

Neurotrophins mediate human embryonic stem cell survival

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Growth of human embryonic stem (hES) cells as a pluripotent population requires a balance between survival, proliferation and self-renewal signals. Here we demonstrate that hES cells express receptors of the tropomyosin-related kinase (TRK) family, which mediate antiapoptotic signals. We show that three TRK ligands, brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4, are survival factors for hES cells. Addition of neurotrophins to hES cell cultures effects a 36-fold improvement in their clonal survival. hES cell cultures maintained in medium containing neurotrophins remain diploid and retain full developmental potency. In the presence of neurotrophins, TRK receptors in hES cells are phosphorylated; TRK receptor inhibition leads to hES cell apoptosis. The survival activity of neurotrophins in hES cells is mediated by the phosphatidylinositol-3-kinase pathway but not the mitogen-activated protein kinase pathway. Neurotrophins improve hES cell survival and may facilitate their manipulation and the development of high-throughput screens to identify factors responsible for hES cell differentiation.

HES cells are pluripotent stem cells that have the dual ability to self-renew and to differentiate into all cell types in the body^{1–3}. Growth and expansion of pluripotent hES cells require a balance between survival, proliferation and self-renewal signals. Although some of the growth factors involved in hES cell self-renewal are known, factors regulating survival have yet to be identified. Basic fibroblast growth factor (bFGF or FGF2) at high concentration maintains hES cells in an undifferentiated state and has a profound effect on self-renewal⁴. Clonal lines of hES cells have been established in the presence of bFGF, albeit at low efficiency (<1%)⁵. The bone morphogenetic protein antagonist Noggin can synergize with bFGF to promote hES cell self-renewal and sustain hES cell proliferation⁴. In addition, the Wnt/ β -catenin and activin/TGF β pathways may also be important for maintaining pluripotency^{6–8}. Despite the importance of these findings, bFGF and Noggin poorly sustain the clonal growth of hES cells⁴. To maintain hES cells in bFGF and Noggin, one must passage the cells as clumps either by scraping manually or by treating with collagenase. The survival of single hES cells under such conditions is extremely low,

which limits the ability to rapidly expand hES cell populations and to apply many methods of genetic selection.

We hypothesized that factors required for hES cell survival would act through receptors present on the hES cell surface. We searched the published hES cell microarray and SAGE data sets for receptor tyrosine kinases that might serve as receptors for antiapoptotic factors^{9–11}. This analysis suggested that hES cells express TRKB and TRKC, the receptors for the nerve growth factor-related (NGFR) family of neurotrophins^{12,13}.

Using RT-PCR analysis, immunocytochemistry and western blotting, we found that hES cells express TRKB and TRKC (Fig. 1). TRKB and TRKC transcripts were present in both H1 and H9 hES cells, whereas transcripts for TRKA and the neurotrophin receptor p75^{NGFR} were either absent or present at low levels (Fig. 1a and Supplementary Fig. 1 online). Immunostaining of H1 and H9 hES cells with antibodies to TRKA, TRKB and TRKC and p75^{NGFR} demonstrated the presence of TRKB and TRKC receptors on the cell surface (Fig. 1b and data not shown). TRKA and p75^{NGFR} receptors were either absent or present at low levels (Fig. 1b). Antibody specificity was confirmed by immunostaining cells that lack neurotrophins (mouse embryonic fibroblast (MEFs) and HeLa cells) and cells that express neurotrophins (primary neurons and PC12 cells) and also by immunostaining hES cells in the presence of a TRKB-blocking peptide (Supplementary Fig. 1 online). We also carried out western blotting of hES cell lysates with antibodies to the TRK receptors as well as to p75^{NGFR}. In both H1 and H9 hES cells, TRKB and TRKC proteins were present, whereas TRKA and p75^{NGFR} proteins were absent or present in much lower amounts (Fig. 1c).

Because hES cells express both TRKB and TRKC at high levels, we tested whether the ligands for these receptors, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4), affect clonal survival of hES cells. H1 and H9 hES cells were trypsinized, and single cells were individually plated into wells of a 96-well plate containing hES medium with or without added neurotrophins (NTs: 50 ng/ml each BDNF, NT3 and NT4) and mitomycin-treated MEFs or Matrigel. After 4–5 d, hES cell colonies were visualized by staining for alkaline phosphatase (AP), an activity characteristic of pluripotent stem cells. When grown in hES medium

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alone, about 6% of hES cells formed AP-positive colonies (Fig. 1d). In contrast, between 27% and 30% of hES cells grown in hES cell medium containing neurotrophins formed AP-positive colonies (Fig. 1d). Thus, clonal survival of hES cells is substantially increased in the presence of neurotrophins. To test whether the effect of neurotrophins on short-term hES cell clonal survival improves the ability to derive clonal lines, we passaged clones derived in the presence or absence of neurotrophins. Clones were passaged twice and the numbers of surviving clones counted. In the absence of neurotrophins, 0.4% of the initial clones survived (Fig. 1f), a number similar to that previously described for clonal derivation⁵. In the presence of neurotrophins, 14.6% of the clones survived (Fig. 1f).

Therefore, addition of neurotrophins results in a 36-fold increase in clonal survival.

