that the histone deacetylase proteins, HDAC1 and HDAC2, are increased in karyotypically abnormal hESCs when compared to their normal counterparts. Importantly, similar to many cancer cell lines, we found that HDAC inhibitors repress proliferation of the karyotypically abnormal hESCs, whereas normal cells are more resistant to the treatment. The decreased proliferation correlates with downregulation of HDAC1 and HDAC2 proteins, induction of the proliferation inhibitor, cyclin-dependent kinase inhibitor 1A (CDKN1A), and altered regulation of tumor suppressor protein Retinoblastoma 1 (RB1). Through genome-wide transcriptome analysis we have identified genes with altered expression and responsiveness to HDAC inhibition in abnormal cells. Most of these genes are linked to severe developmental and neurological diseases and cancers. Our results highlight the importance of epigenetic mechanisms in the regulation of genomic stability of hESCs, and provide valuable candidates for targeted and selective growth inhibition of karyotypically abnormal cells.
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Introduction

Human embryonic stem cells (hESCs), with the capacity to give rise to all the somatic tissues and germ cells, provide a valuable tool for both basic research and future regenerative medicine (Thomson et al., 1998). However, during in vitro maintenance genomic abnormalities can accumulate in hESC lines. Interestingly, the hotspots for the changes lie in chromosomes 1, 8, 12,

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17, 20q10–11 and X, many of which are frequently affected in germ cell tumors and other types of cancers (http://cgap.nci.nih.gov/Chromosomes/RecurrentAberrations) (Baker et al., 2007; Ben-David and Benvenisty, 2011; Draper et al., 2004; International Stem Cell Initiative et al., 2011; Laurent et al., 2011; Lund et al., 2012; Taapken et al., 2011). Human ESC lines with genomic abnormalities display improved survival, proliferation, cloning and self-renewal capacity and decreased spontaneous differentiation making these demanding cells much easier to maintain in culture (Andrews, 2006; Baker et al., 2007; Draper et al., 2004; Enver et al., 2005; Herszfeld et al., 2006; Maitra et al., 2005; Werbowetski-Ogilvie et al., 2009). Accumulation of the genomic alterations in these chromosomal hotspots is likely to give selective advantage to the cells in culture and may indicate transformation of the cells towards a malignant phenotype. Elucidation of the mechanisms implicated in the culture adaptation process may provide valuable information about the signaling mechanisms important for the regulation of stem cell fate and provide insights into the transformation of the stem cells.

Previous study by Enver et al. (2005) on transcriptional profiles of karyotypically normal and abnormal cells provided data about the genes potentially important for the culture adaptation of hESCs (Enver et al., 2005). To analyze further the mechanisms regulating altered growth properties of karyotypically abnormal cells, we have now re-examined these data and identified the histone deacetylase HDAC1 as a possible candidate implicated in the process. HDAC1 together with HDAC2 forms a core component of several multiprotein chromatin remodeling complexes, including Sin3, Nurd, CoRest and NODE (Brunmeir et al., 2009). Of these particularly Nurd and NODE complexes are known to be important in regulation of pluripotency (Kaji et al., 2006, 2007; Liang et al., 2008; Reynolds et al., 2012). Studies with mouse models have shown that Hdac1, rather than Hdac2, is required for the activity of chromatin modeling complexes, early embryonic development and proliferation and differentiation capacities of ESCs (Brunmeir et al., 2009; Dovey et al., 2010). Reduced proliferation of HDAC1-knockout mESCs correlates with decreased cyclin dependent kinase activity and elevated p21 and p27 levels. Furthermore, HDAC1-knockout leads to the decrease in overall histone deacetylase activity and hyperacetylation of histone H3 and H4 subsets (Lagger et al., 2002). HDAC1 does not bind to DNA; however, it can be recruited to chromatin by several factors, such as SP1/SP3, RB1 or p53 (Brunmeir et al., 2009). Through deacetylation of histone proteins HDACs participate in controlling activity and structural dynamics of chromatin. In addition to histone proteins, HDAC1 is involved in regulation of stability of several non-histone proteins, such as tumor suppressor protein p53 (Brunmeir et al., 2009; Luo et al., 2000). Despite the multiple protein interactions, Hdac1 targets a specific set of genes in mESCs (Zupkovitz et al., 2006) and localizes in promoters of genes critical for self-renewal and pluripotency, including Oct4, Nanog and Klf4 (Kidder and Palmer, 2012). In addition to crucial function in the regulation of cellular differentiation, Hdac1 has a key function in the regulation of cell cycle and proliferation through negative regulation of CDKN1A (Zupkovitz et al., 2006). Furthermore, HDAC1 interacts directly with RB1 to repress E2F targets regulating G1–S transition of the cell cycle. This function mediated by HDAC1 and RB1 has been thought to be the key element controlling cell proliferation and differentiation and is target for transforming viruses (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Decrease in NuRD and HDAC1 activity has been associated with aging (Pegoraro et al., 2009; Willis-Martinez et al., 2010). On the other hand, overexpression of HDACs has been linked to different cancers (Brunmeir et al., 2009). Interestingly, HDAC inhibitors are cytotoxic to several types of cancer cells inducing proliferation block, apoptosis and differentiation, whereas normal cells are more resistant. Thereby HDAC inhibitors are actively studied for cancer treatment and vorinostat has been accepted for clinical treatment of cutaneous T cell lymphoma (Dokmanovic et al., 2007; Kelly and Marks, 2005; Coffey et al., 2001; Gottlicher et al., 2001).

In this study we found that, similar to certain cancers, karyotypically abnormal hESCs express increased levels of epigenetic regulator proteins, HDAC1 and HDAC2, and are more sensitive to HDAC inhibitors than their karyotypically normal parent cells. Treatment of abnormal cells with HDAC inhibitors leads to the decrease in proliferation which correlates with dowregulation of HDAC1 and HDAC2 proteins and induction of proliferation inhibitor CDKN1A (p21). Furthermore, we show that HDAC1 binds to RB1 in hESCs and inhibition of HDAC activity selectively induces phosphorylation of RB1 in abnormal hESCs but not in normal hESCs. Finally, we show that the genes with altered regulation in karyotypically abnormal hESCs and selective responsiveness to the HDAC inhibition are associated with severe developmental diseases, neurodegenerative diseases, and cancers particularly those common in childhood.

Materials and methods

Cells and culture conditions

Human embryonic stem cell lines (Supplemental Table SI) were cultured on mitotically inactivated (Mitomycin C, Sigma) mouse embryonic fibroblasts in 80% knockout DMEM, supplemented with 20% knockout serum, 4 ng/mL bFGF, 1% nonessential amino acid solution, 1 mM L-glutamine (all from Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich) (Thomson et al., 1998). Enzymatic passaging with Type IV Collagenase (Life Technologies) and glass beads were carried out for the cells when approximately 80% confluent. In some of the experiments the cells were maintained on human foreskin fibroblast feeders (CRL-2429, ATCC) as previously described (Narva et al., 2012) and then maintained one passage on Matrigel (BD Pharmingen) in mTeSR1 (STEMCELL Technologies). The HDAC inhibition was performed with chemical inhibitors by using indicated concentrations of valproic acid (Cat No. 676380, La Jolla, CA) or CBHA (Cat No. 382148, Calbiochem).

High-content analysis

The high-content analysis was carried out as previously described (Barbaric et al., 2010, 2011). Briefly, cells were harvested with Accutase (Millipore) and 3000 karyotypically abnormal cells per well or 5000 normal cells were plated per well of a 96-well plate (M056-32EA, Sigma-Aldrich). The cells were plated on mitotically inactivated mouse embryonic

feeders in standard hESC medium. On the following day the medium was supplemented with indicated concentrations of two different HDAC inhibitors VPA or CBHA. For each condition four to eight replicates were carried out. At the indicated time points the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by blocking with PBS supplemented with 2% FCS for 1 h at +4 °C. The cells were stained with 10 μg/ml Hoechst 33342, in house produced anti-TRA-1-60 (2 h at +4 °C) and 1:150 goat anti-mouse IgG + IgM (H + L) -FITC (Caltag, M30801). After each step three washes with PBS supplemented with 2% FCS were carried out. Imaging of the stained cells was carried out using the InCell Analyzer 1000 (GE Healthcare) and images were analyzed using Developer Toolbox 1.7 software (GE Healthcare).

Co-immunoprecipitation

For the co-immunoprecipitation experiments the cells were washed with ice cold PBS and were lyzed on ice in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X-100 supplemented with phosphatase and proteinase inhibitor cocktail (Roche). The samples were sonicated and pre-cleared with protein A agarose beads. The indicated antibodies (1–5 μg) were added into the samples and the samples were incubated in gentle rocking for 1 h. Protein A beads were added into the sample suspension and the samples were incubated overnight in gentle rotation. The protein–antibody complexes bound to the beads were collected by centrifugation (12,000 *g*, 20 s). The complexes were washed 4 times for 20 min with ice cold buffer containing 50 mM Tris–HCl (pH 7.5) and 120 mM NaCl. Consequently, a wash with ice cold 10 mM Tris–HCl, pH 7.5 for 20 min was carried out. Finally, the complexes were suspended in SDS buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% Glycerol, 50 mM DTT) and denaturated at 100 °C for 3 min. The beads were removed from the suspension by centrifugation (12,000 *g*, 20 s). To examine the protein interactions, the samples were further analyzed with Western blot assay.

