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Transcriptional profiling in an MPNST-derived cell line and normal human Schwann cells

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Abstract

cDNA microarrays were utilized to identify abnormally expressed genes in a malignant peripheral nerve sheath tumor (MPNST)-derived cell line, T265, by comparing the mRNA abundance profiles with that of normal human Schwann cells (nhSCs). The findings characterize the molecular phenotype of this important cell-line model of MPNSTs, and elucidate the contribution of Schwann cells in MPNSTs. In total, 4608 cDNA sequences were screened and hybridizations replicated on custom cDNA microarrays. In order to verify the microarray data, a large selection of differentially expressed mRNA transcripts were subjected to semi-quantitative reverse transcription PCR (LightCycler). Western blotting was performed to investigate a selection of genes and signal transduction pathways, as a further validation of the microarray data. The data generated from multiple microarray screens, semi-quantitative RT–PCR and Western blotting are in broad agreement. This study represents a comprehensive gene-expression analysis of an MPNST-derived cell line and the first comprehensive global mRNA profile of nhSCs in culture. This study has identified ~900 genes that are expressed abnormally in the T265 cell line and detected many genes not previously reported to be expressed in nhSCs. The results provide crucial information on the T265 cells that is essential for investigation using this cell line in experimental studies in neurofibromatosis type I (NF1), and important information on normal human Schwann cells that is applicable to a wide range of studies on Schwann cells in cell culture.

Keywords

Cancer; NF1; MPNST; T265; Schwann cell; cDNA microarray

INTRODUCTION

The T265 cell line is widely used for cell culture studies of Schwann cells in MPNSTs (Muja *et al*., 2001; Badache and De Vries, 1998; Muir *et al*., 2001; Miller *et al*., 2003; Fieber *et al*., 2003). This cell line has characteristics consistent with Schwann cells that have been

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transformed into a malignant phenotype because of the loss of neurofibromin in the auto-somal-dominant condition NF1; however, many genes are thought to be regulated abnormally in NF1 and in this cell line, which makes it difficult to design properly controlled experiments for studies of NF1 *in vitro*. In order to fully characterize this cell line for use in NF1 research and obtain greater insight into this disease, gene-expression profiling using high-throughput, custom cDNA microarrays, was used to assess of the level of a defined set of mRNA transcripts in T265 cells and nhSCs in culture. The outcome of this study reveals deregulation of an extremely wide range of genes in T265 cells. This comprehensive analysis will be helpful in future molecular and cellular studies of NF1 using T265 cells, and the results indicate several new lines of research into NF1.

In addition to confirming the abnormal expression of many genes that were associated previously with T265 cells and NF1, the study reveals the aberrant expression of many genes that have not previously been associated with this disease. Many of these genes are either associated with malignancy in other forms of cancer or are involved in cellular functions that are essential for tumorigenesis. The results indicate that understanding the biological mechanisms for the transition from benign to malignant phases of NF1 requires a perspective recognizing that wide deregulation of essential biological processes is necessary for the growth and invasion of these malignant cells.

OBJECTIVE

To determine the transcriptional profile of a widely used MPNST cell line and nhSCs in culture, and identify genes expressed in cultured human Schwann cells that have not been previously reported. To achieve this aim we have carried out multiple microarray screens, validated a subset of genes by RT–PCR and performed a series of Western blots to look at key signaling pathways in Schwann cells.

METHODS

cDNA array hybridization

Total cellular RNA (3–5 μg) was prepared from T265 and nhSCs using the TRIzol RNA isolation method (Invitrogen, Life Technologies). The concentration and quality of total RNA was calculated by spectrophotometry (A_{260} nm/A_{280} nm, 1.9–2.1) and by agarose gel electrophoresis. RNA samples were stored at –80°C until needed. RNA samples were radio-labeled and hybridized according to protocols described at http://www.grc.nia.nih.gov/branches/rrb/dna/dna.htm. Probe preparation was accomplished by radiolabeling (α [^{33}P] dCTP) 3–5 μg total RNA in a reverse transcription reaction using Superscript II (Invitrogen). Briefly, RNA was annealed in 10 μl H_2O, with 1 μg of 24-mer poly (dT) primer (Research Genetics) by heating at 65°C for 15 minutes and cooling on ice for 5 minutes. The RT reaction was carried out by adding 8 μl of 5× first-strand RT buffer (Invitrogen), 4 μl 20 mM dNTPs (minus dCTP, Invitrogen), 4 μl of 20 mM DTT, 50 U of rRNasin (Promega), 6 μl of 3000 Ci mmol^{-1} α[^{33}P]dCTP (New England Nuclear) to the RNA/primer mix to a final volume of 40 μl. Superscript II reverse transcriptase was then added and the mix incubated at 42°C for 45 minutes followed by an additional 2 μl of Superscript II reverse tran-scriptase and another 45-minute incubation. The reaction was stopped by the addition of 5 μl of 0.5 M EDTA. Samples were then incubated at 65°C for 30 minutes after the addition of 10 μl of 0.1 M NaOH to hydrolyze and remove any remaining RNA. Samples were purified using Sephadex G-50 columns (Bio-Rad) to remove unincorporated radioactivity.

Briefly, membranes were hybridized with α[^{33}P]dCTP cDNA labeled probes in 10 ml Ultrahyb hybridization solution (Research Genetics) containing 10 μl of 10 mg ml^{-1} polyA for 12 hours at 60°C in a rotating hybridization oven. Hybridized arrays were washed in 10 ml of 2× SSC

and 0.1% SDS twice at 60°C, followed by 2–3 washes in 1× SSC, 0.1% SDS at 65°C. The microarrays were then exposed to phosphorimager screens for 12–24 hours and scanned in a Molecular Dynamics Storm Phosphor imager (Molecular Dynamics) at 50 μm resolution. Scanned images were then processed using either Imagequant (Molecular Dynamics) or Array Pro Software (Media Cybernetics) and the raw intensity values were transferred into Excel spreadsheets.

Microarray design

The dataset presented here was generated from a 4608 cDNA focused human microarray, all cDNA spots are printed in duplicate. Because of the nature of the array construction, several cDNAs are represented multiple times on the array, which gives another level of replication within each array experiment. In the summary table (Table 1), such replicates are reported separately.

Experimental design and statistical analysis of microarray data

Sampling was designed to investigate sources of variation in microarray hybridization consistency, genetic differences in human donors, cell plating passage number, and differences between T265 cells and nhSCs. Human Schwann cells were derived from two different donors (D1 and D2), and were screened by microarray in three independent experiments. Each experiment included a duplicate hybridization of the same sample (technical replicate); these exhibited negligible variation (<5% of genes showed discrepant expression between the replicates). The set of genes selected by statistical analysis (z test) as being expressed at significantly different ($P \leq 0.01$) levels in T265 and human Schwann cells were analyzed by hierarchical cluster analysis (Fig. 1). The results exhibited consistent expression of these genes across human donors, experimental replicates and passage number (either 2 or 3). The differences in mRNA expression were validated by RT–PCR (LightCycler) from mRNA isolated from human Schwann cells derived from D1. Validation at the protein level was tested by Western blot from protein derived from human Schwann cells from D2. With a few exceptions (discussed in Results), the results of Western blots and RT–PCR were in agreement with the microarray hybridization.

Raw hybridization-intensity values were normalized using z-score transformation (Cheadle *et al*., 2003). Transformation of microarray data by the z-score method normalizes data internally within each array hybridization to permit pooling multiple arrays. Using z transformation, each hybridized value for a given gene is expressed as a unit of standard deviation from the mean intensity of all genes on the array, normalized to zero. Every cDNA spot is printed in duplicate on the array, therefore the z transformed value for each gene reported in Table 1 is an average of these two independent readings. The statistical significance of differences in expression of individual genes in human Schwann cells and T265 cells was tested by the z test, using the data from all experiments (six hybridizations). The values for the duplicate hybridizations for the same sample were averaged, so that the sample size in the z test represented the number of independent replicate experiments ($n = 3$).

