

SMARTvector shRNA Lentiviral platform: Elucidating the role of factors important in neurite outgrowth.

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Introduction

Normal sensory, motor and cognitive functions rely heavily on the ability of neurons to develop the intricate neurite architecture that supports a complex signaling network. Abnormalities of neuronal cell development and differentiation are implicated in many diseases including Alzheimer's, Parkinson's and other neurological disorders. Neuronal pathologies may also result from stroke, hypoxia, severe blood loss or physical trauma. Clinically, the prognosis for affected individuals can be poor because the recovery and regeneration of damaged or diseased neurons is poorly understood. Therefore, an important aim of many researchers is to elucidate the molecular mechanisms that direct neuronal development, differentiation and regeneration. This application note describes how the combination of state-of-the art Dharmacon™ SMARTvector™ shRNA lentiviral and high content analysis technologies provides multiple layers of important cellular data. These promise to provide a wealth of information about neuronal cell biology and pave the way for developing effective strategies for clinically relevant treatments.

Researchers often rely on *in vitro* cell culture as model systems for identifying potential therapeutic targets. For example, neuroblastomas are common pediatric solid tumors arising from the neuroectoderm and are characterized by their ability to undergo spontaneous regression to mature differentiated neurons. This tumor type responds positively to treatments that rely on inducers of differentiation such as retinoic acid (RA) and its derivatives¹. To better understand the interplay of factors involved in differentiation and to identify novel therapeutic targets, researchers employ cell lines such as SH-SY5Y that are derived from human neuroblastomas¹⁻². While SH-SY5Y cells represent a



valuable model system for the discovery of novel chemical inducers or inhibitors of neuronal differentiation, application of RNAi-based gene knockdown has been hindered by (1) the inefficient delivery of silencing constructs using traditional lipid-mediated transfection methods, (2) the need for robust, sustained silencing (up to 9 days), and (3) the lack of methods to analyze cellular phenotypes in a mixed population of epithelial and neuronal cells (see “Measuring Neurite Outgrowth” on next page.

In this application note, we describe how the SMARTvector shRNA lentiviral platform can be used to achieve targeted knockdown in SH-SY5Y cells in order to investigate the role of genes involved in neuronal differentiation. In addition, we demonstrate the utility of a high content screening approach which enables the detailed analysis of a neurite outgrowth phenotype in a cell line that exhibits at least two morphologically distinct fates: a neuroblast-like phenotype and an epithelial-like phenotype.

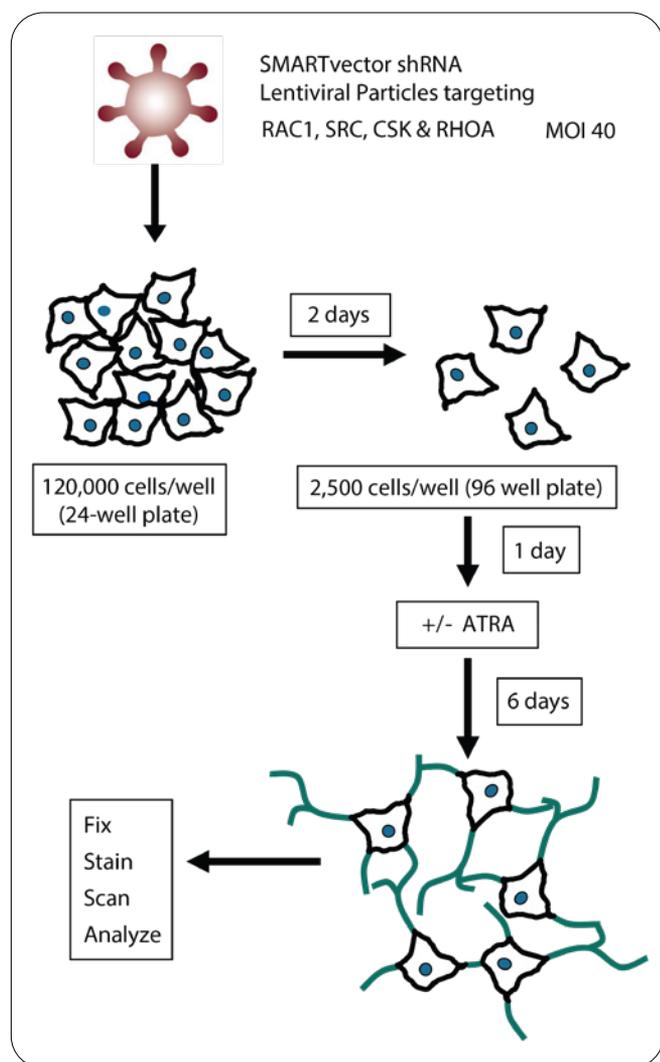


Figure 1: Experimental Workflow of SH-SY5Y Transductions.