Western blot

For the Western blot the cells were lyzed in SDS buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 1% glycerol, 50 mM fresh DTT) and boiled for 5 min at 95–100 °C. The lysates were sonicated and quantitated. Equal amounts of proteins were separated based on mass in 5–10% acryl-amide gel electrophoresis and transferred into nitrocellulose (162-0112, Bio-Rad) or PVDF (RPN303LFP, Amersham Biosciences) membranes for immunoblotting. Membranes were blotted with indicated antibodies and proteins were visualized with Supersignal West Pico chemiluminescent substrate kit (Pierce). See the Supplemental Table SVI for information on the antibodies used in the experiments.

Flow cytometry

For the flow cytometry the cells were harvested with 0.05% Trypsin + 0.4 mM EDTA and blocked with 1× PBS + 2% FCS for 30–60 min. The flow cytometric analysis was carried out using either living cells or cells fixed with 1–4% paraformaldehyde. For intracellular staining the cells were fixed with ice cold 100% methanol for 10 min at −20 °C. The blocking and staining of the cells was carried out in 1× PBS supplemented with 2% FCS. The antibody incubations were carried out for 30–60 min at +4 °C. Primary antibodies used in the analysis (P3X63Ag8, SSEA1, SSEA3 or SSEA4) were derived in house from hybridoma supernatants. See the Supplemental Table SVI for detailed information on the other antibodies used in the experiments. As a secondary antibody anti-rabbit-Alexa647 (A-21245, Molecular Probes) or anti-mouse-Alexa488 (1:500, A-11017, Molecular Probes) or goat anti-mouse IgG + IgM (H + L) -FITC (1:150, M30801, Caltag) was used. The samples were run with ADP CyAN (Beckman Coulter) and data analysis was carried out with Summit 4.0 Software (Beckman Coulter) or Cyflogic (CyFlo Ltd).

Real-time RT-PCR

For the real-time RT-PCR the primers and probes (labeled with FAM and TAMRA) were designed at the Universal Probe Library Assay Design Center (Roche). Total RNAs were extracted with RNAeasy Mini Kit (Qiagen). cDNA was prepared from 0.5 to 1 μg of the DNAse I treated totRNA using Superscript II kit (Invitrogen) and was used as a template for real-time RT-PCR analysis (7900HT, Applied Biosystems). The fold differences ($FD = 2^{(IDCT1-DCT2I)}$) were calculated from the normalized CT values ($DCT = CT_{gene\ X} - CT_{housekeeping\ gene}$) as indicated (Lund et al., 2003). The oligos for real-time RT-PCR were designed with Roche ProbeFinder Software and probes were ordered from Roche Universal ProbeLibrary maintained at Finnish Microarray and Sequencing Centre, Finland. The primers and probe used in the measurements were the following: HDAC1 5′-cca agt acc aca gcg atg ac-3′ and 5′-tgg aca gtc ctc acc aac g-3′, probe #58; LRP8 5′-tgt cca gat ggg agt gat ga-3′ and 5′-gtt gtg cag aca ctc gtt cag-3′, probe #71; SPP1 5′-cgc aga cct gac atc cag t-3′ and 5′-ggc tgt ccc aat cag aag g-3′, probe #61.

Transcriptome analysis

For re-analysis of the data by Enver et al. (2005), the original CEL files were imported to R/Bioconductor (http://www.bioconductor.org). The data was normalized with RMA (Robust Multi-Array) normalization method and probe sets with signal levels less than 100 in all the samples were excluded. Significance analysis of microarrays (SAM), siggenes, package was used to extract the genes showing over 2-fold difference between karyotypically normal and abnormal SSEA3 + cells with false discovery rate of 0 (Holger Schwender, 2009, siggenes: Multiple testing using SAM and Efron's empirical Bayes approaches, R package version 1.22.0). Ingenuity pathway analysis was run to identify putative upstream regulators of the genes differentially regulated in karyotypically normal and abnormal hESCs (Ingenuity Systems).

For the genome-wide transcriptome analysis of karyotypically normal or abnormal cells grown in the presence or absence of HDAC inhibitor (VPA), the RNAs were isolated and DNAse I treated as described above. The quality was controlled with Experion RNA Analysis kit (Bio-Rad, Hercules,

CA). The transcriptome analysis was carried out with Illumina Human HT-12 v3 Expression BeadChip. The samples for the Illumina chips were processed from 200 ng of DNAse I treated totRNA by the Finnish Microarray and Sequencing Centre, Turku Centre for Biotechnology, Finland. The raw data was processed with GeneSpring GX (Agilent). First the data was quantile normalized and quality controlled. The differences between four different conditions were identified through pair-wise comparisons using appropriate T-test statistics. Paired T-test statistics was used for the comparison of untreated vs HDAC inhibitors treated and unpaired T-test statistics was used to compare karyotypically normal cells to abnormal cells. To reduce the number of false positives a cut-off value of 2-fold was applied for the data filtering. In addition to public databases (www.ncbi.nlm.nih.gov, http://ist.medisapiens.com/, www.alzgene.org), Ingenuity Pathway Analysis Tool (Ingenuity Systems) was utilized in the data mining to seek information on the selected genes and associated pathways.

Induced shRNA knockdown of HDAC1

For knockdown of HDAC1, a previously established system (Zafarana et al., 2009) utilizing doxycyclin inducible short hairpin RNA (shRNA) under control of Tet Repressor protein responsive element was used. ShRNA sequences targeting HDAC1 were cloned into the multiple cloning site of pSuperior-GFP-Neo (Oligoengine) vector. The shRNA sequences used in the experiments were from Biomers (Germany): HDAC1 shRNA3: 5′-GAT CCC CCA GAA CAC GAA TGA GTA CTT CAA GAG AGT ACT CAT TCG TGT TCT GGT TTT TA-3′ and 5′-AGC TTA AAA ACC AGA ACA CGA ATG AGT ACT CTC TTG AAG TAC TCA TTC GTG TTC TGG GG-3′; HDAC1 shRNA4: 5′-GAT CCC GGC TGG CAA AGG CAA GTA TTT CAA GAG AAT ACT TGC CTT TGC CAG CCT TTT TA-3′ and 5′-AGC TTA AAA AGG CTG GCA AAG GCA AGT ATT CTC TTG AAA TAC TTG CCT TTG CCA GCC GG-3′. Briefly, the shRNA oligos were annealed and ligated (T4 Ligase, New England Biolabs) into the digested pSuperior vector purified from the 1% Agarose gel (Qiaquick Gel Extraction kit, Qiagen). Competent DH5alphaF cells were transformed with the ligated vectors and screened for positive colonies from the LBA plates. Transformants were identified with PCR directly from the bacterial colonies using forward primer 5′-AGA ATT CGA ACG CTG ACG TC-3′ and reverse primer 5′-GGA ACA AAA GCT GGG TAC CG-3′. Plasmid DNAs were prepared and purified with GenElute™ Endotoxin-free Plasmid Midiprep Kit (Sigma-Aldrich). The positive clones were verified with restriction enzyme analysis and sequencing. The NT2D1 cells carrying pGAC-Rnls vector, for Tet Repressor protein expression, were transfected with linearized plasmids at 50% confluency with Lipofectamine 2000. Puromycin 1 μg/ml and G418 1 μg/ml (both from Sigma) selection was added for selective expansion of the clonal cell lines carrying both vectors pCAG-TetRnls and pSuperior. Doxycyclin 1 ng/ml was added to induce the shRNA expression. Knockdown of HDAC1 was analyzed at mRNA level with TaqMan real-time RT-PCR and at protein level with Western blot analysis. Three clones (shRNA3cl1, shRNA4cl2 and shRNA4cl4) were selected for further experiments, based on efficient knockdown at mRNA and protein levels.

Promoter analysis

Promoter analysis included DNA sequence scanning with transcription factor motif sequences. Here, position weight matrices (PWMs) of various transcription factors available in TRANSFAC Professional database version 2009.3 (BIOBASE GmbH) were used. The gene promoter sequences were obtained from the UCSC upstream sequences (hg18, NCBI Build 36.1). The promoters were scanned 1000 base pairs upstream from the transcription start site. This was done with the PWMs of all the 388 transcription factors that are found in the database for *Homo sapiens*. For each PWM, a MotifLocator score s, as implemented in Lahdesmaki et al. (2008), for each base pair position in each promoter sequence was calculated. A background distribution of scores S_{bg} calculated from permuted promoter sequences was constructed. Then each score s was compared to the background distribution resulting in a p-value. The base pair positions with a $p\text{-value} < 0.001$ were considered as binding sites for the transcription factor at hand.