Changes in gene expression between nhSCs and T265 cells were then calculated by subtracting the average of replicate ($n = 3$) experiments. This value is referred to as the z difference (average z in T265 cells minus the average z in nhSCs). Significance was tested using a two-tailed z-test ($z \geq \pm 2.58$; $P \leq 0.01$). The data reflect three, independent isolations of total RNA from cultured human Schwann cells derived independently from two human donors and T265 cells. Significant changes in gene expression calculated in this manner take into account the variation between replicates on a gene-by-gene basis. This method, based on z distributions, allows combining replicate experiments for a rigorous statistical analysis of the microarray data

without resorting to intensity ratios or subtraction methods to normalize differences in intensity of hybridizations from replicate experiments.

Cell culture

Adult, nhSCs were obtained using cauda equina of organ-donor patients as a peripheral nerve source. The nerves were obtained through the courtesy of Dr. Patrick Wood (Miami Project to Cure Paralysis). All procedures were carried out in full accordance with all legal and ethical guidelines. The cauda equine was removed within one hour of death and maintained at 4°C throughout shipment. All shipments were received and processing began within 12 hours of death. The procedure described by Casella (Casella *et al*., 1996) was utilized to isolate nhSCs from the nerves. Under these procedures it is apparent that the Schwann cells survive in a healthy state, and that the number of cells harvested from the nerve is equal or greater than the number of Schwann cells originally present in the nerve. The cultures of human Schwann cells are 98–99% pure, as identified by S100 staining. The adult, nhSCs had a morphology typical of Schwann cells, being bipolar and spindle shaped with elongated processes. Cells were cultured on 100 mm collagen-coated plates (BD Biosciences) in DMEM supplemented with 5% FBS and 10 ng ml^{-1} neuregulin-1β (R&D Systems). The expansion and purification procedure for obtaining nhSCs takes advantage of the selective effect of the growth factor neuregulin-1β on nhSCs over fibroblasts and other possible cellular contaminants. T265 and nhSCs were grown in the same media (not supplemented with neuregulin) for 24 hours before extracting mRNA. In these studies, the nhSCs were always utilized at passages 2 and 3.

The T265 MPNST cell line was developed by Klein in the laboratory of DeVries (Klein and DeVries, in preparation). Cells were grown in DMEM supplemented with 5% FBS. The cell line was isolated and cloned from a MPNST using the techniques outlined by Muir (Muir *et al*., 2001). The T265 cells had a doubling time of 40 hours and a pleomorphic morphology consistent with their transformed state. Cells were used between passage numbers 24 and 27.

Semi-quantitative, real time RT–PCR

RNA was extracted from cells at a density of 2×10^6 cells ml^{-1} using TRIzol reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed using random hexamers at 42°C for 1 hour and then heated to 94°C for 5 minutes in a total reaction volume of 20 μl (AMV 1st strand cDNA Synthesis Kit; Roche Diagnostics). Following RT, the reaction was diluted and used subsequently in the PCR reaction. Semi-quantitative, real-time PCR was performed on a Roche LightCycler using the Faststart DNA Master SYBR Green 1 PCR reaction mix (Roche Diagnostics). PCR reactions (20 μl were run in capillaries using either 1:10 or 1:1 dilution of the RT reaction, 0.3 μl of the appropriate PCR primers and additional $MgCl_2$ to bring a final concentration of 3.5 mM. The PCR amplification began with a 10 minute denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 58°C for 5 seconds and extension at 72°C for 10 seconds. At the end of the final cycle, PCR products were annealed at 65°C for 15 seconds and then heated slowly to 95°C at 0.1°C $second^{-1}$ under continuous fluorescence measurement to determine the melting curves of PCR products; specific PCR products melted between 80°C and 92°C. Data analysis was performed using Lightcycler Software (Roche Diagnostics) with quantification and melting-curve options. The acquired fluorescence signal was quantified by the second derivative maximum method using LightCycler data analysis software to obtain crossing point values (*Cp*) and slope. Serial dilutions of cDNA template (1:10, 1:10, 1:100 and 1:1000) were prepared and used to determine PCR efficiency. Changes in concentration of the amplified target were detected as differences in threshold cycle (Δ*Cp*) between samples and the relative expression ration (R) of a target genes were calculated based on E and Δ*Cp* of the T265 and nhSC cDNA, and expressed as a ratio to the reference housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). All RT–PCR measurements were performed in duplicate.

Immunoblotting

Total cellular protein was extracted using M-PER mammalian protein extraction reagent (Pierce) supplemented with Complete, EDTA-free protease inhibitor cocktail tablets (Boehringer). Extracts were either flash frozen in liquid N2 and stored at –80°C until needed or protein concentration was determined immediately using Coomassie plus protein assay reagent (Pierce). An equal concentration of protein extract was used and 5× SDS sample buffer added prior to loading 4–20% or 8–16% Tris-glycine SDS gradient gels (Invitrogen). Unless otherwise stated, immunoblotting was performed in duplicate. Equal loading of protein was confirmed in several ways. Protein transfer onto PVDF membrane was checked by staining of the gel after transfer at 4°C either overnight or for 3 hours. Additionally, protein transferred onto the PVDF membrane was stained by Ponceau S to check for equal transfer and loading before Western blotting. Western blot analyses of total protein extract were carried out with anti-p38 (Cell Signalling), anti-phospho-p38 (Cell Signalling), anti-MEK1/2 (Cell Signalling), anti-phospho-MEK1/2 (Cell Signalling), anti-ERK1/2 (Cell Signalling), anti-phospho-ERK1/2 (Cell Signalling), anti-retinoblastoma (Santa Cruz), anti-phospho-retinoblastoma (Cell Signalling), anti-p21 (Santa Cruz), anti-p27 (Santa Cruz), anti-cyclin D1 (Santa Cruz), anti-cyclin D3 (Santa Cruz), anti-EGFR (Santa Cruz), anti-A2a (Alamone), anti-A2b (Alamone) and anti-P2Y2 (Alamone), using the ECL enhanced chemiluminescence detection system (Pierce).

RESULTS

In this mRNA-profiling study we compared global mRNA abundance profiles in T265 cells and nhSCs. Total RNA was extracted from adult, non-myelinating human Schwann cells and a Schwann-cell MPNST-derived cell line (T265) grown in cell culture. The cluster analysis showed remarkable consistency in gene expression across different donors, between 2–3 passages of nhSCs from the same donor, across different culture preparations of T265 cells, and between duplicates of the same sample (Fig. 1). Genes with variable expression or that were differentially expressed on the basis of either passage number or donor origin were screened out by the statistical analysis identifying those genes that differ consistently between normal and T265 cells ($P \leq 0.01$) by a 2-tailed z test ($n = 3$).

The results of this microarray screen of three independent microarray screens were validated at the mRNA and protein levels for several genes of interest that were selected because of their high abnormal expression or possible relevance to tumor development. Validation by RT–PCR was performed on mRNA extracted from D1, validation by Western blot was performed on protein extracts derived from Schwann cells from D2. There was good agreement between microarray screens, RT–PCR and Western blot data.

nhSCs

The expression levels of all genes that were differentially expressed in normal and T265 cells were ranked according to z score, based on the average of all three experimental replicates. The 100 genes expressed most highly in human Schwann cells in culture were selected for analysis (supplemental material 2, http://journals.cambridge.org/jid_NGB). All are expressed with a z value of ≥ 1.5. These results reveal transcripts of several genes not previously reported as being expressed in Schwann cells, such as the $A2_A$ purinergic receptor. As we report elsewhere, further analysis confirms that the $A2_A$ receptor is expressed in Schwann cells; where it signals through the mitogen-acitvated protein kinase (MAPK) pathway to regulate Schwann cell proliferation in culture (Stevens *et al*., 2004). In addition, transcripts for several other categories of genes mediating functions not typically associated with Schwann cells are found by this analysis, indicating intriguing areas for further research.

As a first step in organizing and interpreting this large dataset of gene-expression data, the most highly expressed genes from nhSCs were analyzed using the Pubmatrix multiplex bioinformatics software (Becker *et al*., 2003). This literature-mining tool was used to cross reference each identified gene against published studies indexed in PubMed for associations with the words 'Schwann, Glia, NF1 or MPNST' appearing in the title, abstract and key-word list. Of these 100 most highly expressed genes in nhSCs, 88 appear not to have been identified previously in SCs. The results of this analysis are provided as a frequency matrix table in supplemental material 2. By linking each gene to the literature, this analysis will facilitate interpretation of the data from nhSCs and indicate genes of interest for further studies designed to confirm their expression and function.