Measuring neurite outgrowth

Neurons assemble into functional networks by developing axons and dendrites, together known as neurites. Growth cones at the terminal processes are the sites where a neurite elongates and splits to create branchpoints. Neurite outgrowth was then quantified in neuronal cells and sub-populations were specifically categorized.

After cells are fixed and stained cells can be scanned and analyzed. Figure 2A shows a schematic representation of a neurite-bearing cell indicating parameters that the Neuronal Profiling BioApplication Software is able to measure. The algorithm identifies key structures of the neuronal cell (nucleus, cell body and neurites) and measures multiple morphological parameters of these structures such as intensity, area, length and number. Average neurite count is the number of neurites identified in a field divided by the number of valid neuronal cell bodies. Average neurite length is calculated by taking the total measured lengths of all the neurites in a field and dividing it by the total neurite count. Branchpoint average count is determined by dividing the total branchpoint count by the number of neurites in the field. Finally, the branchpoint's average distance from the cell body can be computed. Together, these parameters can be used to assess the extent of neurite outgrowth in treated and untreated cell populations.

Materials and methods

Experimental setup

The transduction conditions including optimal cell density and multiplicity of infection (MOI) for SH-SY5Y cells were determined previously ([SMARTvector application note](#)). Transduction was performed at a high cell density in the presence of Polybrene [American Bioanalytical, (Cat #AB01643)]. SH-SY5Y cells (120,000 cells per well in a 24-well plate) were transduced with different gene-specific SMARTvector shRNA lentiviral constructs at a MOI of 40 according to the general transduction protocol ([SMARTvector technical manual](#)). Two days following transduction, cells were split and plated at 2,500 cells per well in a 96-well cell culture plate. Cells were allowed to recover from transduction and adhere overnight. Culture medium was then changed to complete growth medium with or without All-Trans Retinoic Acid (ATRA; 10 μ M). Cells were cultured for an additional six days with medium changes every two to three days. Cells were then harvested and prepared for mRNA analysis (QuantiGene™ branched DNA (bDNA), Panomics™, Freemont, CA), cell viability assay (Thermo Scientific™ alamarBlue™) and immunocytochemistry. A schematic of the experimental workflow is shown in Figure 1.

Genes targeted

SMARTvector shRNA Lentiviral Particles targeting human RHOA (Accession #NM_001664), RAC1 (NM_006908), SRC (NM_005417) and CSK (NM_004383) were used for silencing experiments. SMARTvector Non-targeting Control (NTC) Particles (Dharmacon, Cat #S-005000-01) were used as negative controls.

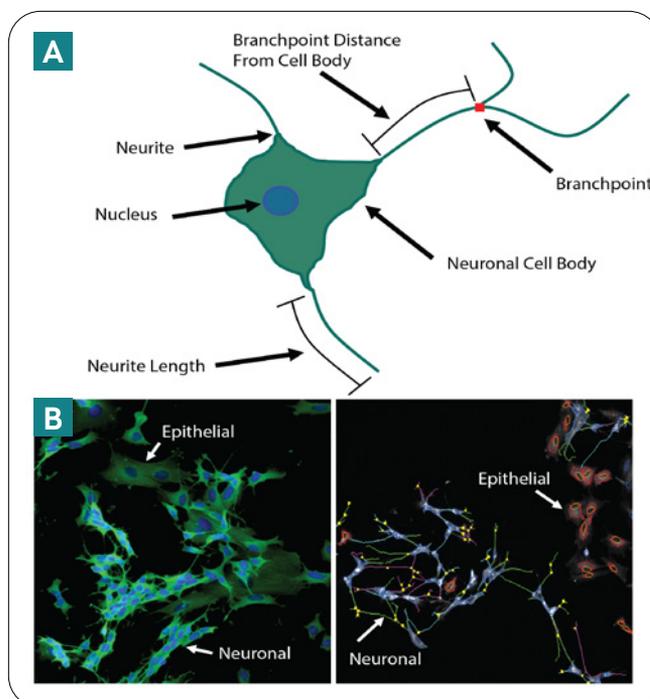


Figure 2: Measurements of Neurite Outgrowth in SH-SY5Y Cells
A. Schematic representation of a neuron and features that are measured by the algorithm. **B.** Composite image of the SH-SY5Y cell line stained with Cellomics Neurite Outgrowth HCS Reagent Kit. Blue indicates nuclei stained with Hoechst 33342. Green represents neuronal bodies (bright green) and epithelial-like cells (faint green) with different intensities. **C.** Raw image with algorithmic overlays from the Cellomics Neuronal Profiling BioApplication indicating the epithelial cells (red demarcations) that were filtered from the analysis based on their cell body average intensity. Blue and light blue demarcations indicate selected neuronal nuclei and cell bodies. Cyan, pink and green demarcations represent neurites and yellow demarcations indicate branchpoints.