To determine whether hES cells maintained in neurotrophin-containing medium remain pluripotent, we assayed the expression of markers characteristic of pluripotent hES cells and differentiation potential. Four clonally derived H1 hES cell lines were established and grown in the presence of neurotrophins for 15–20 passages. The cells retained the morphology of pluripotent hES cells as well as expression of markers of pluripotent hES cells, including OCT4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 and AP (Fig. 1e and data not shown). In addition, the cells retained a normal, diploid karyotype. A total of 106 metaphase karyograms were examined, and all were normal (Fig. 1e).

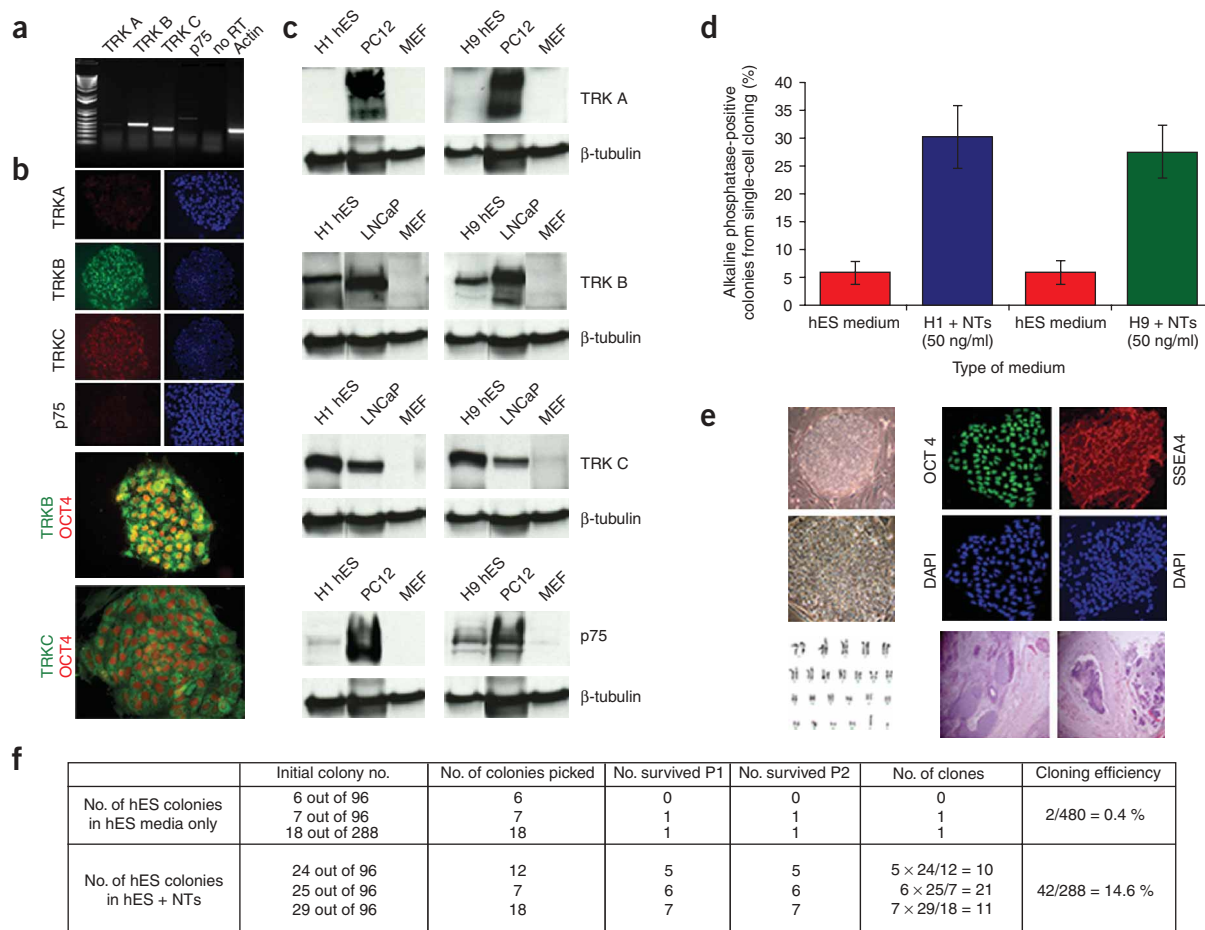


Figure 1 Human embryonic stem (hES) cells express members of the TRK receptor tyrosine kinase family of neurotrophin receptors. (a) RT-PCR analysis of TRKA, TRKB, TRKC and p75^{NGFR} expression in H1 hES cells. Lane 1, molecular weight markers; Lane 2, TRKA; Lane 3, TRKB; Lane 4, TRKC; Lane 5, p75; Lane 6, no RT; Lane 7, β -actin. (b) Immunofluorescence analysis of TRKA, TRKB, TRKC, p75^{NGFR}, and OCT4 expression by H1 hES cells. (c) Western analysis of TRKA, TRK B, TRK C and p75^{NGFR} expression in H1 and H9 hES cells. In each panel, Lane 1, H1 or H9 hES cells; Lane 2, PC12 or LNCaP positive control. Lane 3, MEF negative control. β -tubulin was used as a loading control for all samples. Neurotrophins also support both clonal survival of hES cells and their growth as diploid pluripotent stem cells. (d) H1 and H9 hES cells were plated on MEFs at single-cell density in hES cell medium containing 50 ng/ml each BDNF, NT3 and NT4. hES colonies were counted after visualization by alkaline phosphatase staining. (e) hES cells grown in media with neurotrophins retain the characteristics of pluripotent hES cells for at least 20 passages. Colony morphology remained normal in neurotrophins (see colony at 10 \times and 20 \times). Clones established in neurotrophins retained a diploid karyotype. Out of 106 karyograms analyzed, all were diploid. hES cells maintained in neurotrophins express the POU-domain transcription factor Oct4 and the stem cell marker SSEA4. The middle panels show cells that were counterstained with DAPI. hES cells maintained in neurotrophins formed well-differentiated teratomas when injected into NOD/SCID mice. Sections of teratomas stained with hematoxylin and eosin are shown in the bottom panels. Table in f. hES cells were plated as described in Fig. 2. After 4–5 d, clones (comprised of ~7–11 cells) were picked by manually scraping colonies out of each well in the 96-well plate. All colonies that were seen in regular hES cell medium were scraped (denoted passage 1 (P1)). Only a portion of the colonies grown in neurotrophins were scraped because the colonies were so numerous. After another 5 d, the clones that survived were then passaged again by manual scraping (P2) and allowed to grow for a further 5 d which represents approximately nine population doublings.