Abnormal gene expression in T265 cells

Hundreds of mRNA transcripts were identified whose abundance differs significantly ($P \leq 0.01$) between these two cell types, 21 of which were confirmed by semi-quantitative RT–PCR. Further verification of the deregulation of a subset of genes was confirmed by Western analyses. Because of the large volume of data, results for all of the genes on the microarray that meet our arbitrary z-test threshold of $P \leq 0.01$ are provided as supplemental material 1 (http://journals.cambridge.org/jid_NGB). Results are synthesized below by considering these genes in groups of either known molecular function or on the putative ability of the gene to confer a phenotype that would potentiate the development of MPNSTs.

Cell adhesion molecules

Cell adhesion molecules play a dominant role during several stages of the tumorigenic process. This can be manifest in several ways, including switching types of extracellular matrix (ECM) receptors (integrins) to promote pro-growth signals in highly proliferative cells. Deregulation of expression of adhesion molecules also plays a role in mid-stage events in cancer, such as angiogenesis and tissue invasion and, finally, metastasis. Changes in the expression of integrins and cadherins might contribute to the progression, invasion and metastasis of MPNSTs. One of the most highly deregulated genes in the MPNST Schwann cell line T265 was integrin alpha V (Table 1). This receptor is known to promote cell adhesion and activate signaling pathways that inhibit cellular proliferation (Cruet-Hennequart *et al*., 2003). Downregulation of the integrin alpha V gene in this cancer cell line has not been described previously in NF1, and this might contribute significantly to tumorigenesis by promoting motility and multiple pro-growth signals. Another downregulated integrin in the MPNST cell line is integrin β1 (z test = −2.46, which just falls outside our arbitrary significance cutoff, but is included here), which is known to be involved in facilitating Schwann cell–axon interactions during myelination (Feltri *et al*., 2002). One important feature of T265 cells is their inability to interact with axons (G.H. DeVries, unpublished data). Downregula-tion of integrin β1 in T265 cells might play a role in the disruption of this process. Similarly, expression of other integrins appears to be deregulated in T265 cells (Table 1), and these receptors might contribute significantly to the process of tumorigenesis and malignancy. Several cadherin mRNAs were also deregulated in the T265 cell line, including upregulation of cadherin 11, cadherin 12 and cadherin 19. In a similar manner, cadherin 2, cadherin 3 and neural cell adhesion molecule were downregulated significantly in T265 cells. Deregulation of these receptors has been implicated in various types of cancers (Li *et al*., 2003;Hendrix *et al*., 2003), but has never been identified as having a possible role in NF1. Other genes already known to be involved in the progression of other cancer types, such as CD9 (Funakoshi *et al*., 2003), are also deregulated significantly in T265 cells (Table 1). CD9 has been implicated in lymph node metastasis (Kusukawa *et al*., 2001) and in the invasive and metastatic phenotype of small lung cell cancer (Miyake *et al*., 1999). Deregulation of this gene in a range of cancers might indicate a role for CD9 in the development and maintenance of MPNSTs. Several intracellular signaling molecules associated with integrin signaling were deregulated, in addition to many key, cell-surface receptors. Notable

among these is the integrin-linked kinase, which is upregulated significantly. This kinase has multiple roles in apoptosis and cellular proliferation (Persad and Dedhar, 2003) and, therefore, might have a similar role in MPNST development.

Apoptosis

Mutations in the p53 gene have been identified in MPNSTs but not in benign neurofibromas, indicating that the p53-mediated pathway is involved in tumor development (Legius *et al*., 1994; Menon *et al*., 1990). Altered expression of ligand/receptor pairs, such as insulin-like growth factor 1/2 (IGF1/IGF2) and the receptor IGF-1R, interleukin 3 (IL-3) and IL-3R, FAS and the FAS receptor, and tumor necrosis factor α (TNF-α) and TNF-R1, have key roles in the sensing of the apoptosis (Bhardwaj and Aggarwal, 2003). Deregulation of the caspase family of proteases is crucial if a cell is to avoid apoptosis, because these are the ultimate effectors of apoptosis. Several of these proteases show deregulated expression in T265 cells (Table 1). Another gene of note whose regulation is deregulated significantly in T265 cells is the CD27-binding protein, SIVA-1. This pro-apoptotic protein was thought to be expressed only in T cells (Xue *et al*., 2002). However, it appears to be expressed moderately in normal Schwann cells and is highly downregulated in T265 cells. Transcript analysis in T265 cells has indicated the deregulation of multiple facets of the apoptosis signaling pathway (Table 1 and supplemental material 1). Deregulation of these mRNA transcripts in T265 cells might allow escape of apoptotic mechanisms by perturbation of multiple controlling points in this crucial cellular defense strategy.

Cell cycle

Insensitivity to antigrowth signals achieved by perturbation of key, cell cycle-control points is a characteristic that virtually all cancer cells acquire early in the tumorigenic process. Loss-of-function of the retinoblastoma protein (pRB) pathway plays a significant role in the development of most human cancers, as a deregulated pRB pathway results in aberrant cell proliferation and loss of control of apoptotic mechanisms. The abundance of a subset of key, cell-cycle regulators, including components of this pathway has been studied in MPNSTs and neurofibromas. These studies have revealed a key role for the cyclin-dependent kinase inhibitor, CDKN1B (p27, Kip1), in the malignant transformation of neurofibromas (Kourea *et al*., 1999). We have detected a large group of cell cycle-regulatory proteins whose abundance is deregulated in the T265 cell line. The differences between the two cell types are consistent with a deregulated cell cycle in T265 cells. Most notable was an increase in several cyclin-dependent kinases, including, CDK2-4, and CDK9. Surprisingly, the transcripts for a group of CDK inhibitors, CDKN1A (p21), CDKN2A (p16) and CDKN1C (p57), were higher in the T265 cell line. We have attempted to clarify this issue by examining the protein concentrations of p21, p27, cyclin D1 and cyclin D3, and the phosphorylation state of pRB by Western analyses. The levels of p21 and p27 protein were lower in the T265 line (Fig. 2B), and pRB was hyperphosphorylated (Fig. 2A). Cyclin D1 protein (CCND1) is upregulated highly in MPNST (in agreement with the microarray transcript data). Cyclin D3 (CCND3) mRNA is upregulated, as shown by semi-quantitative RT–PCR, in agreement with the cyclin D3 protein concentration determined by Western analyses (Fig. 2C). In addition, the mRNA transcript levels of CDK2 and CDK4 were abnormally high in T265, which might indicate an abundance of available kinase that can phosphorylate pRB in the absence of suitable inhibitors such as p21 and p27. These data are not unexpected, given the known activities of these enzymes during the cell cycle, but they are novel in the context of NF1 when considering the hyperproliferative phenotype of the T265 cells compared to nhSCs. Hyperphosphorylation of pRB is a common occurrence in a range of malignancies and the data presented here are consistent with previous data implicating deregulation of this pathway as a factor in MPNST progression.

The expression of several other key cell cycle regulators altered in MPNSTs, including CKS1B and CDC20, which are both strongly up regulated in T265 cells (Table 1). Over-expression of these transcripts in T265 cells is likely to have a profound effect on control of mitosis, perhaps leading to the gross chromosomal rearrangements observed in MPNST. In addition, the protein kinase CDC7, which is involved in regulating coupling cell-cycle regulation and genome duplication, is overexpressed highly in T265 cells. This is consistent with data implicating overexpression of CDC7 in hyper-proliferation and neoplastic transformation in a range of tumor types (Hess *et al*., 1998).