Results

HDAC1 and HDAC2 proteins are expressed at increased levels in the karyotypically abnormal hESCs

To elucidate the mechanisms that enable enhanced survival and growth of karyotypically abnormal hESCs, transcriptome data from the study of normal and adapted H7 hESCs by Enver et al. (2005) was analyzed (Enver et al., 2005). From this data set we identified histone deacetylase 1 (HDAC1) as a possible factor involved in the process since it was among the putative upstream regulators of the genes differentially expressed between karyotypically normal and abnormal hESCs (Ingenuity Pathway Analysis, Ingenuity Systems), and the only putative upstream regulator showing over 2-fold increase in gene expression in the abnormal hESC line (Fig. 1A). Several of the known direct downstream targets of HDAC1 or HDAC2 showed altered expression levels in abnormal cells indicating altered activity of these factors (Supplemental Fig. S1). Western blot analysis further validated elevated levels of both HDAC1 and HDAC2 proteins in three different hESC lines with genomic abnormalities (Fig. 1B). The finding was further supported by flow cytometric analysis, which demonstrated that the increased levels of HDAC1 in abnormal cells were observed in both SSEA3+ and negative populations (Fig. 1A) and in POU5F1 high and low populations (Fig. 1C), showing that the increase is not caused by different proportion of spontaneously differentiating cells present in the samples. Based on karyotyping analysis (Supplemental Table SI) in H14 line genomic loci for both *HDAC1* (1p34) and *HDAC2* (6q21), and in Shef5 for *HDAC2*, have diploid dose of genes indicating that other mechanisms than structural changes drive enhanced expression of these proteins.

HDACs are known to negatively regulate acetylation level of histone 3 (AcH3). Thereby we also examined the global levels of this histone modification in normal and abnormal cells. The Western blot analysis revealed increased rather than decreased levels of the denaturated AcH3 protein in

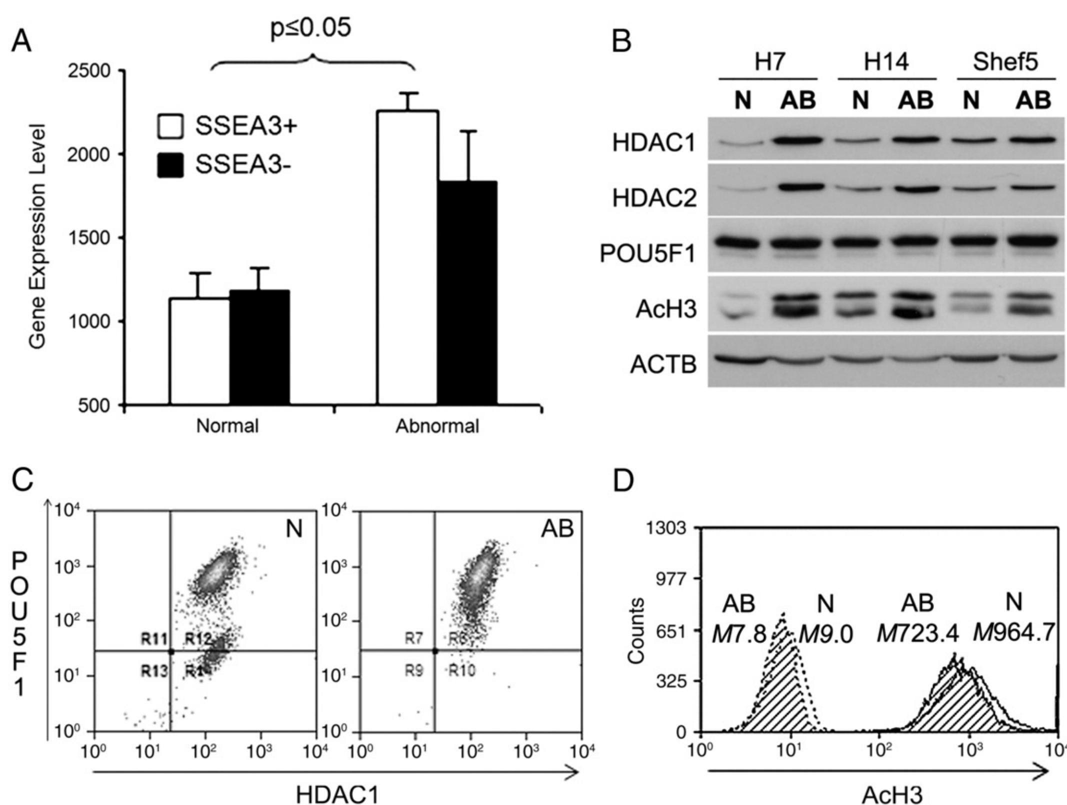


Figure 1 Levels of HDAC1 and HDAC2 proteins are increased in the human embryonic stem cells with karyotypic abnormalities. In panel A are the normalized mean gene expression intensities for HDAC1 gene, extracted from the raw Affymetrix oligonucleotide array data from the study by Enver et al. (2005). The data shows gene expression of HDAC1 in the karyotypically normal vs abnormal cell fractions sorted for SSEA3 marker. The standard deviations and T-test p-values are indicated in the figure. In panel B are the Western blot data on HDAC1, HDAC2, AcH3, stem cell marker POU5F1 and housekeeping gene beta-actin (ACTB) in three different hESC lines (H7, H14 and Shef5) with karyotypically normal (N) and abnormal (AB) counterparts. In panel C is the double staining for HDAC1 and POU5F1 levels as measured with flow cytometric analysis in karyotypically normal (N) or abnormal (AB) H7 hESC lines and in panel D are the levels of AcH3 in normal and abnormal cells as measured with flow cytometric analysis. The median intensity levels are shown in the figure for the secondary antibody controls (dashed lines) and for the normal (N) and abnormal (AB) cells stained for AcH3.

abnormal cells, probably due to increased amount of chromatin present in these cells (Fig. 1B). On the opposite, flow cytometric analysis indicated a slight decrease in the levels of native AcH3 in abnormal cells, which may indicate altered function of HDACs or decreased accessibility of the antibody to chromatin due to more compact chromatin structure (Fig. 1D).

HDAC inhibitors prevent proliferation of hESCs with abnormal karyotype

To assess whether inhibition of HDAC activity has an effect on the growth of karyotypically abnormal hESCs, the cells were cultured under increasing concentrations of two different HDAC inhibitors, valproic acid (VPA) and m-carboxycinnamic acid bis-hydroxamide (CBHA). High content analysis of the cells stained with Hoechst 33342 and stem cell marker TRA-1–60 revealed that treatment with either of these inhibitors leads to a decrease in the number of colonies and TRA-1–60+ cells within the colonies in a dose dependent manner (Fig. 2). With VPA the effect on cell numbers was observed already after one day of treatment with the concentration ≥ 0.75 mM ($p \leq 0.05$); however, higher concentrations were more efficient in maintaining the growth inhibition throughout the experiment. With CBHA the lowest effective concentration was 0.5 μM ($p \leq 0.001$) observed after 3 days of treatment. The dose dependent growth inhibition by HDAC inhibitors and morphology of the treated cells indicated that the effect was rather due to block in the proliferation rather that induction of apoptosis, autophagy or necrosis.

To study whether HDAC inhibitors affect growth of cells with normal karyotype, we grew H7 cells with normal or abnormal karyotype in parallel under increasing concentrations of VPA or CBHA. The results show that whereas HDAC inhibitors strongly prevented high expansion of the karyotypically abnormal hESCs, the cells with normal karyotype grew better under similar conditions. The growth of the normal cells was not decreased by VPA treatment, whereas the growth of the abnormal cells was affected with concentration of ≥ 1 mM of VPA. The calculated average half maximal effecting concentration (EC50) was 1.12 mM of VPA for abnormal cells (Figs. 3A–C). The karyotypically

