

1 **Closing the Phenotypic Gap between Transformed**
2 **Neuronal Cell Lines in Culture and Untransformed Neurons**

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21 Abbreviations used: Bak, Bcl-2-antagonist/killer 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2;
22 BDNF, brain derived neurotrophic factor; CDK, cyclin dependent kinase; NRG1, neuregulin 1; CNS, central
23 nervous system; HDAC, histone deacetylase; IPA, Ingenuity Pathways Analysis; ML, monolayer; N-myc,
24 myelocytomatosis viral related oncogene; PI, propidium iodide; RA, retinoic acid; Rb, retinoblastoma; RWV,
25 rotating wall vessel; SEM, scanning electron micrograph; ST, staurosporine; SY, SH-SY5Y; 3-D, three-
26 dimensional; TG, thapsigargin

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1 **Abstract**

2

3 Studies of neuronal dysfunction in the central nervous system (CNS) are frequently limited by
4 the failure of primary neurons to propagate *in vitro*. Neuronal cell lines can be substituted for
5 primary cells but they often misrepresent normal conditions. We hypothesized that a 3-
6 dimensional (3-D) cell culture system would drive the phenotype of transformed neurons closer
7 to that of untransformed cells. In our studies comparing 3-D versus 2-dimensional (2-D) culture,
8 neuronal SH-SY5Y (SY) cells underwent distinct morphological changes combined with a
9 significant drop in their rate of cell division. Expression of the proto-oncogene N-myc and the
10 RNA binding protein HuD was decreased in 3-D culture as compared to standard 2-D conditions.
11 We observed a decline in the anti-apoptotic protein Bcl-2 in 3-D culture, coupled with increased
12 expression of the pro-apoptotic proteins Bax and Bak. Moreover, thapsigargin (TG)-induced
13 apoptosis was enhanced in the 3-D cells. Microarray analysis demonstrated significantly
14 differing mRNA levels for over 700 genes in the cells of each culture type. These results indicate
15 that a 3-D culture approach narrows the phenotypic gap between neuronal cell lines and primary
16 neurons. The resulting cells may readily be used for *in vitro* research of neuronal pathogenesis.

1 **Introduction**

2
3 *In vitro* studies of disease pathogenesis in the CNS are often conducted with cultures of
4 primary cells, but when the cells in question are neurons, in particular, human neurons, this
5 becomes problematic. Because most post-embryonic neurons do not divide, their usefulness in
6 primary culture is limited (1, 2). Transformed neuronal cell lines of both human and animal
7 origin have thus become a requisite tool in studies of neuronal dysfunction in the CNS.

8 Although transformed neurons in cell lines will divide, they are known to exhibit an arrested
9 state of cellular differentiation (3-7). Expression of the proto-oncogene N-myc is typically
10 elevated and resistance to apoptosis is increased, making the interpretation of experimental
11 results with these cells difficult when compared to their untransformed counterparts (6, 8-11).

12 Differentiation of transformed neurons in 2-D culture can be pursued by the addition of
13 biochemical agents such as phorbol myristate acetate (PMA), retinoic acid (RA), staurosporine
14 (ST) and brain-derived neurotrophic factor (BDNF) (12, 13) but it is not clear how closely these
15 conditions model the cell-cell interactions that drive differentiation *in vivo*. Phorbol esters, for
16 example, have been used to induce SY cells to differentiate toward a noradrenergic phenotype,
17 while treatment with RA effects a cholinergic phenotype (13). Incubation of SY cells with either
18 RA or PMA induces up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L, whereas
19 differentiation obtained with ST has been shown to notably diminish expression of these proteins
20 (12, 14, 15). In each case, changes in resistance to apoptosis follow changes in expression of the
21 markers. This is important because the Bcl-2 family proteins, including pro and anti-apoptotic
22 members such as Bax, Bak and Bcl-2, figure prominently in molecular pathways of programmed
23 cell death and may play a role in the degree of neuroblastoma cell tumorigenicity (16-19).

1 In addition to the limitations introduced by transformed cell lines, traditional monolayer or 2-
2 D culture systems using flasks and multi-well dishes are often themselves inadequate to
3 realistically model *in vivo* conditions (7, 20-22). Gravity induced sedimentation, non-
4 homologous delivery of nutrients and a lack of cell-cell and cell-extra cellular matrix contacts are
5 all potential limitations of 2-D cell culture (4, 5, 22, 23). Perhaps more importantly, 2-D cell
6 culture approaches are also known to alter gene expression, to hinder cellular differentiation and
7 the acquisition of polarity, and to prevent formation of the complex 3-D cellular architecture
8 commonly found in an intact tissue (5, 7, 22, 24-27). While matrigel, collagen, peptide and
9 synthetic nanofiber scaffolds are each being used and developed as more realistic procedures for
10 *in vitro* cell culture (5, 7, 21, 25), NASA engineered rotating wall vessels (RWV) are also being
11 employed to establish a fluid suspension culture that is capable of inducing biologically
12 meaningful 3-D growth *in vitro* (4, 23, 28, 29). During culture in a RWV, individual cells
13 aggregate into 3-D tissue-like assemblies developing enhanced states of differentiation and cross
14 communication through cell-cell contacts. Gas exchange and nutrient delivery are optimized
15 under these conditions, (4, 22) and the cellular phenotypes, as compared to their 2-D cultured
16 counterparts, become functionally and morphologically similar to the parental tissues and organs
17 that they represent (7, 20, 29-32).

18 Our lab is interested in studying the pathogenesis of Lyme neuroborreliosis. This disease is
19 caused by infection with the spirochete *Borrelia burgdorferi*. We and others have argued that
20 the associated neurocognitive symptoms of this disease are precipitated by neuronal dysfunction
21 resulting from inflammatory effects elicited by the spirochete (33-35). The inherent limitations
22 of primary neuronal culture *in vitro* prompted us to incorporate transformed neurons into our
23 research design. As molecular and cellular changes that accompany the conversion of normal

1 cells into states of disease or malignancy are altered by 2-D growth (4, 28, 36), we wanted to
2 establish a 3-D model of *in vitro* neuronal culture, to assess whether this procedure would
3 attenuate the phenotypic differences that exist between transformed and untransformed neurons.