To determine whether hES cells cultured with neurotrophins retain full developmental potency, we tested their ability to make embryoid bodies in culture and to form teratomas after injection into histocompatible mice. hES cells cultured with neurotrophins formed well differentiated, cystic embryoid bodies morphologically indistinguishable from those obtained from hES cells cultured in standard conditions and formed teratomas containing differentiated cells derived from all three primary germ layers (Fig. 1e and data not shown). Because hES cells appear to differentiate easily into cells of the neuroectodermal lineage^{7,14}, we also tested whether hES cells cultured with neurotrophins express neuronal markers. hES cells grown with neurotrophins did not express detectable amounts of the neuronal markers GFAP, MAP2, PAX2 or PAX6, indicating that they did not acquire a neuronal phenotype (Supplementary Fig. 2 online).

hES cells are typically grown on a feeder layer of mitotically inactivated MEFs or on Matrigel in the presence of medium conditioned by MEFs (MEF-CM)^{15,16}. We speculated that the beneficial effects of MEF-CM might be due, at least in part, to the presence of neurotrophins. To test whether MEFs express neurotrophins, we performed RT-PCR analysis, which demonstrated that MEFs express mRNA for NGF, BDNF, NT3 and NT4 (Fig. 2a). To test whether the beneficial effects of MEF-CM on hES cell growth is mediated by neurotrophins, we blocked the action of neurotrophins with anti-neurotrophin antibodies in a low-density survival assay. In this assay, hES cells were dispersed to a single-cell suspension by trypsin treatment and plated at low density (500 cells/well in a 96-well plate). They were cultured for 4–5 d in the presence of neurotrophin-neutralizing or control antibodies; hES colonies were then visualized by AP staining (Fig. 2b). A combination of BDNF, NT3 and NT4 antibodies reduced

cell survival from 21% to 6%, whereas an isotype-matched control antibody or an antibody to NGF had no significant effects (Fig. 2b). These data indicate that a portion of the survival activity of MEF-CM is indeed due to the action of neurotrophins.

BDNF, NT3 and NT4 together act as survival factors for hES cells. To determine whether any of the neurotrophins alone can mediate survival of hES cells, NT3, BDNF, NT4 and NGF were tested individually in the low-density survival assay (Fig. 2c–e). NT3, BDNF and NT4 had potent, dose-dependent effects on hES cell survival when plated on either MEFs or Matrigel (Fig. 2c–e). Furthermore, enhanced survival was observed in cells plated on Matrigel in the absence of MEFs, suggesting that neurotrophins act directly through TRK receptors on the hES cells rather than indirectly through MEFs. Addition of NGF resulted in a slight increase in hES cell survival (Fig. 2c–e), possibly mediated by the low levels of TRKA and p75^{NGFR} present in hES cells. These data demonstrate that BDNF, NT3 and NT4 are potent survival factors for hES cells, presumably acting through TRKB and TRKC.

In neuronal cells, activation of TRK receptors by neurotrophins results in their phosphorylation. To analyze the phosphorylation of TRK receptors in hES cells, we immunoprecipitated TRK proteins from hES cells with TRKB or TRKC antibodies and then blotted with an antiphosphotyrosine (P-Tyr) antibody. In the absence of neurotrophins, phosphorylated TRK proteins of ~145 kDa were present at low levels (Supplementary Fig. 2 online). When hES cells were exposed to neurotrophins for 5 min, phosphorylation of TRKB and TRKC increased ~30- and 1.7-fold, respectively (Supplementary Fig. 2 online), suggesting that TRKB and TRKC receptors on hES cells are activated in the presence of the NT3, BDNF and NT4. We