Gene knockdown and cell viability

The level of target mRNA knockdown was assessed using the bDNA assay specific for the targets of interest at three and nine days post-transduction. Cell viability was determined using the alamarBlue assay according to manufacturer's instructions.

High content analysis of neurite outgrowth

After fixing with 3.7% formaldehyde in phosphate buffered saline (PBS), the cells were stained using antibodies provided in the Cellomics Neurite Outgrowth HCS Reagent Kit (Cat #K07-0001-1). Images were acquired on the Cellomics ArrayScan VTI HCS Reader and analyzed with the Cellomics Neuronal Profiling BioApplication. The mixed population of neuronal and epithelial-like cell types was distinguished with the gating technique provided by the Neuronal Profiling BioApplication. While both the neuronal and epithelial cells are detectable with a primary neural specific antibody supplied in the kit, the fluorescence intensity is generally much stronger in the neuronal cells (Figure 2B). Epithelial-like cells were removed from analysis by demarcation of the cell body based on the lower cell body average intensities (Figure 2C). Four replicate wells for each target and condition were analyzed. Six fields in each well were measured (> 100 cells/field). Parameters used in this analysis included: Mean Neurite Count, Mean Neurite Average Length, Mean Branchpoint Average Count and Mean Branchpoint Average Distance from the Cell Body (see side Bar "Measuring Neurite Outgrowth," Figure 2). These measurements were collected for each of the four replicates. The averages and standard deviations were calculated for each target and condition. All data was normalized to cells transduced with the SMARTvector Non-targeting Control Particles.

Results

ATRA induces neurite outgrowth in SH-SY5Y cells

The addition of ATRA to SH-SY5Y cells induces neurite outgrowth in a dose dependent manner. It was determined that culturing the cells in ATRA for five to six days was the optimal time point for developing significant neurite outgrowth (data not shown). Both the average neurite length and branchpoint count increased with the addition of 1 μ M and 10 μ M ATRA, respectively, compared to untreated and DMSO-treated cells (Figure 3). Addition of ATRA at concentrations greater than 10 μ M was not optimal and resulted in an apparent decrease in neurite outgrowth, possibly due to increased cell death (data not shown). ATRA was added at a 10 μ M concentration for all subsequent experiments.

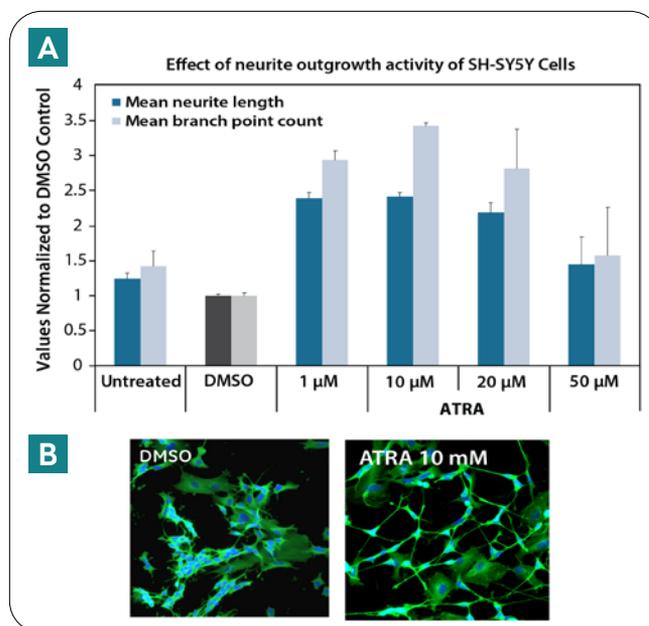


Figure 3: All-Trans Retinoic Acid Induces Neurite Outgrowth in SH-SY5Y Cells. SH-SY5Y cells were treated with increasing concentrations of ATRA or DMSO. Cells were cultured with medium changes every two or three days for seven days and then fixed and stained. A. The Average Neurite Length and Branchpoint Average Count were each determined by the Neuronal Profiling Bioapplication. Values were normalized to DMSO-treated controls (gray bars). B. Representative micrograph of cells treated with DMSO or ATRA. Cells were stained with Neurite Outgrowth HitKit (green) and Hoechst 33342 (blue).