4 The SY cell line is an adrenergic “n” type clone of the “mixed cell” human neuroblastoma
5 line SK-N-SH and has been used extensively in standard 2-D cultures to study neuronal function,
6 growth, damage in response to insult, degeneration and differentiation (3, 13, 14, 37-39). By
7 culturing SY cells under gentle, low-shear conditions in a RWV, we have succeeded in obtaining
8 a cell line that expresses classic morphological and functional patterns of neuronal
9 differentiation. We believe that the enhanced state of differentiation and cell-cell crosstalk events
10 generated by culture in 3-D, bypasses the need for chemical treatment of the cells, and provides a
11 more normalized cellular response during experimental studies.

12
13

1 **Results**

2 **3-D culture changes the morphology and proliferation rate in SY neuronal cells**

3 SY cells cultured for 21 d in the RWV, and then for counting purposes, transferred back to 2-
4 D culture flasks for 5 d, revealed a decrease in the cell doubling rate from 40 h to approximately
5 65 h, with no change in cell viability (Fig.1 A). Because the carrier beads used in our 3-D culture
6 were coated in collagen, we additionally cultured SY cells for 3 and for 4 wks in 2-D flasks
7 coated with collagen. We observed no detectable difference in the morphology, cell viability or
8 doubling rate of 2-D cells cultured on plastic as compared to collagen. Scanning electron
9 microscopy (SEM) revealed important differences in the morphology of SY cells cultured in 2-D
10 or in 3-D. Specifically, only the 3-D-cultured SY cells acquired a parental, tissue-like
11 conformation with dramatic increases in neurite extension, direction and number (Fig. 1 B).

13 **Decreased expression of N-myc and HuD**

14 Human neuroblastoma cells are typically characterized by de-differentiation. They have re-
15 entered S-phase of the cell cycle, and are highly resistant to apoptosis (9, 11). Amplified
16 expression of the proto-oncogene N-myc has been correlated with cellular de-differentiation and
17 increased resistance to apoptosis, and is believed to have a crucial role in maintenance of the
18 cells' malignant phenotype (6, 10, 40, 41). The RNA binding protein HuD functions in
19 stabilizing N-myc mRNA and may consequently enhance steady-state expression levels of this
20 oncogene (40-42). Reduced expression of the HuD protein could therefore contribute, through
21 destabilization of N-myc, to an increase in cellular differentiation.

22 Western analysis confirmed a culture-dependent shift in protein expression of these markers,
23 with the decrease positively correlating to the length of time the cells had spent in 3-D culture

1 (Fig. 2 A). Images obtained with confocal microscopy revealed a diminished level of N-myc and
2 HuD protein expression in SY cells cultured in 3-D as opposed to 2-D (Fig. 2 B).

3 4 **Apoptosis resistance is diminished in 3-D cultured SY and PC12 cells**

5 Cells over-expressing the anti-apoptotic protein Bcl-2 or cells with depleted pro-apoptotic
6 Bax and Bak exhibit resistance to cell death as induced by mitochondrial dysfunction and ER
7 stress (18, 19, 43, 44). Because increased resistance to apoptosis is one hallmark of a
8 transformed phenotype in many cancer cell lines, we were interested in exploring the effects of
9 3-D culture on the expression of key proteins in the apoptosis pathway. We found a decreased
10 expression of Bcl-2 coupled with increased Bax and Bak proteins in 3-D cultured SY cells as
11 compared to those cultured in standard 2-D conditions (Fig. 3 A). While confocal imaging
12 clearly indicated increased Bak protein in 3-D cultured cells, western analysis was not sensitive
13 enough to detect its expression.

14 Our next consideration was to assess apoptosis functionally and to confirm that our findings
15 were not restricted to a single cell line. PC12 is a rat pheochromocytoma cell line that can be
16 stimulated with nerve growth factor to differentiate into sympathetic-like neurons (45) Due to
17 their induced ability to cease division, become electrically excitable and extend neurites, PC12
18 cells have become an extremely well characterized *in vitro* model for studies of neuronal
19 differentiation and survival (20, 46-49).

20 TG is known to induce apoptosis through the passive release of Ca^{2+} from ER stores. These
21 events lead to subsequent increases in cytosolic Ca^{2+} , stressing both the ER and the mitochondria
22 (16-19, 50). In order to determine inherent differences in apoptosis between the 3-D and 2-D
23 cultured cells we used the TUNEL assay. SY cells were incubated with 10 nM TG for 24 hours

1 and for 5 days. The 3-D-cultured SY cells were treated either inside of the RWV, 3-D(RWV) or
2 after transfer back into standard culture flasks (3-D). We additionally incubated PC12 cells with
3 30 nM TG, for 5 d. All of the 3-D-cultured PC12 cells were treated after transfer back into
4 standard culture flasks. The SY and PC12 cells grown in 2-D culture were treated in their
5 respective dishes.

6 In a 5 d comparison of TG-stimulated versus non-stimulated control cells, we found an
7 approximate 4-7 fold increase in the occurrence of apoptosis in 3-D as opposed to 2-D culture. In
8 a similar 5 d comparison, 3-D cultured PC12 cells were approximately 3-fold more susceptible to
9 apoptosis than were the 2-D cells. At 24 h, a noticeable difference in the degree of apoptosis
10 occurring in stimulated versus control cells was found only in the 3-D(RWV) cells (Fig. 3 B).

11

12 **SY cells maintain 3-D culture-induced alterations in the phenotypic markers N-Myc and** 13 **Bcl-2 for at least 5 d after return to 2-D culture**

14 As many studies of neuronal pathogenesis involve co-cultures of neuronal cell lines with
15 primary glia and/or other live organisms propagated in 2-D culture, we wanted to evaluate the
16 length of time that SY cells would retain a 3-D phenotype once they were transferred back into
17 2-D culture. We thus examined the expression of N-myc and Bcl-2, two molecular markers
18 closely related to both differentiation and tumorigenicity (6, 8-11, 14, 15, 19). Assessment of the
19 SY cells that had been “pre-conditioned” in 3-D culture for approximately 3 wks and were then
20 removed to 2-D culture revealed a five-day experimental window during which reversion of the
21 culture-induced changes were minimal (Fig. 4).