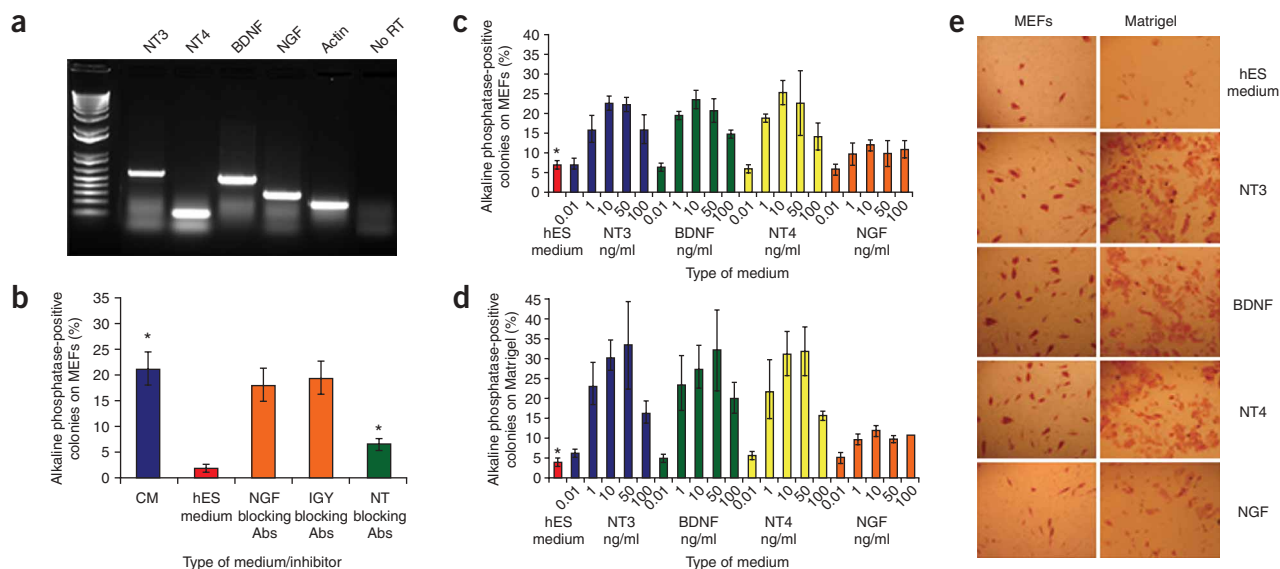


Figure 2 Neurotrophins are expressed by MEFs. (a) RT-PCR analysis of NT3, NT4, BDNF, NGF and actin expression by MEFs. (b) Blocking antibodies to the neurotrophins (NT3, NT4, and BDNF) interfere with hES-cell survival activity of MEF-CM, whereas an NGF blocking antibody and an isotype-matched control IgY do not. Bars, standard deviation of $n = 3$ experiments. Blocking neurotrophin activity is statistically significant at $P < 0.001$ as compared to CM (*).

(c–e) Individual neurotrophins have dose-dependent effects on hES cell survival. (c) Effect of neurotrophins on hES cell survival at low-density on MEFs. hES cells were trypsinized and plated at low density (500 cells per well) on MEFs in 96-well plates in the presence of NT3, BDNF, NT4, NGF or hES medium lacking added growth factor. After 4–5 d, the colonies (comprised of approximately 7–11 cells) were fixed and stained for alkaline phosphatase. (d) Effect of neurotrophins on hES cell survival at low-density on Matrigel. hES cells survive poorly in hES medium alone either on MEFs (c) or on Matrigel (d) but show dose-dependent survival in the presence of the neurotrophins on MEFs (c) or on Matrigel (d). Bar, standard deviation of $n = 3$ experiments.

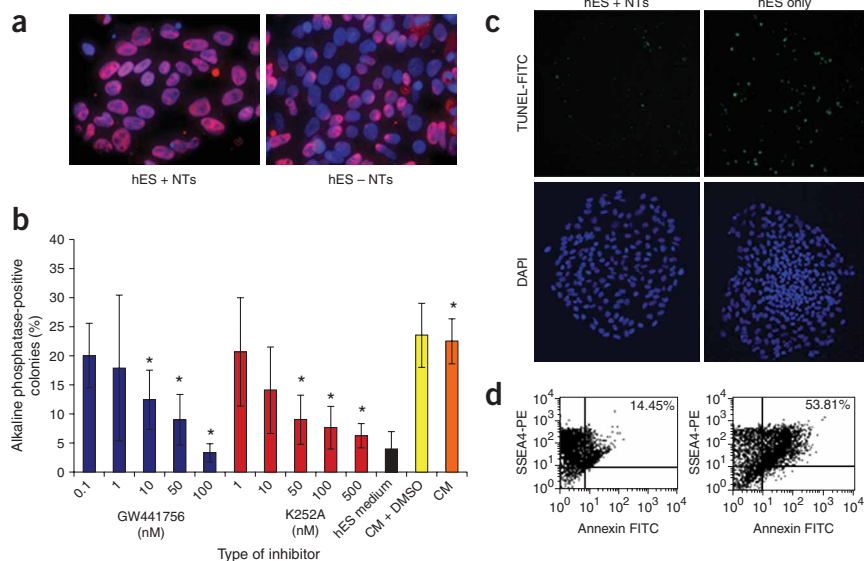
*All neurotrophins tested in c and d showed statistically significant effects on hES cell survival at $P < 0.01$ as compared to hES medium alone. neurotrophins (BDNF, NT4 and NT3) also have statistically significant differences between concentrations of 0.01–100 ng/ml at $P < 0.05$.

(e) Phase contrast images (at 4x) of morphology of hES cell colonies observed on MEFs or Matrigel in the low-density survival assay.