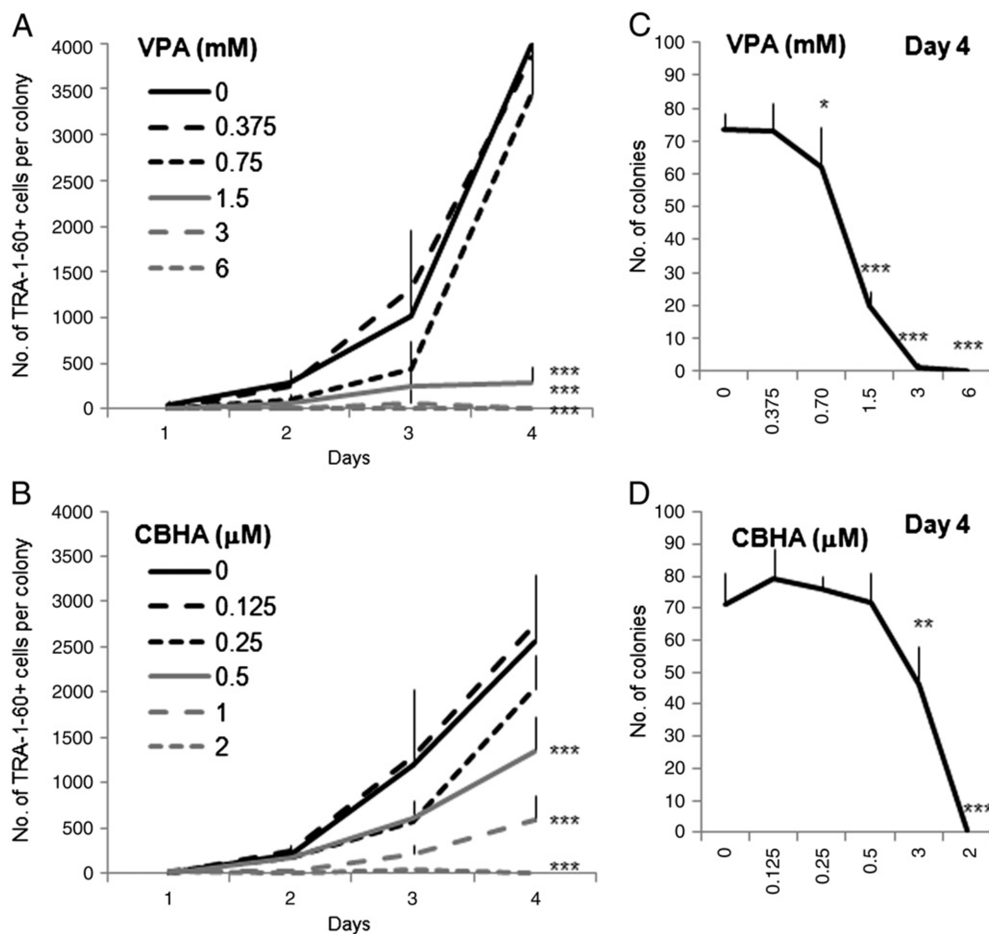


Figure 2 HDAC inhibitors prevent proliferation of human embryonic stem cells with abnormal karyotype. Growth curve data for the abnormal hESCs (H7) grown under increasing concentrations of HDAC inhibitors valproic acid (VPA), or m-carboxycinnamic acid bis-hydroxamide (CBHA) for 4 days. For the assay the cells were harvested with accutase and plated on inactivated MEFs into 96-well plates. On the following day the cultures were supplemented with increasing concentrations of VPA or CBHA, as indicated. For monitoring the growth under different conditions the cells were stained with nuclear stain (Hoechst) and stem cell marker TRA-1-60 and high content analysis of the cell proliferation was carried out (IN Cell Analyzer, GE Healthcare Life Sciences) at indicated time points. The growth curves show the average numbers of TRA-1-60 + cells per colony for the cells grown under VPA (A), or CBHA (B). In panel C is the total number of colonies on day 4 under increasing concentration of VPA, and in panel D for CBHA. The error bars are the standard deviations and asterisk indicates statistical significance with the unpaired T-test p-value * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, shown only for day 4.

abnormal hESCs were also more sensitive to CBHA than normal cells. However, the growth of normal cells was also affected by CBHA, although not as strongly as the growth of abnormal cells. With CBHA EC50 was 0.60 μM for abnormal cells and 1.41 μM for normal cells (Fig. 3D). Thus, VPA seems to be more promising for the selected growth inhibition of abnormal cells, as it is better tolerated by the karyotypically normal cells. Growth inhibition of abnormal cells in response to VPA correlated with downregulation of HDAC1 protein and induction of CDKN1A (p21), a known target of HDAC1 and inducer of cell cycle arrest (Fig. 3E). Induction of CDKN1A was not detected in normal cells or in abnormal cells in response to CBHA (data not shown). Levels of HDAC1 were not repressed in karyotypically normal hESCs (Fig. 3G). As expected, levels of AcH3 were increased in abnormal cells in response to VPA (Figs. 3E–F) as well as CBHA (Supplemental Fig. S2A). In conclusion, similar to the cancer cells and normal cells found in somatic tissues, abnormal hESC is more sensitive to HDAC inhibitors than normal cells.

HDAC inhibition does not induce differentiation of the hESCs

As HDAC inhibition has been reported to induce differentiation of certain cancer cell types, the levels of stem cell markers, SSEA3 and SSEA4, and the differentiation marker SSEA1 were studied after exposure of hESCs to HDAC inhibitors. Analysis of the karyotypically abnormal hESCs grown under effective VPA concentration was challenging due to decreased growth and survival of the cells and in several experiments not enough cells could be harvested for flow cytometric analysis. However, based on the successful experiments HDAC inhibition does not induce differentiation of either karyotypically normal or abnormal cells (Fig. 4A).