22

23 **Microarray analysis of gene expression in SY cells cultured in 3-D and in 2-D**

1 In an effort to expand and to further clarify our findings related to the states of differentiation
2 and morphology in 2-D and 3-D-cultivated SY cells, we employed microarray analysis to
3 observe the culture-induced effects on global gene expression. Because abnormalities in the
4 expression and activity of multiple genes often work in concert to affect a transformed cellular
5 phenotype, (51-54) Ingenuity Pathways Analysis (IPA) software was used to compare the
6 mRNA levels in 44,544 70-mer oligos corresponding to over 24,000 human genes. Cancer, cell
7 morphology and proliferation pathways were among those found to be the most altered (Fig. 5
8 A). The G1/S and G2/M cell cycle check points, as well as the p53 and neuregulin signaling
9 pathways were also significantly affected (Fig. 5 B).

10 Along with abnormalities in the p53 tumor suppressor gene pathway, dysregulation of the cell
11 cycle is one of the most frequent alterations found in tumor development, with the inappropriate
12 progression of G1/S being especially common (52, 53, 55, 56) In the normal dividing cell,
13 cyclin-dependent kinases (CDKs) form a complex with D/E-type cyclins to phosphorylate the
14 retinoblastoma (Rb) gene, causing the release of bound E2F family transcription factors. These
15 now unbound E2F proteins then act to drive G1/S phase transition by the activation (or
16 repression) of multiple gene targets affecting cellular growth and proliferation, nucleotide
17 metabolism and DNA synthesis (53, 54, 57-59). Histone deacetylases (HDAC's) form a complex
18 with bound E2F proteins and are also released upon phosphorylation of Rb. Importantly, HDAC
19 inhibitors have been shown to cause cell cycle arrest in G1 and to function in cellular
20 differentiation and apoptosis (60, 61). Because of its strong ties to transformation, we wanted to
21 look more closely at the actual variance reported in the G1/S pathway.

22 The CDK4/6 inhibitor CDKN2B was found to be significantly up-regulated in 3-D versus 2-D
23 cultured SY cells. At the same time, the transcription factor E2F3, HDAC2 and the neuregulin1

1 (NRG1) gene, whose product promotes growth and proliferation in neuronal cells of the
2 peripheral and central nervous systems (62, 63), were each significantly down regulated (Fig. 5
3 C, Table 1). These events clearly indicate arrest in G1. Rb gene expression was also decreased,
4 but without knowing the phosphorylation state of this gene, correlation to the cell cycle is
5 questionable.

6

7 **RT-PCR confirms the differential expression of G1/S cell-cycle check point genes in 3-D** 8 **versus 2-D cultured SY cells**

9 A significant part of our microarray analysis was focused on exploring culture-induced
10 differential gene expression in a neuronal cell line that could indicate phenotypic reversion
11 toward a more normalized state. Pathways such as growth and proliferation or the cell cycle
12 checkpoints were of interest. We used RT-PCR to confirm our initial array findings. In order to
13 maintain integrity in this experiment as compared to our microarray analysis, we used aliquots of
14 the same SY 3D and 2-D cell RNA that was collected for each of the arrays. Expression changes
15 in 3 of the 4 selected genes known to influence the G1/S cell cycle checkpoint matched our
16 microarray data (Fig. 5 C2, Table 2).

17

18 **Discussion**

19

20 It is generally accepted that once developing neurons leave the ventricular and sub-ventricular
21 zones of the CNS, they are terminally differentiated and become persistently postmitotic (56, 64,
22 65). Although some neurons are generated in the adult brain, neuronal exit from the cell cycle is
23 typically viewed as permanent (56, 64-67). This inability to divide often complicates research
24 requiring primary neuronal cultures. While a handful of human neuronal cell lines are available

1 to researchers, the transformed phenotype of these cells is less than optimal. In consideration of
2 these circumstances, we have applied a transitional cell culture technique to neuronal cell lines
3 that attenuates some of the aberrant features characteristic of transformed neurons.

4 Loss of cellular differentiation combined with an unchecked potential to proliferate has long
5 been a hallmark in the progression of tumorigenesis (53, 54, 64, 67). In this report we have
6 shown that the morphology and proliferation characteristics of 3-D-cultivated SY cells align
7 more with a parental untransformed phenotype than those grown in 2-D. The two classic
8 prognostic markers of tumorigenicity in neuroblastoma, N-myc and HuD expression, are
9 diminished in 3-D as compared to 2-D-cultured SY cells. A decline in the amount of HuD
10 mRNA and protein in various cell lines has been shown to cause a marked reduction in steady-
11 state levels of mature N-myc mRNA and protei, (6, 10, 11, 40-42) thus even small decreases in
12 HuD protein may be contributing, via the effect on N-myc, to increased cellular differentiation in
13 3-D cultured SY cells.

14 Numerous reports verify the ability of 3-D growth to sway cellular characteristics toward
15 those of a less transformed state as compared with that of 2-D culture (5, 7, 14, 20, 22, 23, 26,
16 36, 68, 69). In 1997, Bissell et al showed that breast cancer cells grown in 3-D culture changed
17 their transformed behavior to that of non-cancerous cells, resuming a normalized morphology
18 combined with more typical patterns of growth and proliferation (5, 25). Nickerson et al. further
19 demonstrated that culture in 3-D can promote cellular differentiation and polarity, some of the
20 key elements perturbed in transformation (22, 23, 26, 68, 69). While researchers have followed
21 different protocols for 3-D culture, e.g. growth on gels and matrices versus fluid culture in a
22 RWV, the trend toward a less transformed phenotype is preserved in numerous cell types. In our
23 model, SY cells “normalized” in 3-D culture, were able to maintain a less transformed phenotype

1 for at least 5 d after return to 2-D growth conditions. Given that cell cultures in a 2-D format are
2 generally easier to handle during experimental procedures, than are cells in 3-D culture, this
3 finding adds to the practicality of our model.