Figure 3 Disruption of neurotrophin signaling leads to TRK receptor dephosphorylation and hES cell death. **(a)** Localization of phosphorylated TRK 490 in hES cells in the presence of neurotrophins and upon withdrawal of neurotrophins. hES cells grown in the presence of neurotrophins for 24 h were stained with P-TRK 490 antibody directed against a phosphorylated epitope present in the TRK receptors (red) and nuclei stained with DAPI (blue). Anti-P-TRK 490 antibody staining was fairly uniform in cells throughout the colonies (left panel). In hES cells in which the neurotrophins were removed for 20 min, cells began to lose P-TRK 490.

(b) TRK signaling and survival can be blocked by pharmacological inhibition. TRK inhibitors were added to hES cells plated with MEF-CM in the low-density survival assay. hES cells were exposed to increasing concentrations of a GW441756, K252A, or vehicle (DMSO). Bars, standard deviation of $n = 3$ experiments. *Trk inhibitors are statistically significant at $P < .008$ as compared to CM. K252a is a staurosporine analog that has a broad kinase inhibition profile,

including potent inhibition of TRKs. K252a has been widely used as a TRK inhibitor with specificity for TRKs over FGF, EGF and other signaling pathways previously demonstrated¹³. GW441756 is a 3-anilinomethylene-oxindole analog with a more specific kinase inhibition profile (see compound no. 3 in ref. 19). In a kinase inhibition assay, this compound has tenfold, or in some cases 100-fold, selectivity for TRKs over many kinases including c-Src, VEGFR2, Raf and CDK1¹⁹. **(c–d)** Loss of the TRK signaling pathway leads to hES cell death. **(c)** TUNEL analysis of hES cell apoptosis in the presence or absence of neurotrophins. hES cells were grown in the presence or absence of neurotrophins and apoptosis assayed by TUNEL analysis. More FITC-positive cells (green) were present in cells grown without neurotrophins (right panel) as compared to cells grown in the presence of neurotrophins (left panel). Counterstaining of nuclei in the same colonies with DAPI is shown in the lower panels. **(d)** FACS analysis of apoptosis in hES cells grown in the presence or absence of neurotrophins. After 24 h of culture grown with or without neurotrophins, hES cells were stained with fluorescein isothiocyanate-conjugated Annexin V and analyzed by flow cytometry. hES cells were identified by anti-SSEA-4 staining. hES cells grown in media with neurotrophins (left panel) had significantly fewer Annexin V-positive cells than their counterparts grown in normal hES cell culture medium (right panel).



then asked whether removal of neurotrophins affects phosphorylation of TRK proteins in hES cells grown continuously with neurotrophins. For this study, we visualized TRK receptor phosphorylation immunocytochemically using an antibody that binds to TRK proteins phosphorylated at tyrosine 490 (P-TRK(490)). In the presence of neurotrophins, P-TRK(490) stained hES cells throughout the colonies (Fig. 3a). When neurotrophins were removed from the medium for 20 min, P-TRK(490) staining disappeared from many of the cells (Fig. 3a and Supplementary Fig. 2 online) confirming that NT3, BDNF and NT4 affect TRK receptor phosphorylation in hES cells. Notably, P-TRK(490) staining is often lost from the center of the colonies, the area that often undergoes apoptosis or differentiation (Supplementary Fig. 2 online)⁴. Anti-P-TRK antibodies also stained hES cells growing on MEFs, supporting the idea that part of the effect of MEF-CM is due to TRK receptor activation (Supplementary Fig. 2 online).

If neurotrophins are mediating hES cell survival through activation of TRK signaling pathways, then pharmacological inhibition of TRK signaling pathways should reduce hES cell survival. To test this prediction, hES cell survival was measured using the low-density survival assay in the presence or absence of two different TRK inhibitors, GW441756 and K252a^{17–19}. Both GW441756, which binds to the ATP binding site of the TRK receptors, and K252a, which inhibits the tyrosine phosphorylation and kinase activity of TRK receptors, showed statistically significant ($P < 0.008$) and dose-dependent effects on hES cell survival (Fig. 3b). Although pharmacological inhibitors can often affect multiple signaling pathways, the demonstration that two TRK inhibitors, working through different mechanisms, have the same effect on hES cell survival suggests that the

effect observed arises from inhibition of TRK receptors. Taken together with the ability of neurotrophin-neutralizing antibodies to block hES cell survival (Fig. 2b), these data suggest an important role for neurotrophin-mediated activation of TRK receptor signaling in hES cell survival.

We next asked whether the increase in clonal hES survival mediated by TRK receptors is associated with a decrease in apoptosis. Clumps of hES cells were plated on Matrigel in the presence or absence of neurotrophins. The surviving colonies were analyzed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. In the absence of neurotrophins, a large number of TUNEL-positive apoptotic cells were observed in colonies of hES cells (Fig. 3c). In contrast, few TUNEL-positive cells were observed in hES cells plated in the presence of neurotrophins (Fig. 3c). Flow cytometry was used to quantify apoptosis in hES cells. Clumps of hES cells grown on Matrigel in the presence or absence of neurotrophins for 24 h were stained with the early apoptotic marker Annexin V-fluorescein isothiocyanate (FITC) and the hES cell marker SSEA-4. A substantially greater proportion of hES cells grown without neurotrophins were labeled with Annexin V-FITC (53.81%) compared with hES cells grown with neurotrophins (14.45%; Fig. 3d). Similar results from both TUNEL staining and flow cytometry were observed when trypsin-dispersed hES cells were plated as single cells at low density in the presence or absence of neurotrophins (data not shown). These data suggest that hES cells grown without neurotrophins undergo increased apoptosis. In contrast, growth with neurotrophins dramatically decreases both TUNEL-positive and Annexin V-FITC-positive populations of hES cells, indicating that neurotrophins act in an antiapoptotic fashion to promote hES cell survival. Because