ECM

Changes in ECM proteins and an increase in secreted proteases were detected in the MPNST cell line, which is consistent with increased motility and, possibly, related to the tumorigenic process. An increase in collagen type III and a decrease in type XI was observed. In addition, we observed marked upregula-tion of fibronectin mRNA in T265 cells. Fibronectin has a role in Schwann cell adhesion and might play a key role in Schwann cell motility, attachment and metastasis in the context of MPNSTs. SPARC is an ECM protein that is produced by Schwann cells that exhibits antiangiogenic properties in neuroblastoma (Chlenski *et al*., 2002). In T265 cells, we have shown that SPARC mRNA is downregulated significantly, determined by microarray (Table 1) and semi-quantitative RT–PCR (Table 2). This potent anti-angiogenic signal is likely to give MPNSTs an advantage in either maintaining or initiating angiogenesis, a process that is crucial for tumor growth.

Growth factor receptors

Many growth factor receptors are downregulated in the T265 cell line, a notable exception being fibroblast growth factor (FGF) receptors 2–4. The FGF2 receptor might play a role in MPNST proliferation and malignancy, evidence for this comes from work indicating that neurofibrosarcoma-derived (T265) Schwann cell proliferation is increased two-fold by FGF2 *in vitro* (Badache and De Vries, 1998). The epidermal growth factor receptor (EGFR) has been implicated in malignancy in NF1 (DeClue *et al*., 2000), as have downstream targets of EGFR such as BLBP (Miller *et al*., 2003). We have shown significant deregulation of these components in T265 cells (Table 2).

Growth factors

Deregulated production of growth factors has obvious advantages in the progression of the tumorigenic process. mRNA transcripts for bone morphogenetic protein 2 (BMP2), FGF1, FGF2, FGF5, IGF2, transforming growth factor α (TGFα), leukemia inhibitory factor and IL-1β were all upregulated in T265 cells (supplemental material 1). Although the significance of the overexpression of several growth factors to the progression of MPNSTs is unclear, Schwann cells appear to express many of these molecules at relatively high levels. Endothelin (EDN1) is strongly upregulated in T265 cells, as determined by microarray and semi-quantitative RT–PCR (Tables 1 and 2). This growth factor is known to stimulate proliferation in various cell cancer types (Nelson *et al*., 2003) and has been implicated in the regulation of Schwann cell morphology and proliferation (Berti-Mattera *et al*., 2001). The effect of upregulation of EDN1 on MPNST-derived T265 cells or other cell types present in MPNSTs is unknown, but the available evidence indicates a role in the regulation of proliferation and, therefore, a positive contribution to MPNST development. By contrast, brain-derived neurotrophic factor, BMP1, FGF4, platelet-derived growth factor and TGFβ were downregulated in T265 cells (supplemental material 1), the significance of which is unclear at present.

Neurotransmitter receptors

Of the 23 neurotransmitter receptors determined to be significantly deregulated by microarray analyses, 19 were downregulated. Several isoforms of purinergic receptors were downregulated from microarray data, including adenosine $A2_A$ and A3 receptors and the nucleotide receptor P2Y2. Western analyses of adenosine $A2_A$ and $A2_B$ receptors indicated a small decrease in the expression of both proteins (Fig. 3B) in T265 cells, consistent with the mRNA transcript data. We also analyzed P2Y2 by Western analyses and observed a small decrease in T265 cells, consistent with the decrease in mRNA abundance for this gene (Fig. 3C). This data might be significant in the context of NF1 and MPNSTs, as adenosine receptors and a subset of purinergic receptors have been implicated as having a key role in Schwann cell proliferation. Interestingly, one of the few receptors up regulated is a different purinergic receptor, P2Y6. Recently, this gene has been implicated in a mechanism protecting astrocytoma cells against TNF-induced apoptosis (Kim *et al*., 2003) and it might play a similar role in T265 cells, thus, allowing apoptotic escape in MPNST. Downregulation of several isoforms of glutamate receptors and acetylcholine receptors was observed (Table 1). The AMPA1 receptor (GRIA1) was upregulated, determined by microarray (Table 1) and validated by semi-quantitative RT–PCR (Table 2). The significance of these findings in the context of Schwann cell biology and MPNSTs is unclear at present.

Signal transduction

As expected, many genes that are involved directly in intracel-lular signaling appear to be alternatively regulated in T265 cells, including MAPK signaling, signaling from integrins, and the protein kinase C (PKC) and PKA pathways. Very specific changes in the mRNA abundance of many activator and components of MAPK signaling indicated the potential for differential activation of two such signaling pathways, the ERK1/2 MAPK pathway and the p38 MAPK pathway. Confirmation of this difference in T265 cells using phospho-specific antibodies and Western blotting, demonstrates that signaling through the p38 MAPK pathway is downregulated in MPNST-derived Schwann cells (Fig. 4B), while there is strong signaling (presumably from uncontrolled RAS activity in the context of NF1) through ERK1/2 and MEK1/2 (Fig. 4A). Phosphorylation of p38 MAPK has been associated with inhibition of proliferation of normal Schwann cells in response to application of ATP (B. Stevens and R.D. Fields, unpublished), although the significance of this finding in the context of NF1 awaits further investigation.

Transcription factors

Perturbation of the abundance of mRNA encoding transcription factors would have a major influence on cell function and disease by altering a wide range of proteins, including structural genes, key modifiers of signaling pathways, receptors, growth factors, DNA modifying enzymes, differentiation factors, angiogenesis initiating signals, and factors allowing tissue invasion and metastasis. We have found that many genes in this category are deregulated, including some already implicated in other malignancies, and might have a role in the initiation and development of MPNSTs (Table 1, Table 2 and supplemental material 1). We have confirmed the abnormal expression of transcription factors by semi-quantitative RT–PCR and the data from these analyses is consistent with the changes observed in the microarray dataset (Table 1 and Table 2). EGR1 (Krox-24) is highly upregulated in T265 cells and is known to have multiple roles in many other types of cancer (Adamson and Mercola, 2002). Interestingly, overexpression of EGR1 results in transactivation of the fibro-nectin (FN1) gene and EGFR, with subsequent positive effects on tumor development (Alexander *et al*., 2002;Kobayashi *et al*., 2002;Nishi *et al*., 2002). We also observe an increase in the mRNA abundance of the FN1 gene in T265. This might be an important factor in MPNST development as in other malignancies (Engbring and Kleinman, 2003). Other deregulated transcription factors we have

validated by semi-quantitative RT–PCR include ATF2 and CREB1B, both of which are downregulated in T265 cells. The functional significance of this is unclear, but the ATF2 transcription factor is part of the transcriptional output resulting from signaling through the p38 MAPK pathway. This pathway appears to be shutdown in T265 cells, therefore it might not be too surprising that the expression of ATF2 is also deregulated. Another transcription factor whose expression was altered in T265 cells is the p53-regulated gene DDA3; this was also confirmed by semi-quantitative RT–PCR (Table 1 and Table 2). Upregulation of the DDA3 gene indicates a deregulated, TP53-controlled signaling pathway because transcription of this gene is controlled directly by TP53 and its homolog p73 (Hsieh *et al*., 2002). However, the targets of DDA3 itself are unknown and its contribution to Schwann cell proliferation and MPNST development is not understood.

CONCLUSIONS

- 955 genes have been identified that are expressed abnormally in MPNST-derived T265 cells compared to nhSCs in culture.
- Within this group of genes, the 100 most highly expressed genes in nhSCs were described. A search of the literature indicates that expression of as many as 88 of these genes have not been reported previously in Schwann cells.

DISCUSSION

Cell culture is an essential tool in experimental studies of NF1, but to design and interpret these studies it is essential to know how the cell line differs from nhSCs in culture. Approximately 900 genes were deregulated in T265 cells (Table 1 and supplemental material 1), z-test, $P \leq 0.01$ ($z \geq \pm 2.58$) was chosen arbitrarily as a manageable dataset for analysis and presented here. Using the $P \leq 0.01$ criteria, 14% of cDNAs were upregulated significantly, 13% were downregulated significantly and 73% were unchanged in the T265 cell line, using the human focused 4k array cDNA set. Experimental studies on any one aspect of NF1 must take into consideration the other abnormally expressed genes in T265 cells that have been identified in this study, which might interact with the mechanism under investigation.