4 Further distancing transformed cells from the normal tissues that they are intended to
5 represent is an increased resistance to apoptosis (36, 49, 53). We have found 3-D-cultured SY
6 and PC12 cells to be more susceptible to TG-induced apoptosis than the same cells grown in 2-
7 D. Because of TG's known association to the intrinsic pathway of ER and mitochondrial induced
8 mortality in which Bcl-2 family proteins are highly implicated, changes in the anti-apoptotic
9 protein Bcl-2 and pro-apoptotic family members Bax and Bak, provided a possible mechanism
10 contributing to the enhanced apoptotic response (17-19). Interestingly, while stimulation with ST
11 diminishes the expression of Bcl-2 in 2-D-cultivated SY cells, chemical differentiation with RA
12 or the phorbol ester TPA has been shown to up-regulate Bcl-2 expression in these cells (3, 13,
13 14, 70, 71). While the latter situation could be viewed as a more "protected" condition for the
14 differentiated and thus (now) postmitotic neurons, resistance to signals of apoptosis above that of
15 a cancerous state seems incongruous with a less transformed phenotype.

16 The primary hypothesis addressed in this study was that a 3-D culture system would drive the
17 phenotype of transformed neurons closer to that of untransformed cells. We have noted culture
18 induced changes in the morphology and biomarker expression of our 3-D cultured SY cells,
19 reflecting a more differentiated, and thus a less transformed phenotype. We have shown
20 apoptosis resistance to be diminished and have observed a decline in the 3-D cultured SY cell
21 doubling rate. Microarray analysis comparing 3-D and 2-D-cultivated SY cells provided a strong
22 indication that alterations in the G1/S cell cycle progression were contributing to the diminished
23 doubling rate in the 3-D cultured SY cells. Neuronal cells arrested at this checkpoint are known

1 to either return to G0 and re-differentiate, or they will die by apoptosis (67). Due to the decline in
2 doubling rate and the near 100 percent viability of our 3-D cultured SY cells, we believe that a
3 return to quiescence is occurring. Confirmation of the array results involved in this pathway was
4 obtained using QRT-PCR. Lending added support to our hypothesis, culture-induced variance in
5 several other prominent pathways leading to transformation and cancer, were also identified on
6 the array.

7 The use of microarray analysis to screen for the differential expression of SY cell genes
8 propagated in 2-D and 3-D culture conditions has been employed by other investigators (39, 72).
9 While focusing on differences in morphology and neurite outgrowth, Li et al, found more than
10 1700 of 14,564 genes to be differentially regulated in SY cells propagated on collagen matrices.
11 Based on the number of genes being modified, it is reasonable that phenotypic changes beyond
12 those of morphology and neurite extension are occurring. These results, like our own, indicate
13 that continued research in this area will be needed to fully characterize the culture induced
14 changes in gene expression that define 3-D versus 2-D-cultured cells.

15 The generation of purified primary neurons in numbers satisfactory for experimental study is
16 difficult to achieve with animal cells, and is nearly impossible with human cells. Researchers
17 must therefore rely on transformed cell lines for many studies of pathogenesis in the CNS. The
18 ability to provide a more normalized cellular environment *in vitro* has improved dramatically
19 over the past decades, particularly with the use of 3-D culture systems (4, 5, 7, 22, 23, 26, 73,
20 74). The application of this technique to human neuronal culture is particularly attractive in view
21 of the cells' post-mitotic constraints in primary culture. We have shown here that 3-D culture
22 evokes changes in SY cell morphology, proliferation, apoptosis resistance and differentiation
23 states in a manner that narrows the phenotypic gap between those cells and their non-transformed

1 counterparts. As studies involving human neuronal pathogenesis remain largely dependent on *in*
2 *vitro* cell culture, this approach may easily be further exploited to create a more realistic
3 environment in which to model nerve cell function and response.

1 **Material and Methods**

2 **Cell Culture and Reagents**

3 2-D System

4 Human SY neuroblastoma cells (American Type Tissue Culture Collection ATCC CRL-
5 2266) and PC12 rat pheochromocytoma cells (ATCC CRL-1721) were each seeded into separate
6 T75 flasks with medium renewal every 3-7 d. The culture flasks for PC12 cells were coated with
7 PureCol collagen (Inamed). Cell propagation was performed as per the ATCC product sheet.
8 Nerve Growth Factor (Sigma) was added to the PC12 medium at 50 ng/2-D. Penicillin (100
9 units/ml), streptomycin (100 units/ml) and amphotericin B (0.25 µg/ml) (Gibco/Invitrogen) were
10 added to all media. Trypsin(2.5%)/EDTA(0.38 g/L) was used to dislodge the cells, and Trypan
11 Blue stain was used in assessing cell viability, (Gibco/Invitrogen). 2-D samples were harvested
12 at a passage ≤ 20 .

13 3-D System

14 Approximately 10^7 viable 2-D-cultured SY or PC12 cells were dislodged by trypsin and
15 loaded into 50 ml RWVs (Synthecon) containing 200 mg of Cytodex-3 micro-carrier beads
16 (Amersham Biosciences) suspended in complete growth medium (ATCC product sheet). Entirely
17 filled vessels were then attached to a rotator base (Synthecon) with initial speeds typically set at
18 18-22 RPM. The RPM were adjusted during cultivation to maintain the cell aggregates in
19 suspension. Complete removal of all bubbles was addressed upon initial rotation and daily
20 thereafter. Cell viability assays and medium replacement were performed every 2-5 d. 3-D cells
21 were collected after 2-4 wk (see individual results) of culture. Although minimal changes were
22 noted at 2 wk, we typically found significant molecular marker differences at the 3 wk time
23 points with small additional changes at 4 wk. For efficiency, 3 wk was used as our standard.

1

2 **Cell Counting and Proliferation Assays**

3 3-D cultures were removed from the RWV, dislodged from the cytodex beads by treatment
4 with trypsin/EDTA, and then dissociated from the beads with 40 μm cell strainers (Becton
5 Dickenson). 10^6 2-D and 3-D cultured SY cells were independently seeded into 10 ml of
6 complete growth medium in T75 culture dishes and allowed to propagate for 5 d. Cells were
7 then removed from the dish, (trypsin/EDTA), and counted in a BrightLine Hemocytometer.