Table 1: Target Gene Knockdown and Viability of SH-SY5Y Cells Transduced with SMART-vector shRNA Lentiviral Constructs. Cells were transduced with SMARTvector constructs at an MOI of 40, split after two days, maintained in culture medium without selection and then harvested at three days and nine days post-transduction. The Thermo Scientific™ alamarBlue™ assay was used to assess viability and normalized to the SMARTvector NTC. mRNA levels were determined by the Panomics bDNA assay and normalized to a house-keeping gene (GAPDH) and to the SMARTvector Non-targeting Control.

	Target gene knockdown		% Viability
	72 hours	216 hours	216 hours
SMART vector SRC	65%	51%	86%
SMART vector RAC1	96%	86%	80%
SMART vector CSK	75%	70%	111%
SMART vector RHOA	76%	64%	127%

Sustained silencing of specific gene targets

SH-SY5Y cells were transduced with SMARTvector shRNA Lentiviral Particles targeting RAC1, RHOA, SRC or CSK, as well as a NTC construct. Knockdown of mRNA was assessed at three and nine days post-transduction. All genes were sufficiently silenced at both three days and nine days (Table 1) with the slightly better knockdown at the earlier time point (three days). Since cells were cultured in the absence of puromycin, untransduced cells may have had a growth advantage over transduced cells thus leading

to an underestimation of knockdown after nine days. Depleting RAC1 and SRC mRNA had a negative effect on the growth rate of the cells: 80% and 86% viability compared to the cells transduced with the SMARTvector Non-targeting Control. In contrast, depleting RHOA and CSK mRNA resulted in a positive effect on growth rates: 111% and 127% compared to the cells transduced with the SMARTvector Non-targeting Control (Table 1, Figure 4).