It is clear that many of the normal regulatory circuits involved in control of cell growth and division are perturbed in T265 cells. Some of these might be specific to this cellular model of NF1, but multiple abnormalities are to be expected in tumor cells. In addition to genes previously known to be deregulated in NF1, such as EGFR (DeClue *et al*., 2000), FABP7 (Miller *et al*., 2003) and elements of the RAS signaling pathway (Ingram *et al*., 2001), many genes that are associated with the multiple stages of tumor pathogenesis have been found to be expressed abnormally in T265 cells by this gene expression-profiling method. These include cell cycle-regulatory proteins, adhesion and ECM molecules, oncogenes, tumor suppressor genes, growth factor receptors, intracellular signaling molecules and regulators of transcription. This cell line was derived from a patient with a highly malignant peripheral nerve sheath tumor. Considering the multiple biological processes involved in malignancy, such widespread abnormalities in gene expression might be required for the transition from a ‘normal’ state of growth to a malignant, life-threatening tumor.

To confirm changes observed by microarray analyses, a large subset of mRNAs were subjected to semi-quantitative RT–PCR (LightCycler) analyses (Table 2). In total, 21 genes have been analyzed by semi-quantitative RT–PCR. Validated transcripts include genes already implicated in MPNST, key cell-surface receptors, intracellular signaling molecules that are implicated as having a role in other cancer types, and abnormally expressed genes that might be involved in MPNST development and progression on the basis of this global gene-expression analysis. The validation of array data was taken a stage further by analysis of the protein products of 16

transcripts by Western blotting. The protein abundance data for several products, as measured by Western analysis, was in general agreement with mRNA abundance data generated from microarray and RT–PCR. Array data indicated that many signal transduction pathways and the cell-cycle apparatus was deregulated, therefore the phosphorylation state of several key components of selected signaling pathways was investigated. This provides insights into the cell cycle and signal transduction pathways that are disrupted in T265 cells.

The 100 most highly expressed genes in nhSCs in culture that were differentially expressed in T265 cells revealed a number of genes not previously identified in Schwann cells, based on a Pubmatrix search of the literature. Although, the data represents a subset of the expression of the ~4000 genes assayed during this study, it is a valuable resource that is not available currently to researchers in the neuroscience community.

Aberrations in normal gene expression in T265 cells can result from three general sources: (1) genetic mutations in the tumor cells; (2) secondary mutations that may develop in any cell line over time; (3) changes in expression level of specific mRNAs that are secondary to genetic mutation either *in situ* or *in vitro*, such as cellular responses to changes in growth factor secretion. Regardless of which of these factors influences the abnormal expression of any given gene, it is essential that the differences in specific mRNAs in T265 cells are known in order to properly design and interpret experimental studies of NF1 using T265 cells in culture. This study provides this essential information, and it opens new insights into the pathways and molecular mechanisms that might be important in Schwann cell biology, and relationships to MPNST development and progression in the context of NF1.

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We are grateful to Dr. David M. Levi, Director of the Life Alliance Organ Recovery Agency, Department of Surgery, University of Miami School of Medicine for the human cauda equina. We would also like to thank Chris Cheadle for help with statistics, Bill Wood III for preparation of the microarrays and Beth Stevens for helpful discussions.

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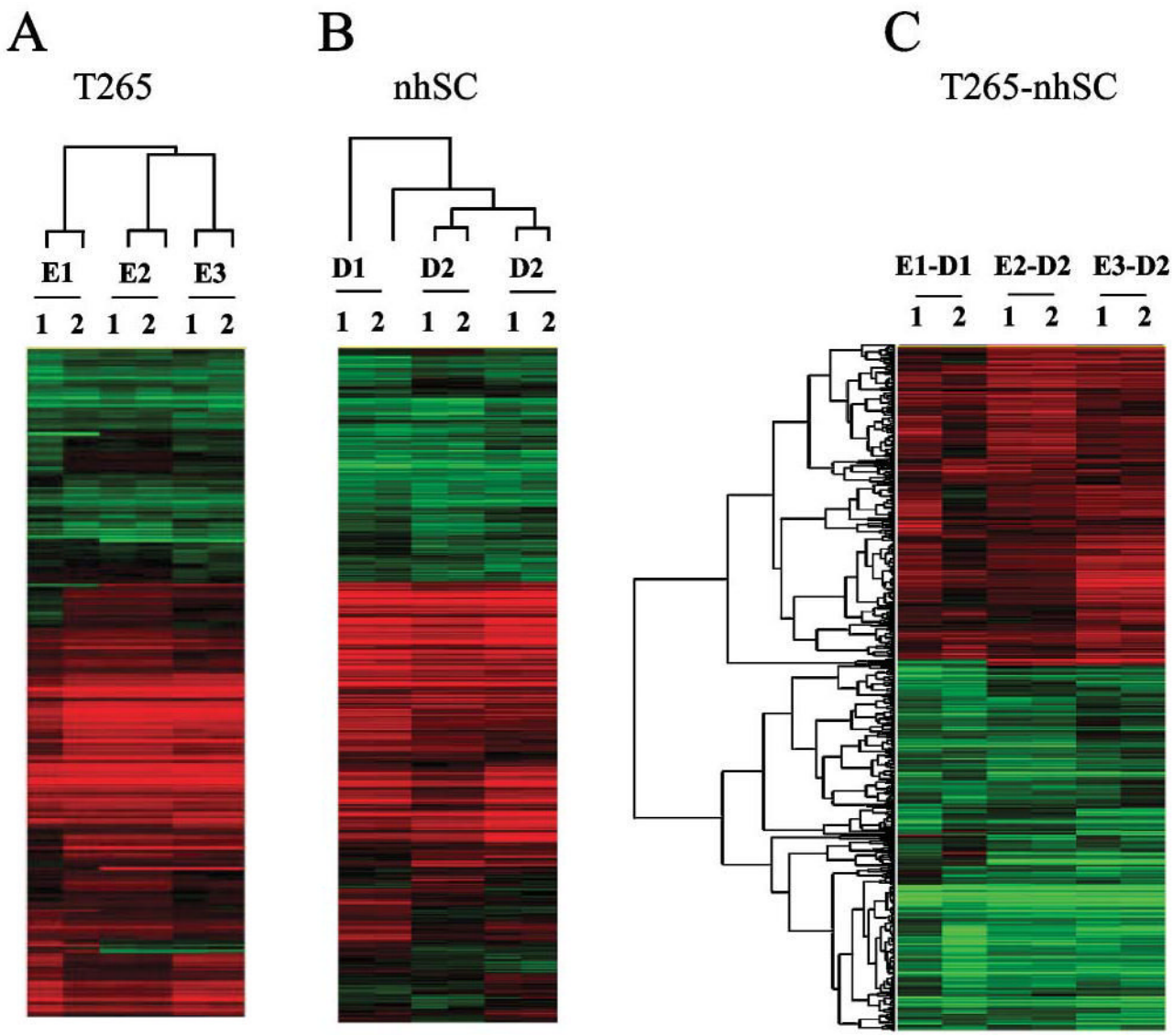

Fig. 1. Hierarchical cluster analysis of microarray data showing replication between nhSC donors and T265 cell platings, and expression differences between nhSCs and T265 cells.

Individual genes are represented in rows and hybridizations in columns. nhSCs were derived from two donors (D1 and D2) and screened by microarray in three independent experiments. Each experiment included a duplicate hybridization (1 and 2) of the same sample (technical replicate); these exhibited negligible variation (>5% of the genes showed discrepancies in expression between the technical replicates). (A) Cluster analysis of T265 gene expression (z-score) showed good agreement among experiments (E1–E3) and between technical replicates (1, 2). (B) Similarly in nhSCs, good agreement was observed between technical replicates (1, 2) and between donors (D1, D2). Cells used in the two experiments from D2 were expanded 2 and 3 passages respectively. (C) Analysis of the z-differences of 955 genes whose expression changed significantly ($P \leq 0.01$) between nhSCs and T265 cells. The identity and expression level of 955 genes is provided in a table as supplemental material 1.