8

9 **Morphology: Light and Electron Microscopy**

10

11 Live cell photographs were imaged with a Sony Cyber Shot digital still camera (DSCF717)
12 attached to a Nikon TMS light microscope. SEM was used to examine changes in the
13 morphology of SY cells as described previously with minor modifications (22). 2-D cells and 3-
14 D cell aggregates were fixed in 3% glutaraldehyde, 0.5% paraformaldehyde, in PBS pH 7.2 for a
15 minimum of 24 h. The samples were flushed in triplicate with filter-sterilized deionized water to
16 remove salts and then transferred for observation to a Philips XL 30 ESEM (FEI Co.) Chamber
17 pressure was adjusted between 1 and 2 torr to optimize image quality.

18

19 **Confocal Microscopy**

20 2-D and 3-D cells removed from culture were washed once in PBS and fixed in 2%
21 paraformaldehyde (PFA) (USB Corporation) for 5-10 min, permeablized in PBS with fish skin
22 gelatin (Sigma-Aldrich) and Triton X-100 (ICN Biomedicals) (PBS/FSG/Triton) and blocked in
23 10% normal goat serum (Gibco). The fixed 2-D and 3-D cultured cells were equally stained with

1 primary antibodies for 1 h, washed 3 times in PBS and then stained with corresponding
2 secondary antibodies for 45 min. Nuclear stains were combined with the secondary antibodies at
3 a concentration of 0.05 µg/ml. Primary antibodies used include anti-N-myc, HuD, Bcl-2, Bax
4 and Bak, (Santa Cruz Biotechnology). Alexa-488 (green)-conjugated secondary antibodies, and
5 the To-Pro (blue) nuclear stains were from Invitrogen. Propidium Iodide (PI) (red) (Sigma-
6 Aldrich) was used as an alternative nuclear stain. Imaging was performed using a Leica TCS SP2
7 confocal microscope equipped with three lasers (Leica Microsystems). Six to eighteen 0.2 µm
8 optical slices per image were collected at 512 x 512 pixel resolution. The pinhole size, gain and
9 contrast, filter settings, and laser output were held constant for each comparison of the 2-D and
10 3-D image sets.

11

12 **Western Blot Analysis**

13 Cells were lysed on ice for 10 min using buffer (0.15 M NaCl, 5 mM EDTA, pH 8, 1% Triton
14 X-100, 10 mM Tris-HCl, pH 7.4) containing 5 mM DTT and a Protease Inhibitor Cocktail for
15 mammalian cells (Sigma-Aldrich). Protein concentrations were measured with the BCA assay
16 (Pierce Biotechnology). After optimization for each sample, total protein (40 µg/lane for N-myc,
17 HuD, Bcl-2, and Bak, and 50 µg/lane for Bax) was resolved in 12% Tris-Hcl pre-cast gels
18 (BioRad), and electrophoretically transferred to nitrocellulose Protran membranes (Schleicher
19 and Schuell BioSciences). Non-specific binding was blocked with 3% BSA fraction V (Sigma-
20 Aldrich) in PBS-tween (PBST) at 4°C over night. Target proteins were detected with rabbit or
21 mouse primary antibodies for 2 h at room temperature or at 4°C over night (all antibodies were
22 from Santa Cruz Biotech. except for β-actin (Abcam). The blots were washed 3 times in PBST
23 and incubated for 45 min with horseradish peroxidase-conjugated anti-rabbit or anti-mouse

1 secondary antibodies (Santa Cruz Biotech.) The blots were again washed 3 times in PBST,
2 developed for 1-2 min in Western Blot Luminol Reagent (SCB) and visualized using a Kodak
3 Imager 2000 and Kodak Image Analysis Software.

4

5 **Apoptosis assays**

6 SY cells (1×10^6) cultured in 2-D or in 3-D were incubated with or without 10 nM TG. The 2-
7 D and 3-D cells were harvested using trypsin, washed in PBS, and fixed for 5-10 min in 2%
8 PFA. Prior to fixation, the 3-D-cultured cells treated inside of the RWV were separated from the
9 beads using a 40 μm cell strainer (Becton Dickinson). The fixed cells were permeablized in
10 PBS/FSG/Triton and blocked with 10% NGS. Apoptosis was evaluated using the Apoptag
11 TUNEL assay kit (Chemicon). The results were analyzed using a Leica TCS SP2 confocal
12 microscope as described above. Cell morphology consistent with apoptosis including cell
13 shrinkage, nuclear condensation and membrane blebbing were assessed along with the
14 fluorescein staining for TUNEL. The number of apoptotic cells counted was divided by the total
15 (500 minimum) number of cells counted. This protocol was also followed for evaluation of
16 apoptosis in PC12 cells. Due to an increased drug tolerance, 30 nM TG was used in the PC12
17 assay. 3-D-cultured PC12 samples were stimulated for 5 d after removal from the RWV to multi-
18 well dishes.

19

20 **Microarray**

21 Microarray experiments and analysis of data was performed according to previously
22 described protocols (75, 76). Further details are available in the Supplemental data section.

23

1 **QRT-PCR**

2 RNA was collected as for the microarray analysis. The QuantiFast SYBR Green RT-PCR kit
3 (Qiagen) was used for the QRT-PCR. All assays were performed as per manufacturer's
4 instruction with Qiagen QuantiTect primer pairs in a 96-well block ABI 7700 RT cyclor.

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1 **Figure 1. 3-D culture-induced changes in cell division rates and morphology.** (A) After 3
2 wk in RWV culture, the doubling rate of SY cells that were transferred back into 2-D culture for
3 5 d, drops from 1x/40 h to 1x/65 h, with no change in viability. Data are shown as the mean
4 (n=4) ± SD. * P<0.001 (B) SY cells grown in standard 2-D tissue culture flasks, sediment to the
5 bottom surface with a flattened morphology. Culture in a RWV promotes 3-D assembly of the
6 individual cells into large tissue-like aggregates. SEM: scanning electron micrograph.