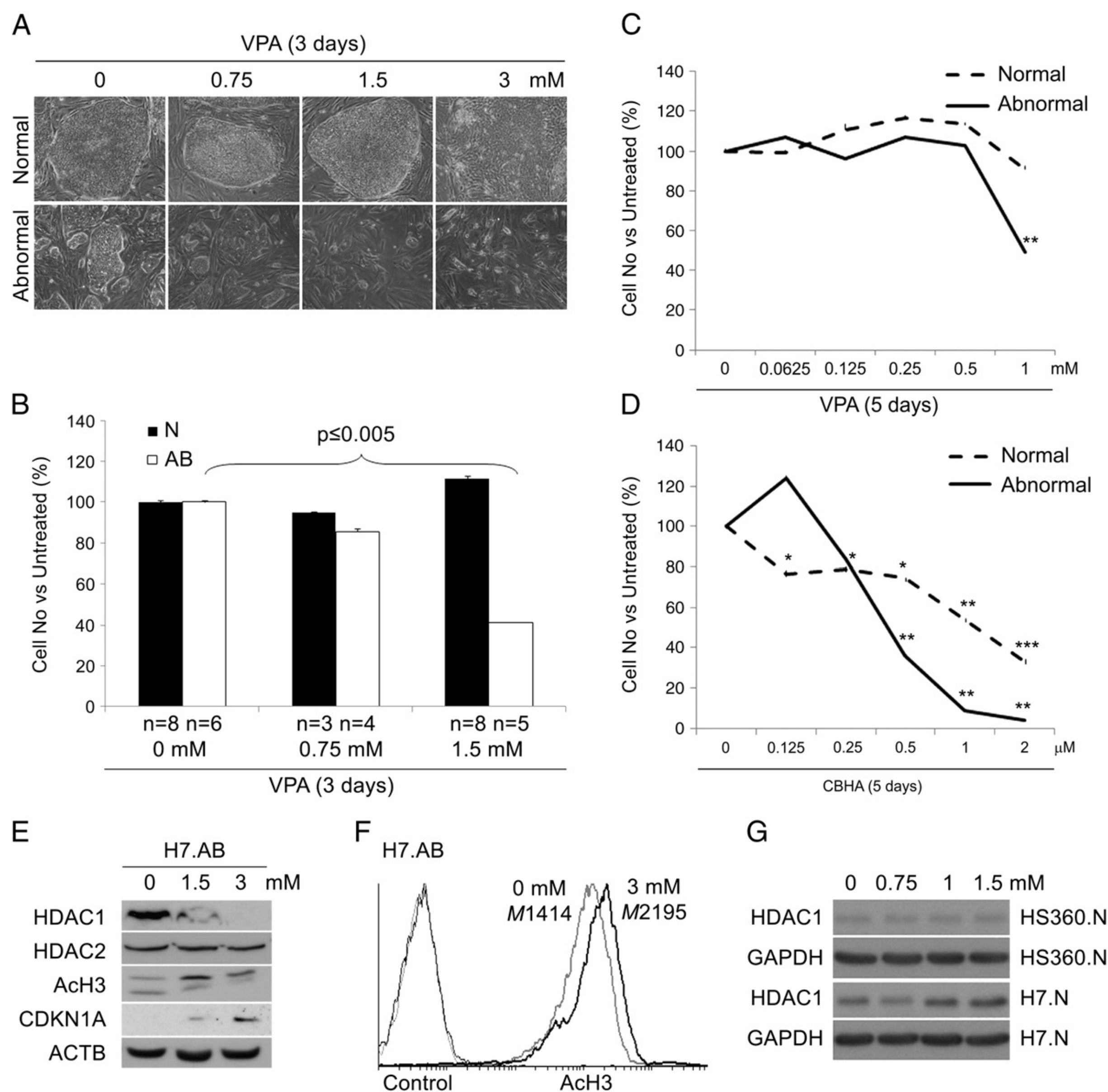


Figure 3 Normal human embryonic stem cells are more resistant to HDAC inhibition. Karyotypically normal (N) or abnormal (AB) hESCs (H7) were plated on mitotically inactive mouse embryonic fibroblast feeders. One day after plating the cultures were supplemented with HDAC inhibitor VPA as indicated. In panel A is the cell morphology after 3 days under increasing concentrations of VPA. In panel B is the proportional number of cells grown under VPA as compared to the untreated control. For growth curve assay the cells were dissociated into single cells with accutase and were plated in equal numbers on mouse feeders. One day after plating the cultures were supplemented with VPA or CBHA. For monitoring the growth under different conditions the cells were stained with nuclear stain (Hoechst) and high content analysis of the cell proliferation was carried out. The percentage of average cell numbers as compared to untreated control cells, after 5 days of VPA treatment is shown in panel C, and that of CBHA in panel D. The error bars indicate the standard deviations between four replicates. The asterisk indicates statistical significance with the unpaired T-test p-value * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001. In panel E are the levels of HDAC1, HDAC2, acetylated H3 protein (AcH3), CDKN1A (p21) and housekeeping gene ACTB proteins under increasing concentrations of VPA in abnormal (H7). In Fig. 1F is the flow cytometric measurement of the AcH3 levels in abnormal H7 cells grown in the absence or presence of 3 mM VPA for 3 days. The mean intensity values for the samples are indicated in the figure. See Supplemental Fig. S2 for CBHA treatment. In panel G is the Western blot data of HDAC1 and housekeeping gene GAPDH in the karyotypically normal HS360 and H7 hESC lines grown under valproic acid treatment as indicated in the figure for 3 days.

This was supported by the observed morphology of the cells under phase contrast microscope (Fig. 3A). Also no clear and consistent changes in POU5F1 protein levels were observed in response to VPA, although in some of the cultures slight decrease with high concentrations of inhibitor was observed (Fig. 4B). Similar observations were made with CBHA (Supplemental Fig. S3). In conclusion, HDAC inhibition does not induce differentiation of hESCs.

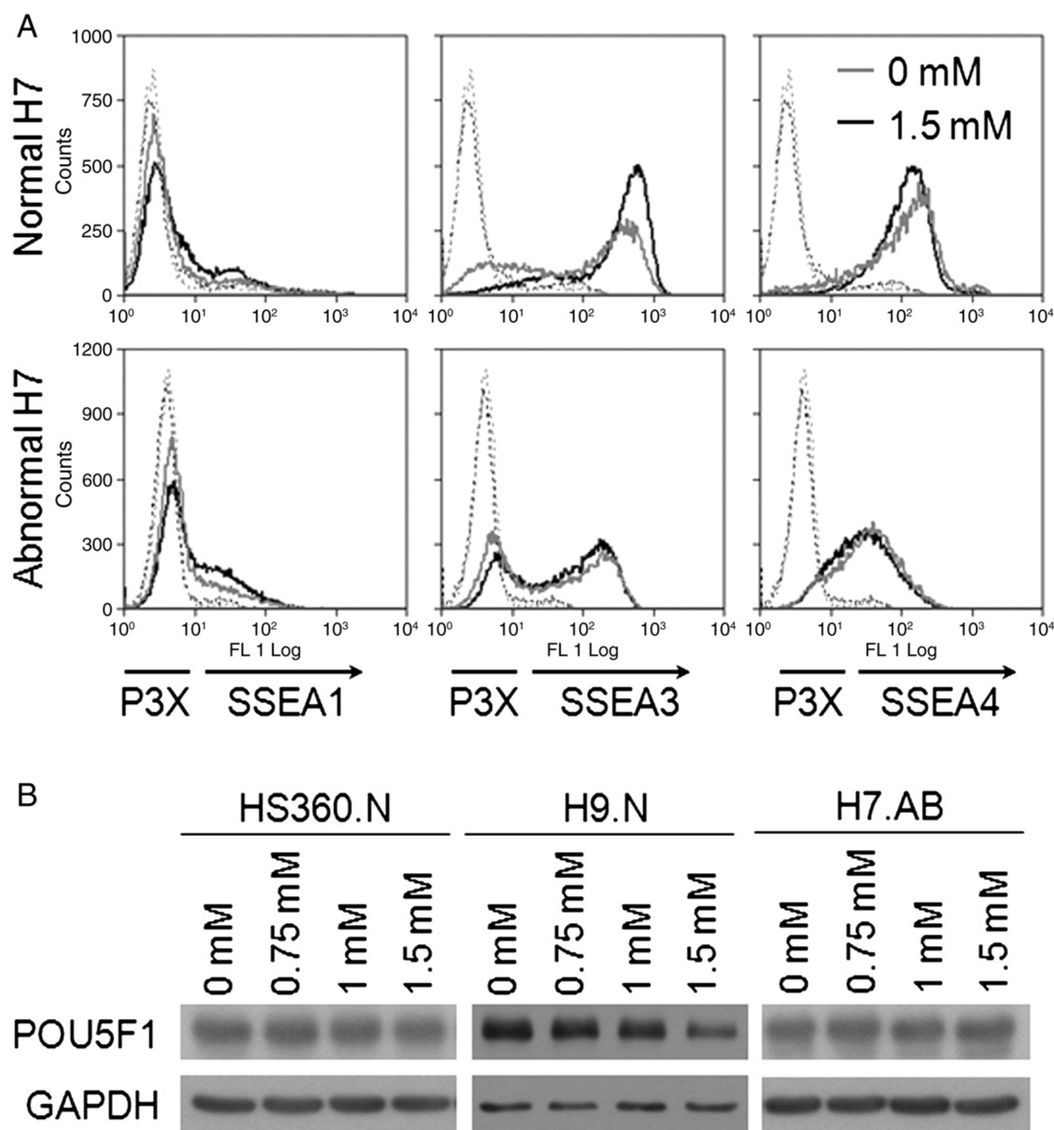


Figure 4 HDAC inhibition does not induce differentiation of human embryonic stem cells. The karyotypically normal (N) or abnormal (AB) H7 cells were grown with or without HDAC inhibitor (valproic acid) for 3 days as indicated in the figures. The levels of (A) SSEA1, SSEA3 or SSEA4 were measured with flow cytometric analysis and (B) POU5F1 and housekeeping gene GAPDH with Western blot analysis to determine the differentiation status of the cell. See also Fig. S3 for CBHA treatment.

Altered regulation and binding of RB1 with HDAC1 in karyotypically abnormal hESCs

Previous studies have indicated a key function for the RB–HDAC1 complex in the regulation of cell proliferation and differentiation through repression of E2F target genes (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Thus we studied how RB1 is regulated in these conditions. Enhanced levels of RB1 were repeatedly, though not exclusively observed in the abnormal hESCs when compared to the normal cells, particularly in H7 line (Figs. 5A–B). The variation in the levels may be partly explained by the differentiation status of the cells, as in karyotypically normal cells POU5F1 low and negative cells clustered into several different populations with variable levels of RB1 proteins. In contrast, abnormal cells had more homogenous cell population positive for both POU5F1 and RB1 (Fig. 5C). Interestingly, although no clear differences in the phosphorylation status of RB1 protein were observed in the normal and abnormal cells, our data shows that in response to HDAC inhibition RB1 became differentially modified in abnormal cells, but not in normal cells (Fig. 5D). We then next examined whether HDAC1 binds to RB1 in hESCs. As HDAC1/2 has been reported to form the NODE complex with Pou5F1 in mouse ESCs, we also included this protein in the analysis. Co-immunoprecipitation experiments revealed interaction of HDAC1 with RB1 in abnormal hESCs (Fig. 5E). Interaction of HDAC1 and RB1 in normal cells was also observed in one of the replicates; however, it could not be confirmed as level of HDAC1 is often weak in normal cells. Similar observations were made for HDAC2. POU5F1 clearly co-precipitated with RB1 in both normal and abnormal cells.