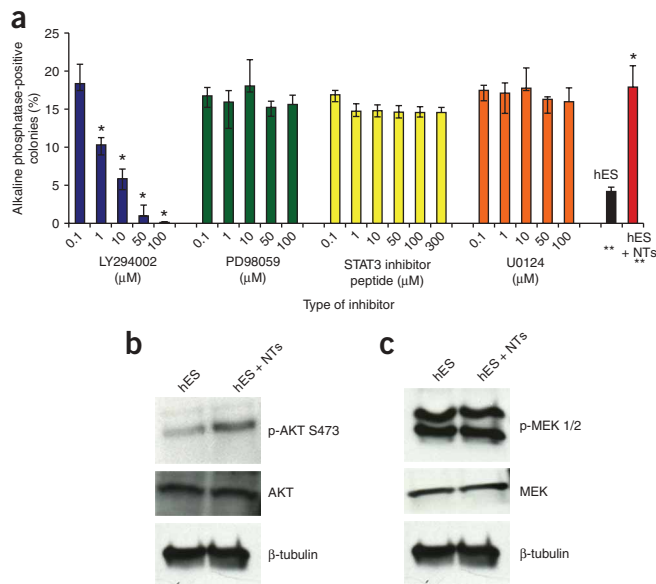


Figure 4 PI-3K activity is required for hES cell survival. **(a)** Effect of inhibitors on the survival of hES cells. hES cells were plated as single cells at low-density in neurotrophins but in the absence of bFGF. Cells were plated in the presence or absence of increasing concentrations of inhibitors of PI-3K (LY294002), MAPK (PD98059) and STAT3 (Inhibitor peptide) as well as an inactive form of a MAPK kinase inhibitor (UO124) as a control. As further controls, hES cells were plated in hES cell medium. The effect of the PI-3K inhibitor LY294002 on hES cell survival was statistically different from the control (*) ($p < 0.006$) at all concentrations tested. The STAT3, MAPK kinase, and UO124 control inhibitors had no effect on hES cell survival. bFGF was omitted from the medium in all conditions tested. **(b–c)** Neurotrophins have differential effects on AKT and MEK1/2 phosphorylation. hES cells were grown in the presence or absence of neurotrophins (but without bFGF) and cell lysates probed with antibodies to phospho-AKT or phospho-MEK1/2. In the absence of neurotrophins, a weak phospho-AKT band is observed. Upon stimulation of hES cells with neurotrophins a stronger band is observed. The intensity of the bands were compared to the total level of AKT and to a loading control (β -tubulin). **(c)** In contrast no change in the level of phospho-MEK1/2 was observed by comparison with the controls.

neurotrophins stimulate DNA synthesis in some cells¹², we also examined the mitotic index in cells grown with or without neurotrophins. No differences in mitotic index were observed (data not shown). In contrast, we did observe a decrease in the population doubling time of hES cells cultured in the presence of neurotrophins (**Supplementary Fig. 3** online), a result most likely due to the suppression of apoptosis. The decrease in population doubling time was more pronounced in hES cells grown on Matrigel than on MEFs, presumably because MEFs produce some neurotrophins (**Fig. 2**) and the addition of neurotrophins, therefore, had less effect (**Supplementary Fig. 3** online). In both conditions (Matrigel and MEFs), neurotrophins improved the initial bulk survival of trypsinized hES cells, in agreement with the effect seen on clonal cell survival. The combined action of neurotrophins on initial survival and on population doubling time of hES cells results in a substantial improvement in their expansion in culture (**Supplementary Fig. 3** online).

Activation and phosphorylation of TRK receptors leads to activation of a number of downstream effectors, including phosphatidylinositol-3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK). To test the role of these molecules in neurotrophin-mediated

survival of hES cells, we perturbed their action with pharmacological inhibitors. We used inhibitors to the JAK/STAT signaling pathway, a pathway known to have little, if any, role in hES cell growth^{20,21}, as a control. bFGF was omitted in these cultures to eliminate any effects resulting from inhibition of bFGF signaling. Inhibition of the PI-3K pathway had a dramatic effect on hES cell survival in medium with neurotrophins, whereas inhibition of the MAPK and JAK/STAT pathways had little or no effect (**Fig. 4a**). Consistent with the observed effects of PI-3K and MAPK inhibitors of hES cell survival, phosphorylation of AKT, a downstream effector of PI-3K, increased upon addition of neurotrophins to hES cells (**Fig. 4b**). In contrast, phosphorylation of MEK1/2, a downstream effector of MAPK, was unaffected by the addition of neurotrophins (**Fig. 4c**). These data suggest that activation of PI-3K, presumably acting through AKT, is a critical event in neurotrophin-mediated hES cell survival.

Our studies demonstrate a role for neurotrophins in hES cell survival and provide new insights into the molecular mechanisms controlling the growth of these cells. Although previous studies identified growth factors that affect self-renewal of hES cells, few, if any, have identified factors that act through specific receptors present on hES cells and activate the receptors at physiological concentrations. Furthermore, a physiological response, namely induction of apoptosis, results from blockade of the signaling pathway. The survival effect of neurotrophins appears to be mediated through TRK activation of the PI-3K pathway.