7
8 **Figure 2. Decreased expression of N-myc and HuD in 3-D versus 2-D-cultured SY cells.** (A)
9 Western blot analysis reveals a progressive decrease in the expression of N-myc and HuD
10 proteins after 2 and 4 wk in 3-D culture that does not occur during growth in 2-D. (B) Confocal
11 images showing expression of the N-myc oncogene and the neuron-specific RNA binding
12 protein HuD. The 3-D culture was maintained for 4 wk. The secondary antibody to N-myc and
13 HuD is labeled with Alexa 488 (green). PI (red) was used as the nuclear stain. The scale bar on
14 each image represents 20 μm.

15
16 **Figure 3. Resistance to apoptosis is diminished in 3-D cultured SY cells.** (A) 1- Confocal
17 microscopy reveals diminished expression of the anti-apoptotic protein Bcl-2 in SY cells
18 cultured for 3 wk in a RWV. Pro-apoptotic Bax and Bak proteins are up-regulated in 3-D culture.
19 The secondary AB to Bcl-2, Bax and Bak is labeled with Alexa 488 (green). PI (red) or To-Pro
20 (blue) was used to stain the nucleus. Scale bars on the images are: Bcl-2 20 μm, Bax 23.81 μm,
21 Bak 40 μm. (A) 2- Western analysis of whole-cell lysates collected from the 3-D and 2-D
22 cultures at 3 wk confirms that Bcl-2 expression is down-regulated in 3-D cells, and expression of
23 Bax is up. (B) 1- The percent of TUNEL-positive SY cells in 3-D culture increased 4 to 7 fold

1 above those in 2-D when treated with TG (10 nM). (B) 2- TUNEL positive PC12 cells in 3-D
2 increased 3 fold above those in 2-D. For (B) 1 and 2: 3-D pre-tx = 3-D cells from RWV just
3 before transfer to dish. 2-D+0 = 2-D cells, unstimulated, 2-D+TG = 2-D cells stimulated with
4 TG, 3-D+0 = 3-D cells, unstimulated, 3-D+TG = 3-D cells removed from RWV to dish,
5 stimulated with TG, 3-D(RWV) +TG = 3-D cells treated with TG inside of the RWV. Data are
6 shown as the mean (n=3) \pm SD. *P<0.01 in SY (except for the 3-DRWV+TG where n=1)
7 *P<0.035 in PC12. L axis: actual percent apoptosis R axis: arbitrary units of fold change
8 representing the actual apoptosis.

9

10 **Figure 4. 3-D culture driven changes in the phenotypic differentiation markers N-myc and**
11 **Bcl-2 are still apparent after 5 d of return to 2-D growth in tissue culture flasks.** After 10 d
12 of re-introduction to 2-D growth, marker expression in the 3-D cultured cells has returned to a
13 level more analogous to those of the 2-D-cultured cell line. The secondary AB to N-myc and
14 Bcl-2 is labeled with Alexa 488 (green). PI (red) was used as the nuclear stain. The scale bars on
15 the 2-D and 3-D images represent 20 μ m except for 5 d which represents 40 μ m.

16

17 **Figure 5. Comparison of gene expression in 2-D and 3-Dcultured SY cells using microarray**
18 **analysis.** (A) Changes in gene expression due to cell culture conditions affect cellular disease-
19 related pathways (top three of 63, shown in order of significance). Threshold = cutoff for P<
20 0.05. (B) Ten canonical pathways most affected in SY cells grown in 3-D rather than 2-D . Bar
21 graph = ratio of gene expression in 3-D cultured cells as compared to those grown in 2-D. Line
22 graph represents significance as $-\log(p\text{-value})$ with P<0.05. (C) 1- (Table 1) Gene expression
23 pathway for G1/S cell cycle progression. Values were obtained using IPA software, version 5.0.

1 Minimum fold change ≥ 1.5 . 2- (Table 2) Confirmation of array results using QRT-PCR.
2 Reactions were run in triplicate with GAPDH gene expression being used as the reference. PCR
3 efficiencies, average fold change and statistical analysis was performed using the REST©
4 software program. All genes in this pathway were represented on the chips. Red: increased gene
5 expression. Green: decreased gene expression. mRNA for the QRT-PCR and array analysis was
6 collected at passage 8 (2-D and 3-D cultures) with $n = 2$ for each culture type.

7
8 **Supplemental Data:**

9
10 Microarray data are annotated both in terms of universal gene symbols (Gene Symbol) and
11 known gene function (Gene Description). Microarray experiments were performed on three
12 biologically replicate Human Exonic Evidence based Oligonucleotide arrays (#s 53383, 53384
13 and 52791). Differentially expressed genes were selected on the basis of statistical significance
14 using One-way Analysis of Variance (p value) and magnitude of change in gene-expression on a
15 \log_2 scale (M). We considered a magnitude change of 50% (1.5 –fold) along with $P < 0.05$ as
16 significant.

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C (1)

Table 1

HUGO Gene Symbol	Description	Log Ratio	Location	Type	Entrez Gene ID (H)
CDKN2B	cyclin-dependent kinase inhibitor 2B (INK4, p15, inhibits CDK4)	+3.348	Nucleus	transcription regulator	1030
E2F3	E2F transcription factor 3	-2.15	Nucleus	transcription regulator	1871
HDAC2	histone deacetylase 2	-2.236	Nucleus	transcription regulator	3066
NRG1	neuregulin 1	-4.403	Nucleus	Extracellular Space	3084
RB1	retinoblastoma 1 (including osteocarcinoma)	-1.574	Nucleus	transcription regulator	5925
SKP1A	S-phase kinase-associated protein 1A (p19A)	-1.325	Nucleus	transcription regulator	6500

Molecules associated with cell cycle G1/S checkpoint regulation in SY 2-D and 3-D cells above 50% change.

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C (2)
Table 2

Gene	3-D (fold change)	p-value
* CDKN2	+4.04	0.001
E2F3	+1.00	0.947
* HDAC2	-1.57	0.050
* NRG1	-2.39	0.001

Differential expression of selected SY genes in 3-D culture as compared to 2-D. * p≤ 0.05

Figure 1.