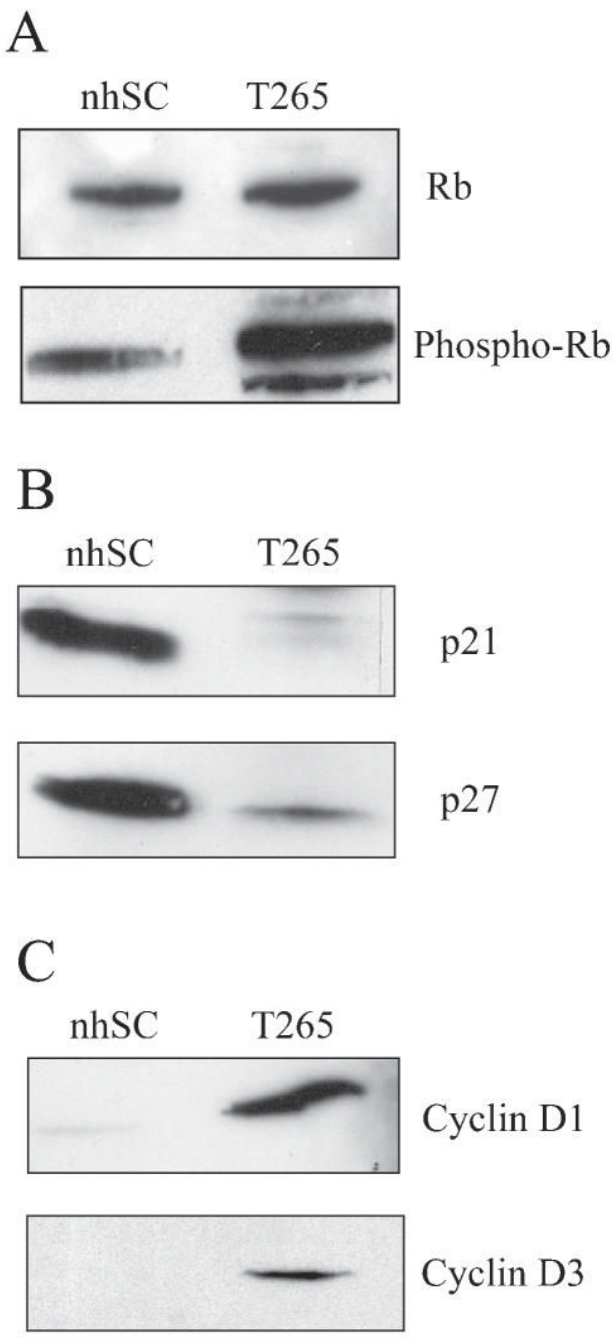

Fig. 2. Western analysis of cell cycle-regulatory proteins in MPNST-derived Schwann cells (T265) and nhSCs.

The cell cycle G1-S checkpoint apparatus is deregulated in T265 cells. Cell lysates were prepared from nhSCs and T265 cells, 35 μg of total cellular lysate was added to each lane and equal protein loading was confirmed by Coomassie staining of gels or staining of PVDF membranes. (A) pRB is hyperphosphorylated in T265 cells. (B) Cyclin dependent kinase inhibitors p21 and p27 are strongly downregulated in T265 cells. (C) Cyclins D1 and D3 are upregulated in MPNST-derived Schwann cells (T265).

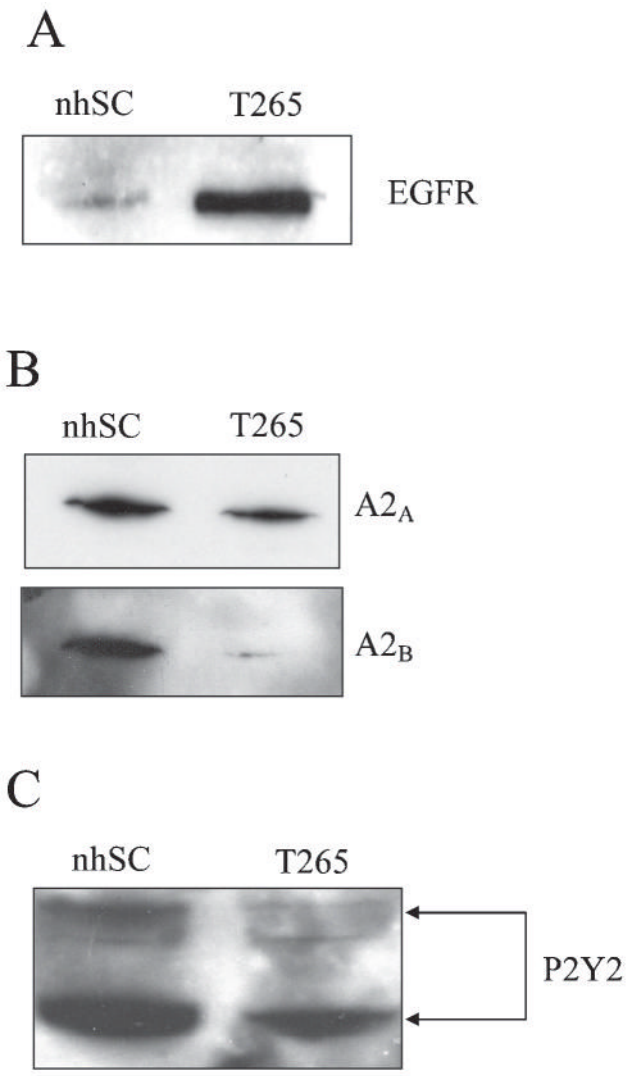

Fig. 3. Western analysis of cell surface receptors in nhSCs and T265 cells.

EGFR is upregulated in T265 cells. Adenosine receptors $A2_A$ and $A2_B$ are downregulated in T265 cells. The P2Y2 purinergic receptor is downregulated in T265 cells. Total cell lysates were prepared and 35 μg added to each lane, equal protein loading was confirmed by Coomassie staining of the gel. (A) EGFR protein is upregulated in T265 cells. (B) $A2_A$ and $A2_B$ adenosine receptors are downregulated in T265 cells. (C) The P2Y2 receptor is downregulated in T265 cells.

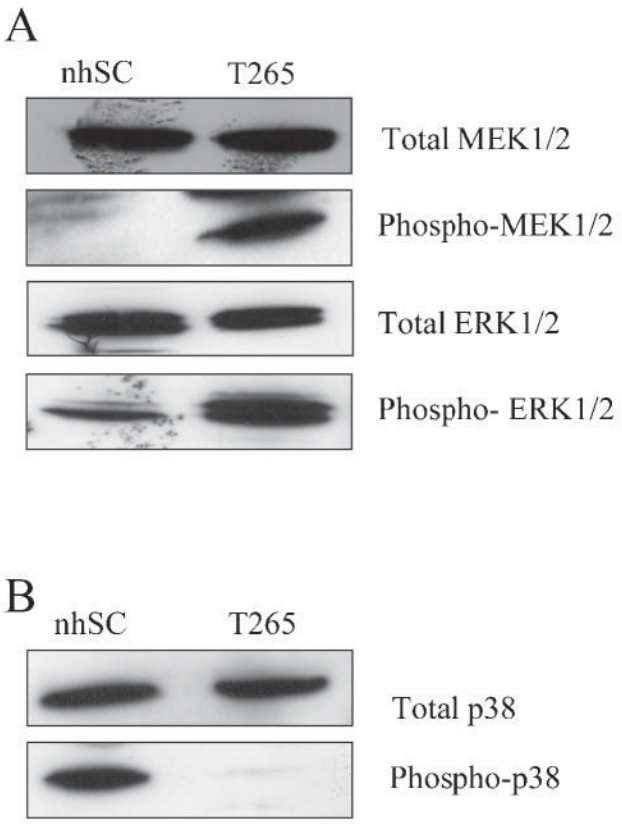

Fig. 4. Western analysis of MAPK signal transduction pathways in T265 cells and nhSCs.

p38 MAPK pathway and ERK1/2 MAPK pathways are differentially activated in the T265 MPNST-derived cell line and nhSCs. Cell lysates were prepared from human Schwann cells and T265 cells, 35 μg of lysate was added to each lane and equal loading was confirmed by either Coomassie staining of gels or loading control antibodies. (A) Phosphorylation of MEK1/2 and ERK1/2 is reduced in nhSCs compared with MPNST. (B) Phosphorylation of p38 MAPK is undetectable in T265 cells compared with nhSCs.