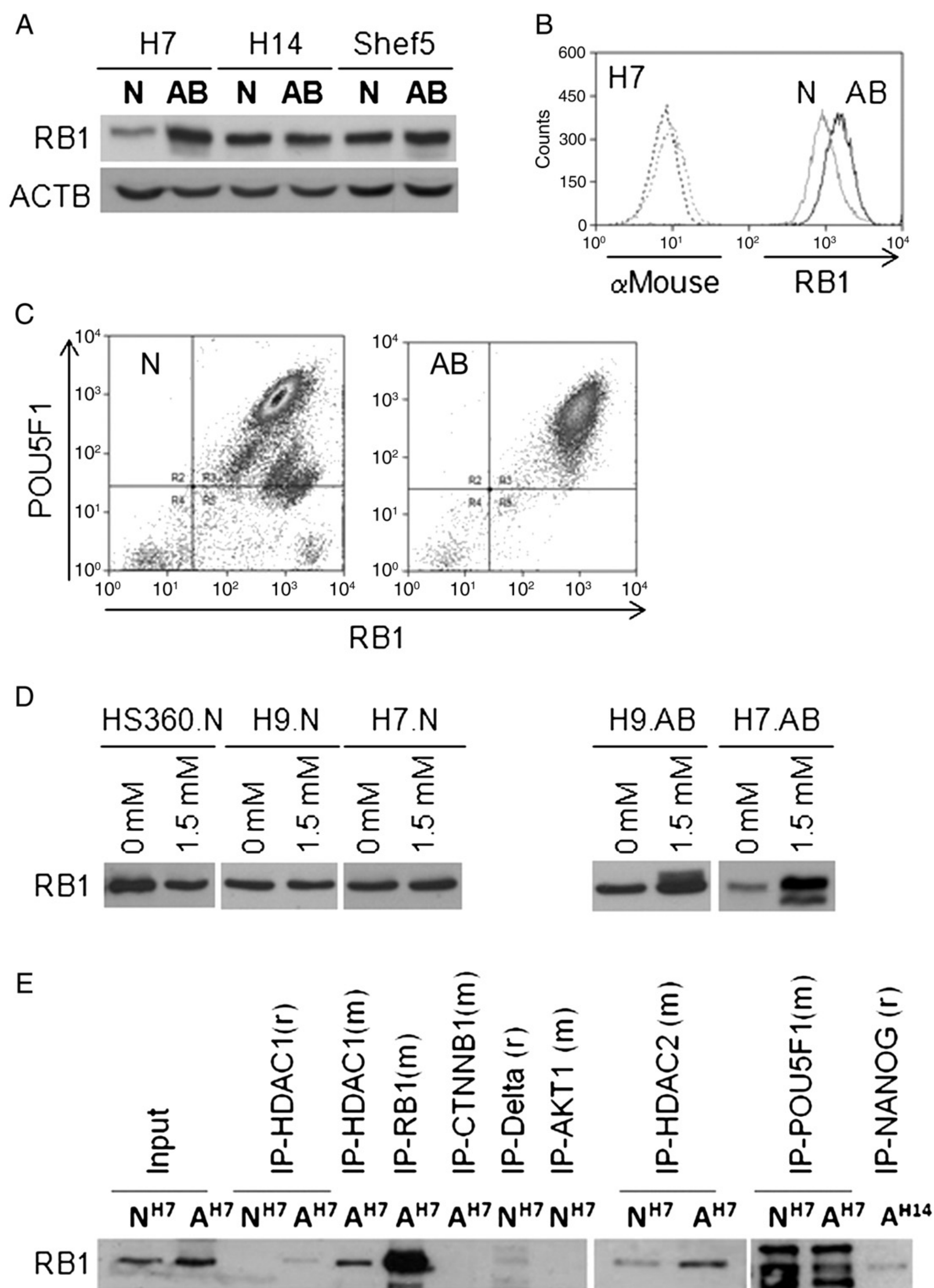


Figure 5 Altered regulation and binding of RB1 with HDAC1 in karyotypically abnormal human embryonic stem cells. Expression level of RB1 protein was measured in karyotypically normal (N) or abnormal (AB) hESCs as indicated with Western blot analysis (A), flow cytometric analysis with single staining (B) and co-staining with stem cell marker POU5F1. In the panel D is the Western blot data for RB1 protein levels in the karyotypically normal or abnormal cells grown in the absence or presence of valproic acid (VPA) for 3 days. In panel E binding of RB1 with HDAC1, and a panel of indicated proteins, were examined with antibody based co-immunoprecipitation in karyotypically normal (N) or abnormal (A) hESCs. In the figure is the representative Western blot data from several co-immunoprecipitation experiments with a panel of antibody controls originated in r = rabbit or m = mouse hosts.

In conclusion, these data indicate that HDACs enable survival and regulate the altered growth properties of abnormal hESCs possibly in a RB1 dependent manner. HDACs bind to RB1 and HDAC inhibition selectively affects phosphorylation status of RB1 in karyotypically abnormal hESCs and induces an antagonist of RB1, CDKN1A protein enabling proliferation block.

Transcriptome analysis reveals the genes with altered regulation and sensitivity to HDAC inhibition in karyotypically abnormal hESCs

To examine further the mechanisms of HDAC mediated signaling and to clarify how HDACs support survival and growth of the abnormal cells, whole genome transcriptome analysis was carried out on the normal and abnormal cells cultured in the presence or absence of HDAC inhibitor. In concordance with the previous data by Enver et al. (2005), differential expression of HDAC1 (2.28-fold, $p < 0.001$) was observed in untreated karyotypically normal vs abnormal hESCs, whereas no major differences were observed in the mRNA levels of stem cell markers POU5F1, NANOG and SOX2 (Supplemental Table SII). Transcriptional profiling revealed that in karyotypically normal hESCs 45 entities (probes), corresponding to 44 genes, were responding to HDAC inhibition with the chosen filtering criteria (Fig. 6A, Supplemental Table SIII). Of these genes, 41 were upregulated and only 3 were downregulated indicating that the HDAC mediated gene regulation in normal hESCs was mostly repressive rather than inductive. Notably, nearly all of the genes regulated by HDAC inhibition in normal cells (n = 41) showed consistent changes also in the karyotypically abnormal hESCs, although for many genes the changes in abnormal cells were not as strong as in normal cells.

Interestingly, in the karyotypically abnormal hESCs, there was a significantly higher number of entities (n = 212, corresponding to 203 genes) responding to HDAC inhibition with the selected filtering criteria (Supplemental Table SIV). This was in line with the observations that abnormal cells are more sensitive to HDAC inhibition than normal cells. HDAC1 or HDAC2 transcripts did not change in response to HDAC inhibition in either normal or abnormal cells indicating that the inhibition does not affect the transcription of these genes. Pathway analysis of the genes regulated by HDAC inhibitor in abnormal cells links these factors with cancer (69 genes) and functional networks regulating cellular growth, proliferation, development and death. Among the genes regulated in response to HDAC inhibition were several previously known mouse Hdac1 target genes, such as *H19* (imprinted endoderm marker), *STMN2* (neuronal growth associated), *ACTG2*, *SOX12*, *GBX2*, *TUBB3* (ectoderm marker) and *FHL1* (Zupkovitz et al., 2006). All of these were induced in response to VPA treatment, except *ACTG2* which was repressed. Strikingly, a stem cell marker *DPPA5* was highly induced (average 24-fold increase) in abnormal cells in response to HDAC inhibition. Although this gene is known to be highly expressed by ESCs, embryonic stem cells or mouse deficient for *Dppa5* are perfectly normal (Amano et al., 2006). Taken together, in accord with the growth curve data, karyotypically abnormal hESCs are more sensitive to HDAC inhibition having increased number of genes responding to the treatment. The genes regulated in response to HDAC inhibition include both stem cell and differentiation markers, although, no clear signs of differentiation were observed in response to the treatment.

To understand how HDAC inhibition selectively affects the growth and survival of the abnormal cells, the genes responding to HDAC inhibition in the abnormal cells and differentially expressed by the normal and abnormal cells were extracted revealing 31 entities for 29 different genes. Among these genes, two patterns of expression profiles were observed. In the first profile pattern, the differences in gene expression between normal and abnormal cells were potentiated by the HDAC inhibition in both conditions thereby not explaining the selective growth inhibition of abnormal cells (Fig. 6B). More interestingly, in the second profile pattern the genes with altered expression in the abnormal cells were selectively affected by HDAC inhibition, which reversed expression of these genes back to the levels observed in karyotypically normal cells (Fig. 6C). Among these the genes *ARHGEF6*, *ATP6V1B1*, *MBD3L3*, *DAZL* and *LRP8* were expressed at increased levels in abnormal cells and were downregulated in response to HDAC inhibition. The genes including *TNFRSF19*, *NLRP1*, *PMP22*, *ID2*, *ARID5B*, *PROM1*, *NEFM*, *EPHA4* and *SPP1* showed decreased expression in the abnormal cells and were upregulated in response to HDAC inhibition. Functional analysis of these genes, with altered expression and selective sensitivity to HDAC inhibition in abnormal cells, revealed link to germ cell differentiation, neuronal development and regulation of apoptosis. Importantly, nearly all of the genes are associated with severe developmental and neuronal diseases, in particular neurodegenerative diseases, such as Alzheimer's disease. Furthermore, nearly all of the genes were linked to cancers, including leukemia, neuroblastoma, glioma and sarcomas, which are cancer types particularly common in childhood (www.cancer.gov) (Table 1).