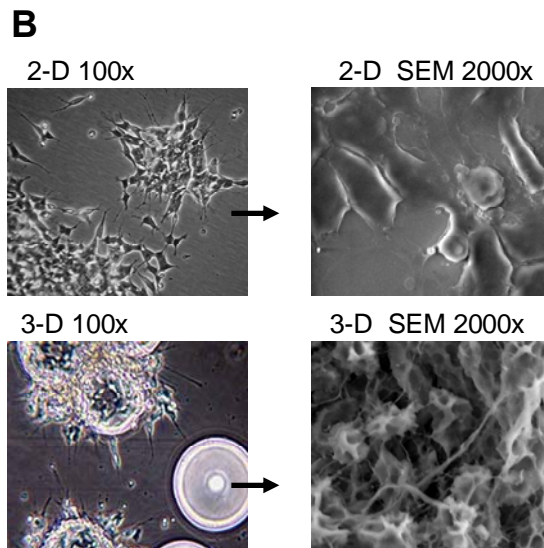
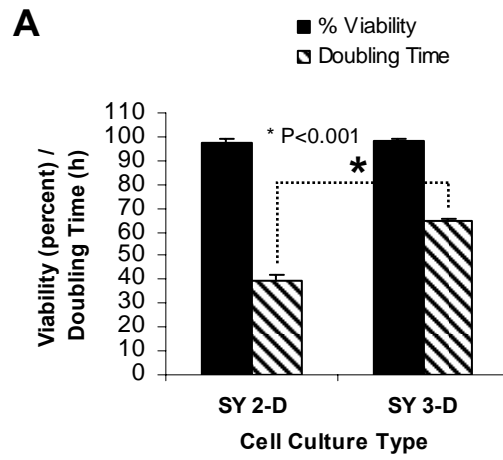


Figure 2.

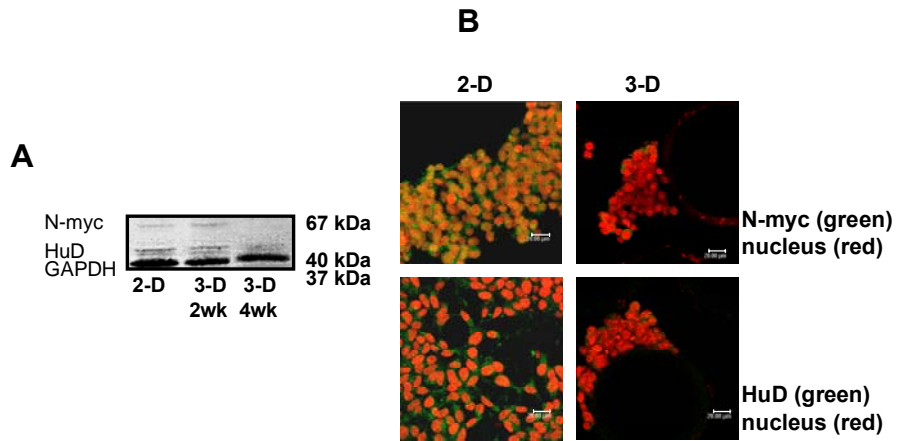
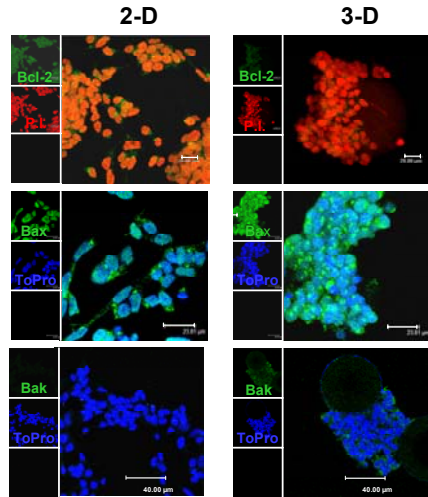
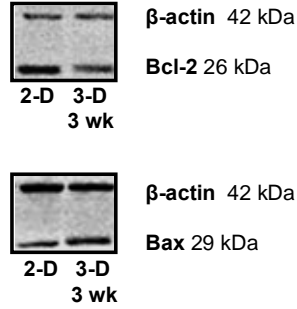


Figure 3.

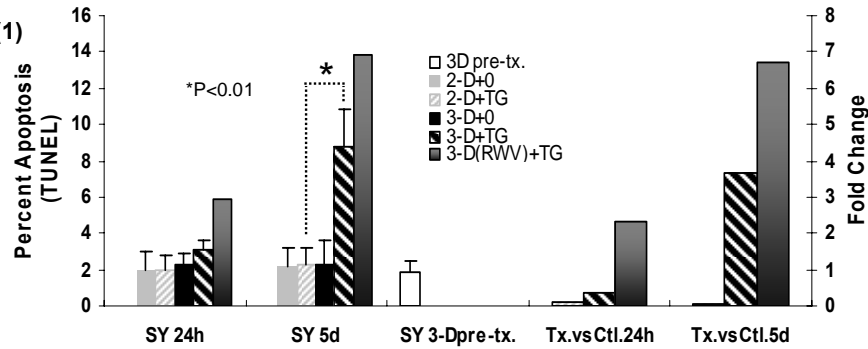
A (1)



(2)



B (1)



(2)

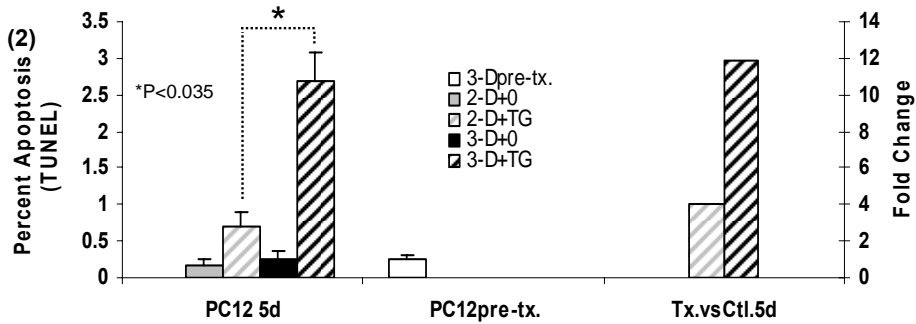


Figure 4.