Table 1

Selection of deregulated genes in MPNST-derived Schwann cell line T265, meeting the criteria $P \leq 0.01$ (±2.58 Z-Test) from three independent microarray experiments. Differences are given as a Z Difference (Z diff = $Z_{T265} - Z_{hSC}$) between T265 cells and nhSCs, significance of this difference in mRNA abundance is given as a Z-Test value (see Methods). Multiple copies of the same cDNA on the array are numbered consecutively (superscript). The full set of genes meeting the significance criteria $P \leq 0.01$ can be viewed in the supplemental material.

Gene name	Gene symbol	Z-test (z?)	Z diff = $Z_{T265} - Z_{hSC}$
			-2.5 -1.5 0 1.5 2.5
Cell cycle			
CDC28 protein kinase 1B	*CKS1B*	7.50	
CDC20 cell division cycle 20 homolog (*S. cerevisiae*)[1]	*CDC20*	4.68	
CDC20 cell division cycle 20 homolog (*S. cerevisiae*)[2]	*CDC20*	3.70	
CDC7 cell division cycle 7-like (*S. cerevisiae*)	*CDC7L1*	4.87	
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*CDKN1A*	3.66	
cyclin-dependent kinase 4	*CDK4*	3.79	
CDC6 cell division cycle 6	*CDC6*	3.77	
tumor protein p53 (Li-Fraumeni syndrome)[1]	*TP53*	3.43	
tumor protein p53 (Li-Fraumeni syndrome)[2]	*TP53*	3.30	
cyclin A1	*CCNA1*	−5.00	
ribosomal protein S6 kinase, 70kDa, polypeptide 2	*RPS6KB2*	−6.75	
SFRS protein kinase 1	*SRPK1*	−7.41	
Apoptosis			
cytochrome C	*HCS*	10.05	
caspase 3, apoptosis-related cysteine protease[1]	*CASP3*	3.97	
caspase 3, apoptosis-related cysteine protease[2]	*CASP3*	3.20	
p53-regulated DDA3[1]	*DDA3*	6.48	
p53-regulated DDA3[2]	*DDA3*	5.00	
p53-regulated DDA3[3]	*DDA3*	4.02	
death-associated protein	*DAP*	4.01	
CD14 antigen	*CD14*	−5.06	
nuclear factor of kappa light polypeptide gene enhancer in B cells (p105)[1]	*NFKB1*	−6.65	
nuclear factor of kappa light polypeptide gene enhancer in B cells (p105)[2]	*NFKB1*	−4.26	
transforming growth factor, beta 1[1]	*TGFB1*	−9.29	
transforming growth factor, beta 1[2]	*TGFB1*	−2.89	
BCL2-related protein A1	*BCL2A1*	−8.23	
CD27-binding (Siva) protein	*SIVA*	−11.83	
Adhesion/cell matrix			
matrix metalloproteinase 1	*MMP1*	7.17	
fibronectin 1[1]	*FN1*	7.62	
fibronectin 1[2]	*FN1*	7.05	
fibronectin 1[3]	*FN1*	6.02	
fibronectin 1[4]	*FN1*	5.74	
fibronectin 1[5]	*FN1*	5.69	
fibronectin 1[6]	*FN1*	5.56	
cadherin 11, type 2, OB-cadherin (osteoblast)	*CDH11*	5.77	
catenin (cadherin-associated protein), alpha 1 (102kDa)[1]	*CTNNA1*	5.32	
collagen, type III, alpha 1[1]	*COL3A1*	4.96	
collagen, type III, alpha 1[2]	*COL3A1*	2.92	
collagen, type III, alpha 1[3]	*COL3A1*	2.71	
selectin E (endothelial adhesion molecule)[1]	*SELE*	3.05	
integrin beta 1 (fibronectin receptor beta polypeptide)*	*ITGB1**	−2.46*	
CD9 antigen (p24)[1]	*CD9*	−3.53	
CD9 antigen (p24)[2]	*CD9*	−5.17	
cadherin 2, type 1, N-cadherin (neuronal)[1]	*CDH2*	−6.01	
cadherin 2, type 1, N-cadherin (neuronal)[2]	*CDH2*	−3.05	
collagen, type XI, alpha 1[1]	*COL11A1*	−5.72	
collagen, type XI, alpha 1[2]	*COL11A1*	−3.36	
peripheral myelin protein 22	*PMP22*	−6.62	
integrin cytoplasmic domain-associated protein 1	*ICAP-1A*	−6.48	
integrin, alphaV (vitronectin receptor, alpha polypeptide, antigen CD51)	*ITGAV*	−19.41	
Signal transduction			
src family associated phosphoprotein 1[1]	*SCAP1*	6.69	
src family associated phosphoprotein 1[2]	*SCAP1*	5.07	
mitogen-activated protein kinase kinase[1]	*MAP2K1*	3.57	
caveolin 1, caveolae protein, 22kDa[1]	*CAV1*	2.59	
WD40 protein Ciao1	*CIAO1*	2.59	
adaptor-related protein complex 2, sigma 1 subunit	*AP2S1*	−9.81	
Ras and Rab interactor 1	*RIN1*	−10.31	

Gene name	Gene symbol	Z-test (z?)	Z diff = $Z_{T265} - Z_{ksc}$
Transcription			-2.5 -1.5 0 1.5 2.5
basic transcription factor 3[1]	*BTF3*	9.75	
basic transcription factor 3[2]	*BTF3*	7.51	
basic transcription factor 3[3]	*BTF3*	3.48	
p53-regulated DDA3[1]	*DDA3*	6.48	
p53-regulated DDA3[2]	*DDA3*	5.00	
p53-regulated DDA3[3]	*DDA3*	4.02	
early growth response 1	*EGR1*	2.92	
E2F transcription factor 5, p130 binding	*E2F5*	5.59	
DNA damage-inducible transcript 3	*DDIT3*	2.92	
CREB binding protein	*CREBBP*	3.02	
activating transcription factor 2[1]	*ATF2*	−5.95	
activating transcription factor 2[2]	*ATF2*	−3.22	
distal-less homeo box 5	*DLX5*	−5.69	
aryl hydrocarbon receptor nuclear translocator-like	*ARNTL*	−8.12	
Structural genes			
destrin (actin depolmerizing factor)	*DSTN*	5.04	
axonal transport of synaptic vesicles	*ATSV*	3.17	
tubulin, gamma 1	*TUBG1*	3.04	
glial fibrillary acidic protein	*GFAP*	−2.97	
amyloid beta (A4) precursor protein[1]	*APP*	−5.52	
amyloid beta (A4) precursor protein[2]	*APP*	−4.18	
gelsolin[1]	*GSN*	−7.25	
gelsolin[2]	*GSN*	−7.86	
actin, alpha 2	*ACTA2*	−9.60	
Growth factors			
connective tissue growth factor	*CTGF*	6.80	
interleukin 1, beta[1]	*IL1B*	5.41	
interleukin 1, beta[2]	*IL1B*	5.03	
interleukin 1, beta[3]	*IL1B*	3.75	
insulin-like growth factor 2[1]	*IGF2*	4.62	
insulin-like growth factor 2[2]	*IGF2*	4.37	
brain-derived neurotrphic factor	*BDNF*	−6.19	
transforming growth factor, beta 1[1]	*TGFB1*	−2.89	
transforming growth factor, beta 1[2]	*TGFB1*	−9.29	
Growth factor receptors			
fibroblast growth factor receptor 4[1]	*FGFR4*	8.90	
fibroblast growth factor receptor 4[2]	*FGFR4*	5.69	
fibroblast growth factor receptor 2	*FGFR2*	4.23	
ephrin receptor A7	*EPHA7*	−7.80	
ephrin receptor B1	*EPHB1*	−10.68	
interleukin 10 receptor, beta	*IL10RB*	−17.48	
Other receptors			
potassium channel, subfamily K, member 1	*KCNK1*	3.48	
adenosine A2a receptor	*ADORA2A*	−5.84	
purinergic receptor P2Y, G-protein coupled, 2	*P2RY2*	−5.79	
cholinergic receptor, nicotinic, epsilon polypeptide[1]	*CHRNE*	−5.89	
cholinergic receptor, nicotinic, epsilon polypeptide[2]	*CHRNE*	−8.78	
glutamate receptor, ionotropic, kainate 1[1]	*GRIK1*	−3.20	
glutamate receptor, ionotropic, kainate 1[2]	*GRIK1*	−3.20	
glutamate receptor, ionotropic, kainate 1[3]	*GRIK1*	−19.08	
Immune function			
complement component 1, r subcomponent	*C1R*	3.98	
CD8 antigen, alpha polypeptide (p32)[1]	*CD8A*	−3.27	
CD8 antigen, alpha polypeptide (p32)[2]	*CD8A*	−3.35	
CD4 antigen (p55)	*CD4*	−7.38	
major histocompatability complex, class II, DO alpha[1]	*HLA-DOA*	−8.01	
major histocompatability complex, class II, DO alpha[2]	*HLA-DOA*	−12.67	
major histocompatability complex, class I, C[1]	*HLA-C*	−14.58	
major histocompatability complex, class I, C[2]	*HLA-C*	−20.92	
major histocompatability complex, class II, DQ beta[1]	*HLA-DQB*	−10.74	
major histocompatability complex, class II, DQ beta[2]	*HLA-DQB*	−16.27	
major histocompatability complex, class II, DQ beta[3]	*HLA-DQB*	−27.61	
major histocompatability complex, class I, A	*HLA-A*	−22.88	