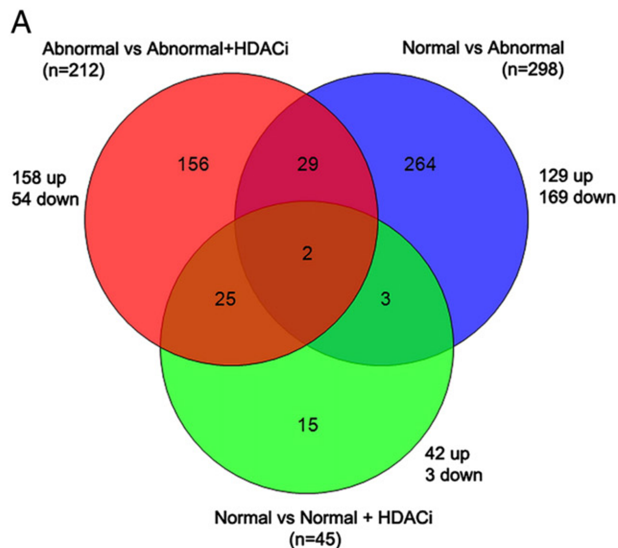
At least 6 of these 14 genes are known to be aberrantly regulated in leukemias, particularly in acute lymphoblastic leukemia (ALL). Thus, it was studied whether HDAC inhibition could also prevent proliferation of T-ALL cells (CCRF-CEM line). Indeed, similar to the abnormal hESCs, proliferation of these cells was decreased when grown in the presence of HDAC inhibitor (Supplemental Fig. S4A). Moreover, RT-PCR analysis showed that levels of SPP1 were induced whereas LRP8 was repressed in response to HDAC inhibition indicating that similar mechanisms are supporting growth of both abnormal hESCs and T-ALL cells derived from somatic tissue (Supplemental Fig. S4B).

Proliferation of NT2D1 cells and transcription of SPP1 and LRP8 are dependent of HDAC1

In order to examine whether the inhibition of growth and transcriptional effects were HDAC1 dependent, we used NT2D1 cell line and created clones expressing doxycyclin inducible shRNAs targeting HDAC1. Three clones with efficient knockdown of HDAC1 in response to doxycyclin treatment were selected for the experiments (Supplemental Fig. S5A–B). As was observed with the hESC lines, HDAC1 knockdown did not induce differentiation of NT2D1 cells (data not shown). However, induction of HDAC1 knockdown in NT2D1 cells induced similar enhancement of SPP1 gene and repression of LRP8 gene within 2 days, as was observed in response to VPA treatment in abnormal hESC cells or CCRF-CEM cell line (Supplemental Fig. S5C–D). Also the growth of the NT2D1 cells was reduced but not fully blocked by HDAC1 knockdown (Supplemental Fig. S5E).

Promoter analysis of the HDAC inhibitor responsive genes

The HDAC inhibitor responsive genes with altered regulation in transformed cells together with their upstream regulators provide valuable candidates for targeted inhibition of abnormal growth. To obtain further information of the potential regulators of these selected genes of interest (Table 1), the proximal promoters were studied up to 1000 bp upstream of the transcription starting site. Putative transcription factor binding sites, present in the promoters of all 14 genes, were extracted and consisted altogether of 28 factors (Supplemental Table SV). Functional analysis of the putative upstream regulators revealed that 27 of 28 factors, excluding AR, are linked to development of the hematological system (Ingenuity Pathway Analysis, Ingenuity Systems). Among these 28 factors, binding sites for SP1 protein (gene at 12q13.1), were the most prominent in the promoters of the genes listed in Table 1. SP1 is known to mediate attachment of HDAC1 into the genomic DNA (Brunmeir et al., 2009). Database search revealed that SP1 protein is known to directly interact with all HDAC1, HDAC2, RB1, POU5F1 and NANOG proteins (Ingenuity Pathway Analysis). Thus, SP1 probably acts as a common mediator of DNA binding to regulate activity and expression of shared target genes of these proteins. Binding sites for POU5F1 were found in the promoters of all of the genes except LRP8, TNFRSF19, EPHA4 and NEFM. Among the putative upstream regulators were also proteins, TP53, YY1 and E2F1, with well known roles in transformation and oncogenesis. Similar to RB1, these proteins are known to be bound and deacetylated by HDACs (Brunmeir et al., 2009). These data together with the co-immunoprecipitation results suggest that HDACs co-operate with RB1, POU5F1 and possibly with NANOG and SP1 to regulate expression of these 14 HDAC inhibitor responsive genes differentially expressed in karyotypically normal and abnormal hESCs. Further studies are needed to clarify how these proteins target chromatin and regulate genes in normal and transformed cells.



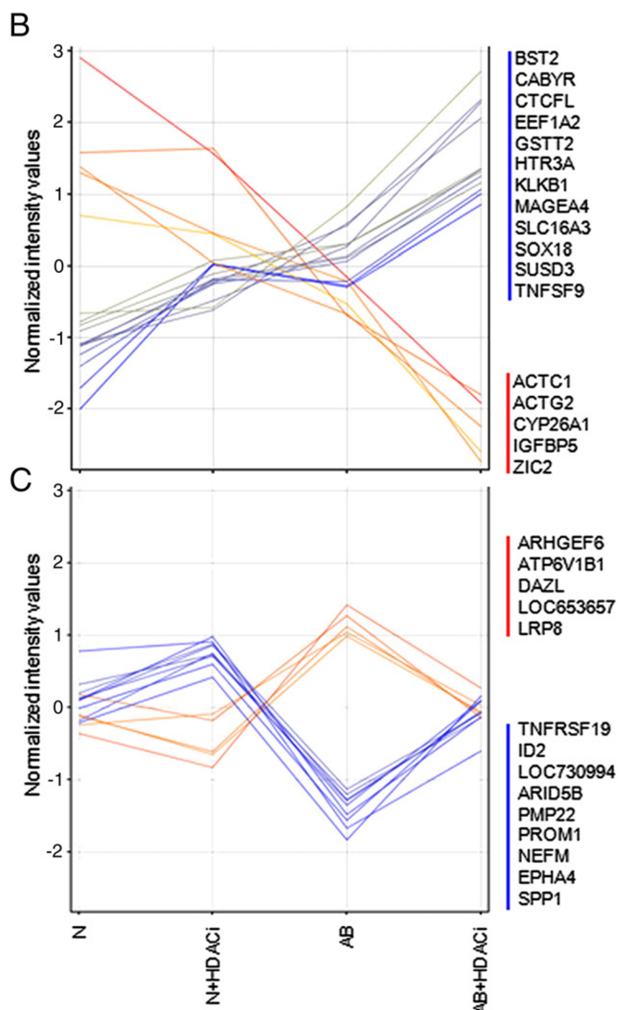


Figure 6 Transcriptome analysis reveals the genes with altered regulation and sensitivity to HDAC inhibition in karyotypically abnormal hESCs (see also Supplemental Tables SII–IV). Whole genome transcriptome analysis was carried out on the karyotypically normal (N) or abnormal (AB) H7 hESCs grown in the presence or absence of HDAC inhibitor (HDACi: valproic acid: 0, 1.5 mM) for 3 days. T-test statistics and fold change cut-off value 2 were applied to extract the genes differentially expressed between indicated sample groups. In panel A is a Venn diagram showing the numbers of genes differentially regulated in the indicated conditions. In panels B–C are the expression profiles of the 31 genes differentially expressed in normal and abnormal cells and affected by HDAC inhibition. In panel B are the genes where the differential expression between normal and abnormal cells was potentiated by HDAC inhibition. In panel C are the genes of interest with altered expression in abnormal cells and differential responsiveness to HDAC inhibition when compared to karyotypically normal cells. See also Supplemental Figs. S4 and S5 for HDAC inhibitor and HDAC1 mediated proliferation effect and transcriptional regulation of SPP1 and LRP8 genes in cancer cells.

Discussion

Characterization of the karyotypically abnormal cells and culture adaptation process is important for stem cell biology and can provide valuable insights into the mechanisms regulating transformation of cells towards malignant fate.

Table 1 Function and disease associations of the HDAC inhibition sensitive genes with altered regulation in karyotypically abnormal hESCs.