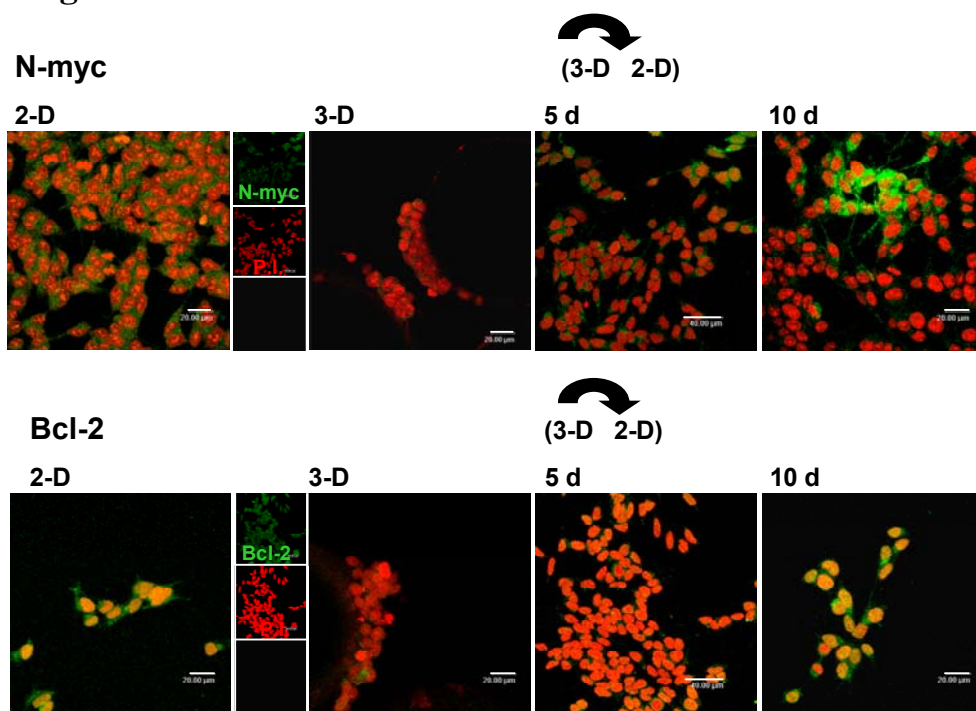
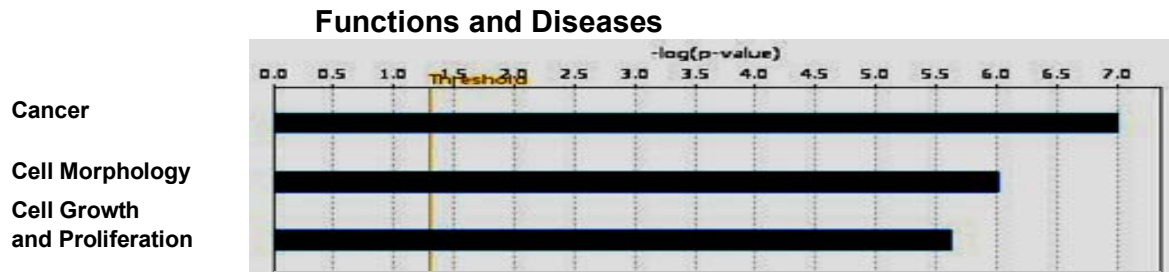
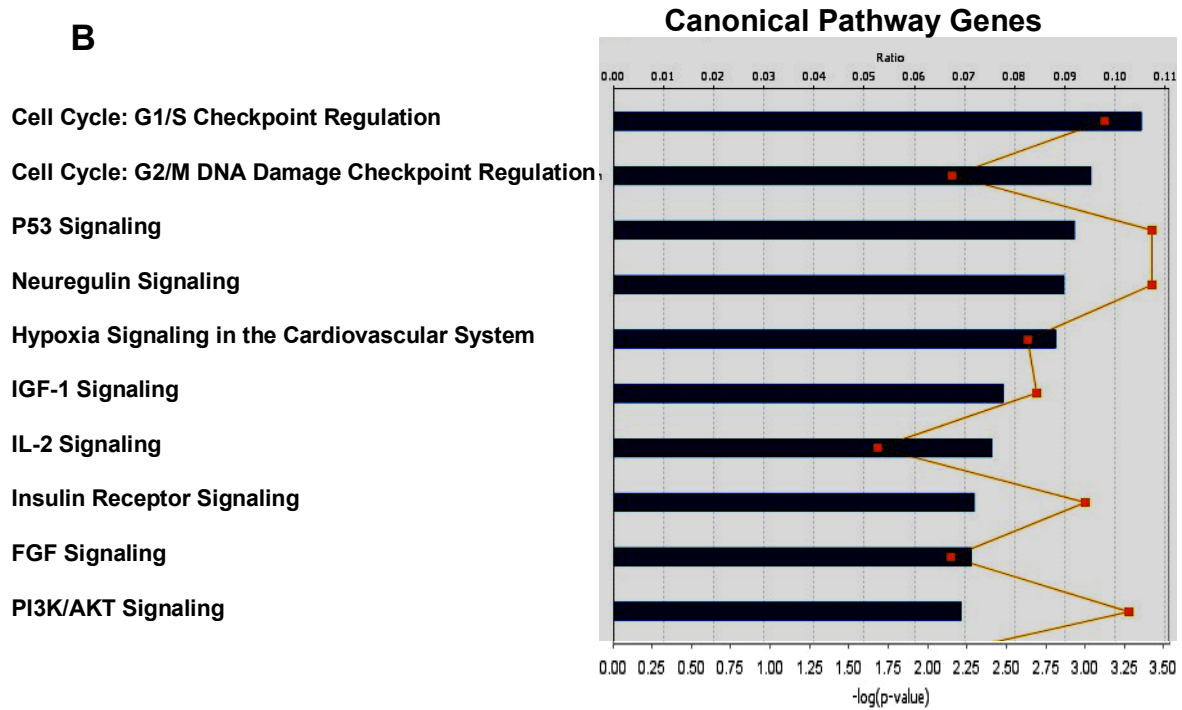


Figure 5.

A



B



C (1)

Cell Cycle: G1/S Checkpoint Regulation