The ability of neurotrophins to support high-efficiency clonal survival of hES cells should facilitate many uses of hES cells that are currently difficult or impossible, such as genetic selection or high-throughput screening. The ability of neurotrophins to support hES cell survival has important implications for cell-based therapies. Transplanted hES cells could conceivably survive in tissues rich in neurotrophins and ultimately form tumors. However, selective killing of pluripotent hES cells before transplantation using pharmacological inhibitors of the TRK signaling pathway (as demonstrated here) could overcome this problem.

Recent reports suggest that aneuploidy occurs in hES cells cultured at clonal density²². Standard conditions currently used to grow hES cells at clonal density could be deficient in a factor (or factors) required for hES cell survival. Our finding that neurotrophins mediate hES cell survival suggests that one of the limiting factors may be a neurotrophin. Interestingly, NT3 and p75^{NGFR} are encoded on chromosomes 12 and 17, respectively, chromosomes that are reported to be commonly amplified in hES cells¹¹. Therefore, acquisition of chromosomal alterations could result in enhanced neurotrophin signaling, allowing the cells to overcome apoptosis and gain a selective advantage.

The mode of action of neurotrophins has been thoroughly studied in a variety of cell types, notably neurons^{12,13,23}. Understanding how neurotrophins act on hES cells could yield new insights into the signaling pathways required for hES cell survival and growth and into the role of this growth factor family in the survival of pluripotent stem cells of the early mammalian embryo. Recent studies suggest a role for neurotrophins in oocyte maturation leading to improved embryo development in mouse, bovine and human^{24–28}. In this regard, addition of neurotrophins to cultures may also improve methods for hES cell derivation.

METHODS

Cell culture, survival assays and inhibitor assays. Human ES cell lines H1 and H9 were cultured as described¹⁵ in high glucose DMEM supplemented with

l-glutamine, nonessential amino acids, serum replacement (Invitrogen) and 4 ng/ml basic Fibroblast growth factor (Invitrogen). MEFs (Specialty Media) were plated at $5-7 \times 10^6$ cells/ml. To prepare single cell suspensions of hES cells, we washed the cells twice with PBS, then incubated them in 0.05% trypsin/EDTA (Invitrogen) for 5 min at 37 °C. The cells were triturated with a 5-ml pipette until a single cell suspension was obtained. Trypsin inhibitor (Invitrogen) was added and the cell suspension filtered through a 0.4- μ m filter (Fisher Scientific) to remove clumps and MEFs. For clonal survival assays, single hES cells were diluted to clonal density and plated into a well of a 96-well plate containing MEFs. For low-density survival assays, trypsinized cells were counted using a hemocytometer and 500 cells were plated in each well of 96-well plate containing MEFs or coated with Matrigel (BD Biosciences). To visualize hES colonies, cultures were fixed in 4% paraformaldehyde in PBS for 30 min, washed once in PBS, once in distilled H₂O, then stained for alkaline phosphatase activity as described previously²⁵. Individual hES cells or hES cell colonies were counted manually on an inverted microscope. NGF, BDNF, NT3 and NT4 were purchased from Peprtech. The TRK inhibitors GW441756 (Sigma) and K252A (Calbiochem), PI-3K inhibitor, MAPK inhibitors, STAT3 inhibitor and control peptides (all from Calbiochem) were prepared according to the manufacturer's instructions. For the antibody-blocking experiments, antibodies to NGF, NT3, NT4 and BDNF as well as a control antibody were obtained from Promega. Antibodies were added to culture medium at a concentration of 20 μ g/ml. After culture, the cultures were fixed and stained for alkaline phosphatase activity as described above. Each experiment consisted of at least four replicates per treatment and each experiment was repeated at least three times. Statistical analysis was carried out using Students *t*-test analysis.

Immunocytochemistry. Immunocytochemical analysis of surface antigens was carried out as described previously²⁵. For staining of intracellular antigens, the cells were fixed in 4% paraformaldehyde in PBS, treated for 3–5 min with 0.05% Triton X-100 in PBS, then washed three times in PBS. Antibodies to SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, Oct-4, TRKA, TRKB, and p75^{NGFR} were obtained from R and D Systems. Antibodies to Pax2 and Pax6 were obtained from Covance, MAP2 and GFAP were from Chemicon, and TRKB from Promega. Antibodies were diluted in blocking buffer containing 4% BSA and 10% heat-inactivated serum of the same species as the secondary antibody. Fluorescein- or rhodamine-conjugated secondary antibodies (Pierce) were diluted in the same blocking buffer. Positive controls for the reactivity of the neuronal antibodies (TRKA, TRKB, TRKC, p75^{NGFR}, Pax2, Pax6, MAP2 and GFAP) were primary rat neurons, astrocytes and glia as well as rat pheochromocytoma cells (PC12; generous gift from David Ginty). TUNEL staining was carried out using the ApopTag Plus FITC *in situ* kit (Chemicon) according to the manufacturer's instructions. Peptide inhibition of antibody binding was carried out as described previously²⁸. Immunocytochemical staining of cells was observed on a Nikon E1000 microscope equipped with fluorescence optics.