*Integrin B1 is included despite having a Z-Test value of −2.46, therefore lies slightly outside our arbitary threshold cutoff of +/−2.58.

Gene name	Gene symbol	Z-test (z?)	Z diff = $Z_{T265} - Z_{kSC}$
Transcription			-2.5 -1.5 0 1.5 2.5
basic transcription factor 3[1]	*BTF3*	9.75	
basic transcription factor 3[2]	*BTF3*	7.51	
basic transcription factor 3[3]	*BTF3*	3.48	
p53-regulated DDA3[1]	*DDA3*	6.48	
p53-regulated DDA3[2]	*DDA3*	5.00	
p53-regulated DDA3[3]	*DDA3*	4.02	
early growth response 1	*EGR1*	2.92	
E2F transcription factor 5, p130 binding	*E2F5*	5.59	
DNA damage-inducible transcript 3	*DDIT3*	2.92	
CREB binding protein	*CREBBP*	3.02	
activating transcription factor 2[1]	*ATF2*	−5.95	
activating transcription factor 2[2]	*ATF2*	−3.22	
distal-less homeo box 5	*DLX5*	−5.69	
aryl hydrocarbon receptor nuclear translocator-like	*ARNTL*	−8.12	
Structural genes			
destrin (actin depolmerizing factor)	*DSTN*	5.04	
axonal transport of synaptic vesicles	*ATSV*	3.17	
tubulin, gamma 1	*TUBG1*	3.04	
glial fibrillary acidic protein	*GFAP*	−2.97	
amyloid beta (A4) precursor protein[1]	*APP*	−5.52	
amyloid beta (A4) precursor protein[2]	*APP*	−4.18	
gelsolin[1]	*GSN*	−7.25	
gelsolin[2]	*GSN*	−7.86	
actin, alpha 2	*ACTA2*	−9.60	
Growth factors			
connective tissue growth factor	*CTGF*	6.80	
interleukin 1, beta[1]	*IL1B*	5.41	
interleukin 1, beta[2]	*IL1B*	5.03	
interleukin 1, beta[3]	*IL1B*	3.75	
insulin-like growth factor 2[1]	*IGF2*	4.62	
insulin-like growth factor 2[2]	*IGF2*	4.37	
brain-derived neurotrphic factor	*BDNF*	−6.19	
transforming growth factor, beta 1[1]	*TGFB1*	−2.89	
transforming growth factor, beta 1[2]	*TGFB1*	−9.29	
Growth factor receptors			
fibroblast growth factor receptor 4[1]	*FGFR4*	8.90	
fibroblast growth factor receptor 4[2]	*FGFR4*	5.69	
fibroblast growth factor receptor 2	*FGFR2*	4.23	
ephrin receptor A7	*EPHA7*	−7.80	
ephrin receptor B1	*EPHB1*	−10.68	
interleukin 10 receptor, beta	*IL10RB*	−17.48	
Other receptors			
potassium channel, subfamily K, member 1	*KCNK1*	3.48	
adenosine A2a receptor	*ADORA2A*	−5.84	
purinergic receptor P2Y, G-protein coupled, 2	*P2RY2*	−5.79	
cholinergic receptor, nicotinic, epsilon polypeptide[1]	*CHRNE*	−5.89	
cholinergic receptor, nicotinic, epsilon polypeptide[2]	*CHRNE*	−8.78	
glutamate receptor, ionotropic, kainate 1[1]	*GRIK1*	−3.20	
glutamate receptor, ionotropic, kainate 1[2]	*GRIK1*	−3.20	
glutamate receptor, ionotropic, kainate 1[3]	*GRIK1*	−19.08	
Immune function			
complement component 1, r subcomponent	*C1R*	3.98	
CD8 antigen, alpha polypeptide (p32)[1]	*CD8A*	−3.27	
CD8 antigen, alpha polypeptide (p32)[2]	*CD8A*	−3.35	
CD4 antigen (p55)	*CD4*	−7.38	
major histocompatability complex, class II, DO alpha[1]	*HLA-DOA*	−8.01	
major histocompatability complex, class II, DO alpha[2]	*HLA-DOA*	−12.67	
major histocompatability complex, class I, C[1]	*HLA-C*	−14.58	
major histocompatability complex, class I, C[2]	*HLA-C*	−20.92	
major histocompatability complex, class II, DQ beta[1]	*HLA-DQB*	−10.74	
major histocompatability complex, class II, DQ beta[2]	*HLA-DQB*	−16.27	
major histocompatability complex, class II, DQ beta[3]	*HLA-DQB*	−27.61	
major histocompatability complex, class I, A	*HLA-A*	−22.88	

*Integrin B1 is included despite having a Z-Test value of −2.46, therefore lies slightly outside our arbitary threshold cutoff of +/−2.58.

Table 2

Semi-quantitative RT–PCR (LightCycler) validation of the abundance of 21 mRNAs indicated from microarray analysis.

Gene name	Gene symbol	Fold change[a,b]
activating transcription factor 2	*BUB1B*	−2.5
budding uninhibited by benzimidazoles 1 homolog beta (yeast)	*CDC6*	+3.4
cell division cycle 6 homolog (S. cerevisiae)	*CDK5R1*	+3.9
cyclin-dependent kinase 5, regulatory subunit 1 (p35)	*CDKN1B*	+1.4
cyclin-dependent kinase inhibitor 1B (p27, Kip1)	*CKS1B*	−3.6
CDC28 protein kinase 1B	*CCND1*	+3.6
cyclin D1	*CCND3*	+1.8
cyclin D3	*DDA3*	+2.4
p53-regulated DDA3	*EDN1*	+2.6
endothelin 1	*EGFR*	+120.2
epidermal growth factor receptor	*ENTPD6*	+96.2
ectonucleoside triphosphate diphosphohydrolase 6	*ERBB2*	−1.9
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	*FABP7*	−15.5
fatty acid binding protein 7, brain	*FGF2*	−35.7
fibroblast growth factor 2 (basic)	*FN1*	+20.2
fibronectin	*GRIA1*	+4.1
glutamate receptor, ionotropic, AMPA 1	*KNCK1*	+20.1
potassium channel, subfamily K, member 1	*MAP4K2/GCK*	+25.4
mitogen-activated protein kinase kinase kinase kinase 2	*SPARC*	−2.8
secreted protein, acidic, cysteine-rich (osteonectin)	*UBE2I*	−166.7
ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	*ATF2*	+3.4

[a] Fold change represents a change in the abundance of a transcript in the T265 cells with respect to nhSCs.

[b] mRNA abundance in nhSCs and T265 cells compared with a control, housekeeping gene, glyceraldehyde phosphate dehydrogenase, for relative quantification (see Methods).