Symbol	Full name	Genomic locus	Disease associations, relevant functions	Association to cancers (MediSapiens)
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	Xq26.3	Apoptosis, X-chromosomal non-specific mental retardation.	ALL, AML, CML
ATP6V1B1	ATPase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B1	2p13.1	Acidification, distal renal tubular acidosis, hearing loss, sperm maturation.	Breast, ovarian and peritoneal cancers, other urogenital tumors
DAZL	Deleted in azoospermia-like	3p24.3	Translation, differentiation, severe spermatogenic failure and infertility in males.	CLL, testicular cancer
MBD3L3	Methyl-CpG-binding domain protein 3-like	19p13.2	NA	NA
LRP8	Low density lipoprotein receptor-related protein 8 (APOER2)	1p34	Cell death, Alzheimer's disease risk, increased fetal growth.	ALL, skin, squamous cell carcinoma, other neuro-ectodermal or thyroid cancers
TNFRSF19	TNF receptor superfamily, member 19	13q12	Apoptosis, biomarker for melanoma.	Head and neck, liver, prostate, uterine, other neuroectodermal cancers, sarcoma, melanoma, skin, squamous cell carcinoma, lung carcinoid tumors
ID2	Inhibitor of DNA binding 2, dominant negative helix–loop–helix protein	2p25	Apoptosis, malignancy, hypoxia, inhibitor of differentiation.	ALL, glioma
NLRP1	NLR family, pyrin domain containing 1	17p13.2	Apoptosis, vitiligo, autoimmune diseases.	CML, sarcoma, skin, squamous cell carcinoma, lung, glioma, neuroblastoma, other neuroectodermal cancers
ARID5B	AT rich interactive domain 5B	10q21.2	Transcriptional repression, B-hyperdiploid pediatric acute lymphoblastic leukemia, CAD, Alzheimer's disease	ALL, CLL, myeloma
PMP22	Peripheral myelin protein 22	17p12-p11.2	Differentiation, proliferation, Charcot–Marie–Tooth disease IA, Dejerine–Sottas synd., hereditary neuropathy.	Glioma, sarcoma, testicular, neuroblastoma, breast or pancreatic cancers, melanoma
PROM1	Prominin 1 (CD133)	4p15.32	Hematopoietic stem cell antigen, inhibitor of differentiation, retinitis pigmentosa, Stargardt disease.	Sarcoma, colorectal, pancreatic, other gi, kidney, ovarian and uterine cancers
NEFM	Neurofilament, medium polypeptide	8p21	Cytoskeleton, biomarker of neuronal damage, schizophrenia.	Sarcoma, lung tumor, glioma, neuroblastoma, head and neck, other neuroectodermal, adrenal gland, kidney, testicular or cervical cancers, other urogenital tumors
EPHA4	EPH receptor A4	2q36.1	Survival, differentiation, neuronal development, potentiates FGFR signaling, Parkinson's, Alzheimer's and Huntington's diseases.	Sarcoma, mesothelioma, melanoma, skin, squamous cell carcinoma, lung cancer, glioma, other neuroectodermal, thyroid or testicular cancers
SPP1	Secreted phosphoprotein 1	4q21–q25	Proliferation, growth, anti-apoptotic, inflammatory diseases, Parkinson's disease, cancers.	Glioma, lung cancer, mesothelioma, kidney cancer

In this study we show that the levels of the epigenetic regulators, HDAC1 and HDAC2 proteins, are increased in the hESCs with genomic abnormalities. Importantly, we further demonstrate that, similar to many cancer cell lines, HDAC inhibitors repress proliferation of the karyotypically abnormal hESCs, whereas normal cells are more resistant to the

treatment. The reduced growth of abnormal hESCs in response to HDAC inhibition correlated with increased levels of AcH3 and CDKN1A and with altered regulation of RB1. Interestingly, levels of both HDAC1 and HDAC2 proteins were decreased in response to both VPA and CBHA treatments. Valproic acid prevents catalytic activity of type I HDACs; however, it does not necessarily induce degradation of the target protein. Previously, in HEK293T human embryonic kidney carcinoma cells and mouse F9 teratocarcinoma cells VPA has been shown to specifically induce proteosomal degradation of HDAC2, whereas HDAC1 levels were not reduced (Gottlicher et al., 2001; Kramer et al., 2003). Taken together, our findings suggest that HDACs are implicated in the stem cell transformation and support self-renewal of abnormal cells.

Although HDAC1 has been reported to be crucial for the growth and normal development of the mouse embryos (Brunmeir et al., 2009), our observation is that HDAC depletion with valproic acid, at least up to the concentration of 1.5 mM, does not have any major impact on the self-renewal and proliferation of the karyotypically normal hESCs. This is consistent with the findings in a recent study where HDAC1 deficiency did not affect proliferation of the mouse embryonic stem cells, but rather was required for the efficient differentiation of the cells (Dovey et al., 2010). HDACs are indeed known to be key factors in directing differentiation of the stem cells to certain lineages, such as neuronal and hematopoietic lineages (Brunmeir et al., 2009; Cunliffe and Casaccia-Bonnefil, 2006; Dovey et al., 2010; Humphrey et al., 2008; Wada et al., 2009), probably indirectly by targeting non-histone proteins and directly through epigenetic modifications of the regulatory areas of genes important for differentiation and development. While our study was in progress another study by Ware et al. (2009) reported that HDAC inhibitors support self-renewal of both human and mouse ESCs in the absence of FGF2 or LIF, respectively. In contrast, they also reported that karyotypic abnormalities were observed at equal frequencies under HDAC inhibitor when using butyrate, vorinostat, trichostatin or butyryl than in the absence of these compounds. However, the karyotypic results for cells cultured under valproic acid were not reported and importantly it did not become clear whether changing the concentrations of HDAC inhibitors under well controlled setup would reveal a concentration for selective suppression of abnormal cell growth. Ware et al. used only 0.5 mM valproic acid, which in our hands is too low concentration to prevent growth of abnormal cells.

Our data shows that HDACs communicate with well known proteins implicated in oncogenic processes, such as RB1 and POU5F1 in hESCs. Possibly through co-operation with RB1 and other factors, HDACs modulate activity of genes implicated in development, cell growth and transformation enabling survival of cells with genomic abnormalities. According to our results HDAC inhibition is cytotoxic to abnormal hESCs, whereas normal cells are more resistant to the treatment. This selective sensitivity of the transformed cells is similar to the cells in somatic tissues as reported by several preclinical studies for a wide range of different cancer types. HDAC inhibitor vorinostat has been approved for clinical use in the treatment of cutaneous T cell lymphoma and several others are currently under evaluation (Chateauvieux et al., 2010). We have also here confirmed the growth inhibitory effect of HDAC inhibition on human acute lymphocytic leukemia cell line (CCRF-CEM) and embryonal carcinoma cells (NT2D1) derived from germ cell tumor. The mechanism behind the antitumor effect of HDAC inhibition has remained unclear.

A whole genome transcriptome analysis was carried out to clarify the mechanisms of the cell type specific cytotoxicity of HDAC inhibition on the abnormal cells. The transcriptome analysis revealed the HDAC inhibitor responsive genes in the normal and abnormal cells. The genes with altered levels in abnormal hESCs and reciprocal regulation in response to HDAC inhibition were identified as putative factors explaining selective sensitivity of abnormal hESCs to HDAC inhibition. Previous studies with mouse cells have shown that many of these genes are direct targets of Hdac1, including Tnfrsf19, Id2, Arid5B and Epha4 in mouse ESCs and in Lrp8 and Pmp22 in mouse trophoblast cells (Kidder and Palmer, 2012) and SPP1 in human fibroblasts (Pazolli et al., 2012). We have further validated that LRP8 and SPP1 are regulated in HDAC1 dependent manner in human embryonal carcinoma cells (NT2D1) correlating with the reduced growth of the cells after HDAC1 knockdown. The genes with altered expression and selective sensitivity to HDAC inhibition in abnormal cells are linked to development and age associated to severe developmental and neurological diseases and cancers, such as Alzheimer's disease, Parkinson's disease, gliomas or leukemias. This indicates that the genes showing altered expression and selective sensitivity in HDAC inhibition in abnormal cells are important for the normal cellular differentiation, development and aging. On the other hand these genes link functionally to neuronal and hematological development and seem to be vulnerable to culture induced changes. Consistently, including HDAC1, most of the putative upstream regulators of these genes have previously known function in hematological development. In addition, many of them, such as TP53 and YY1, link to transformation and oncogenesis. These observations may partly explain why HDAC inhibitors are promising or effective in the treatment in leukemias. These candidate genes with altered regulation in abnormal cells and their upstream regulators are likely to be important for the improved survival and proliferation of the abnormal hESCs and are highly valuable as potential targets for prevention of abnormal or malignant growth.

Whether the activity of HDACs is increased before or after genomic alterations occur in stem cells remains to be elucidated. Interestingly, a recent study describes a key function of HDAC1 and HDAC2 in DNA damage response to promote DNA nonhomologous end-joining. Moreover, hypersensitization of HDAC1/2 deficient cells to DNA damage inducing agents was demonstrated (Miller et al., 2010). Thus, it is possible that accumulation of abnormalities in the HDAC1/2 proteins themselves may pronounce the cells to DNA damage and genetic instability. This is further supported by a recent study showing that HDAC1 and Nurd complex are the key modulators of aging related chromatin defects in primary fibroblasts and activity of HDAC1 is decreased during aging (Pegoraro et al., 2009). Thus, defective function of HDACs and accumulation of genomic abnormalities in the stem cells during extended culture may mimic senescence of the cells and in particular long-lived stem cells residing in aging somatic tissues. Similar to ESCs, changes

occurring in the chromatin of long-lived somatic stem cells are likely to affect the growth, differentiation and regenerative potential of these cells exposing the tissues to malignant growth and degenerative diseases. Future studies determining the importance of HDAC mediated protein complexes in the maintenance of somatic stem cells in aging tissues and their contribution to malignant and age related degenerative diseases will be of great interest.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.07.002.

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