Immunoblotting and immunoprecipitation. Immunoblotting of hES cell antigens was carried out using published methods appropriate for each antibody. Antibodies to TRKA, TRKB, TRKC, p75^{NGFR} and the antiphosphotyrosine monoclonal antibody 4G10 were obtained from Upstate. Lysates of MEFs were used as a negative control. Cells known to express the specific neurotrophin receptors were used as positive controls. PC12 express TRKA and p75^{NGFR} and human metastatic prostate carcinoma cells (LNCaP; ATCC) express TRKB and TRKC^{26,27}. PC12 cells were grown as described²⁸. LNCaP cells were grown according to ATCC instructions (see <http://www.atcc.org/>). Cell lysates were prepared as described previously²⁹. Immunoprecipitation of TRKB and TRKC in hES cell lysates was carried out as described³⁰. Briefly, hES cells were treated with or without neurotrophins for 5 min, washed briefly in ice-cold PBS, then lysed in TBS plus 1% Nonidet P-40, 10 μ g/ml aprotinin, 1 mM PMSF, 1 mg/ml leupeptin and 500 μ M Na Orthovanadate. Cells were rocked on ice and frozen and thawed three times. Lysates were centrifuged to remove debris, incubated with primary antibody overnight at 4 °C, and then incubated with Protein-A-sepharose (Pharmacia) for 1–2 h at 4 °C. The beads were washed 3 times in lysis buffer, once in distilled H₂O, then incubated with SDS-PAGE sample buffer for 5 min at 90–100 °C. The sample buffer was

carefully removed and run on a 7.5% SDS-PAGE gel (BioRad) as described previously²⁹. Western blotting was carried out essentially as described previously²⁹. The resolved proteins were transferred overnight at 4 °C onto Immobilon PVDF membranes (Millipore) and then the PVDF membrane blocked in 1% BSA, 1% nonfat milk and 0.05% Tween 20 in PBS for 1–2 h. Anti-P-Tyr antibody (4G10) was used according to the manufacturer's instructions. Primary antibodies were detected with species-specific horseradish peroxidase-conjugated secondary antibodies (Upstate) and a chemiluminescent kit (Cell Signaling).

Flow cytometry. Analysis of apoptosis in hES cell cultures was carried out by flow cytometry using Annexin-V-FITC (BD Biosciences) according to the manufacturer's instructions. hES cells were identified by staining with anti-SSEA-4 antibody (R and D Systems). Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Karyotyping and teratoma assays. Karyotype analysis was carried out routinely every 2 to 3 months as described previously³¹. For each analysis 10 to 20 karyograms were examined. Teratoma assays were carried out as described¹⁵. Briefly, hES cells from one nearly confluent 6-well plate were harvested then injected subcutaneously into the hind limb or rear flank of a SCID/Beige mouse (The Jackson Labs). Animals were monitored regularly for signs of discomfort and distress. All animal work was carried out under protocols approved by the Johns Hopkins University IACUC. When tumors were visible, animals were killed, the tumor material excised, then fixed and processed for histochemistry as described previously³¹. Differentiation of hES cells into embryoid bodies was carried out essentially as described by Thomson and colleagues⁵.

RT-PCR analysis. mRNA was prepared from hES cells or MEFs as previously described²⁹. Primers for the human TRKA, TRKB, TRKC and p75^{NGFR} genes as well as for the mouse NGF, NT3, NT4 and BDNF are described below. RT-PCR analysis was carried out using the Superscript One-Step RT-PCR with Platinum Taq Kit (Invitrogen) according to the manufacturer's instructions for 30–32 cycles. Amplification of β -actin or omission of RT served as positive and negative controls. The primers used for the individual genes were:

Human TRKA: Forward primer: TTC CAT TTC ACT CCT CGG CTC AGT
Reverse primer: ACG TCA CGT TCT TCC TGT TGA GGT
Human TRKB Forward primer: TCA ATG CCA GGC AGG TCT CCT AAA
Reverse primer: TTG GTG CAG AAT TCC CAG CAA AGG
Human TRKC Forward primer: TGC AGT CCA TCA ACA CTC ACC AGA
Reverse primer: TGT AGT GGG TGG GCT TGT TGA AGA
Human p75^{NGFR} Forward primer: TTC AAG GGC TTA CAC GTG GAG GAA
Reverse primer: TGT GTG TAA GTT TCA GGA GGG CCA
Mouse NT3 Forward primer: CTT ATC TCC GTG GCA TCC AAG G
Reverse primer: TCT GAA GTC AGT GCT CGG ACG T
Mouse NT4 Forward primer: TTC TGG CTC CTG AGT GGA C
Reverse primer: AGT CAA CGC CCG CAC ATA G
Mouse BDNF Forward primer: ATG GGA CTC TGG AGA GCG TGA A
Reverse primer: CGC CAG CCA ATT CTC TTT TTG C
Mouse NGF Forward primer: GGT GCA TGG CGT AAT GTC CAT GTT
Reverse primer: ATT GTA CCA TGG GCC TGG AAG TCT

The expected amplified products were: TRKA (463 bp), TRKB (493 bp), TRKC (372 bp), p75^{NGFR} (721 bp), NT3 (486 bp), NT4 (252 bp), BDNF (501 bp) and NGF (345 bp). For each of the genes the RT-PCR products were sequenced and verified by NCBI blast analysis.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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