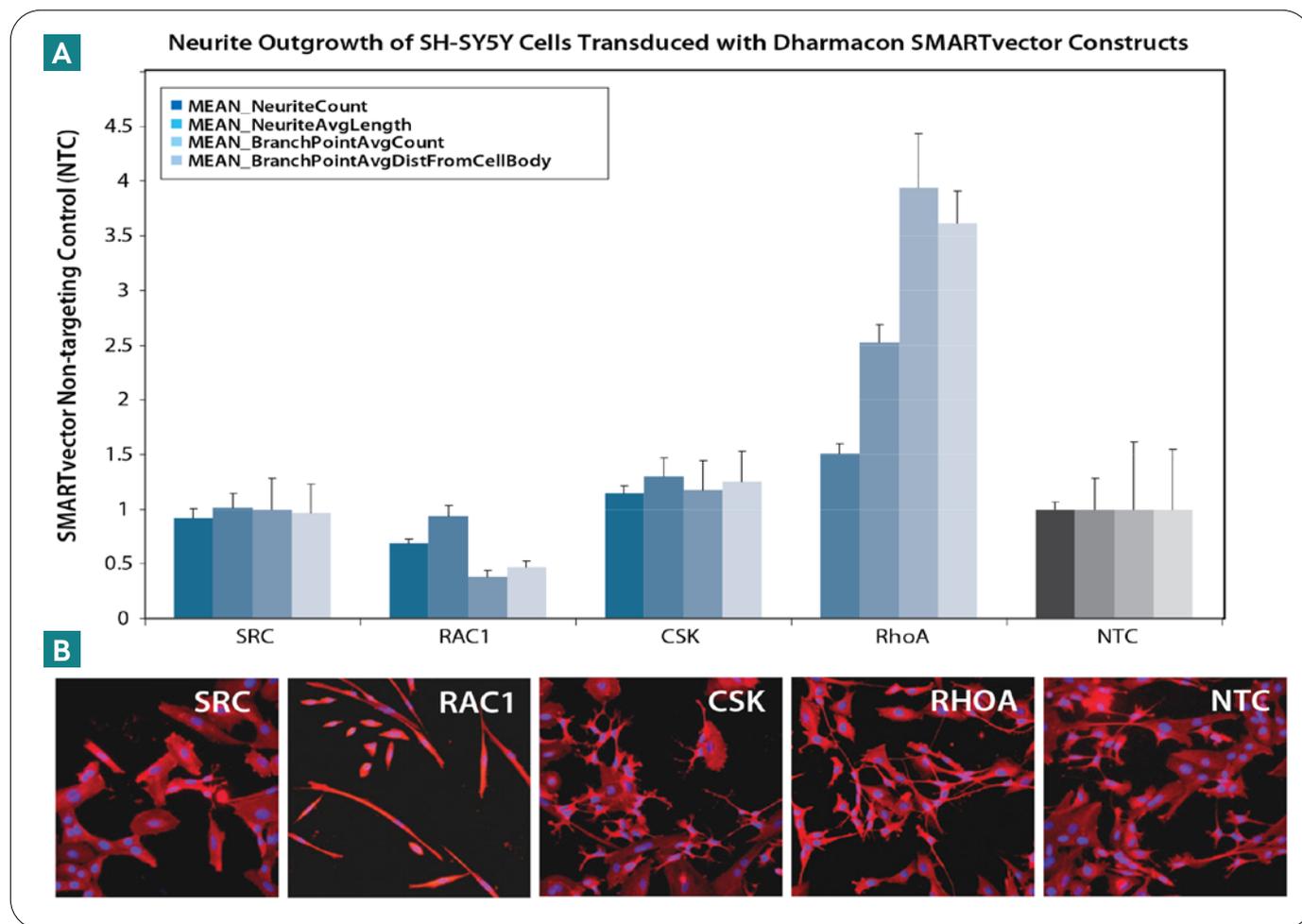


Figure 4: Neurite Outgrowth Nine Days Post-Transduction. A. Neurite outgrowth parameters were measured with the Cellomics™ Neuronal Profiling BioApplication (Mean Neurite Count, Mean Neurite Average Length, Mean Branchpoint Average Count and Mean Branchpoint Average Distance from the Cell Body) in untreated SH-SY5Y cells. Data for targeted genes (SRC, RAC1, CSK and RHOA) was normalized to SMARTvector NTC treated under similar conditions. B. Representative micrographs of SH-SY5Y cells transduced with SMARTvector constructs. Cells were stained with Neurite Outgrowth HitKit (red) and Hoechst 33342 (blue).

Targeted gene knockdown results in differences in neurite outgrowth and response to retinoic acid

SH-SY5Y cells transduced with target knockdown constructs were cultured with or without ATRA for six days. Nine days post-transduction, cells were fixed and stained according to the Cellomics Neurite Outgrowth HCS Reagent Kit protocol. Plates were then scanned on the Cellomics ArrayScan VTI using the Neuronal Profiling BioApplication. Results from untreated and ATRA-treated transduced cells are shown in Figure 4 and 5, respectively. Representative micrographs illustrating the phenotypes of SH-SY5Y cells transduced with different target-specific SMARTvector shRNA Lentiviral Particles and subsequently treated with ATRA or untreated are also shown.

In the absence of an ATRA signal, depletion of RHOA expression in SH-SY5Y cells led to neurite outgrowth phenotypes that had ~ 2.5-fold longer neurites with a ~ 4-fold increase in branchpoints compared to the SMARTvector Non-targeting Control-transduced cells (Figure 4). Knockdown of RHOA in ATRA-treated cells did not lead to a significant increase in neurite outgrowth (Figure 5). In contrast, silencing RAC1 expression in untreated cells resulted in fewer neurites and any associated branching was blocked. Furthermore, depletion of RAC1 strongly inhibited ATRA-induced neurite outgrowth so that branching was diminished ~ 10-fold, while neurite count and length was decreased ~ 2-fold compared to the SMARTvector NTC (Figure 5). Depletion of SRC also significantly inhibited neurite outgrowth induced by ATRA. However, knockdown of SRC and CSK expression in untreated cells had little to no effect on neurite formation. CSK silencing had no effect on neuronal differentiation in ATRA-treated cells.

Conclusion

One of the aims of neurobiology is to identify those factors that promote differentiation and inhibit proliferation to apply as possible therapeutics for treatment of neuronal disease, brain injury and cancers. To achieve this goal requires a better understanding of complexities involved in neuronal differentiation. A critical challenge for the use of *in vitro* neuronal model systems is that the relevant cell types tend to be refractory to standard RNAi delivery methods. In addition, the neuronal phenotypes under investigation require potent and sustained target gene knockdown in order to observe the morphological consequences. SMARTvector shRNA Lentiviral Particles successfully address both challenges by permitting sustained and extended knockdown in difficult-to-transfect cell types, like SH-SY5Y, which have historically been inaccessible to RNAi approaches.

The work described here demonstrates the application of the SMARTvector shRNA lentiviral platform to investigate the specific roles of individual genes in neuronal differentiation. Neurite outgrowth represents a key indicator of neuronal differentiation and requires synchronized changes between the actin cytoskeleton and the microtubule network. These changes are regulated by signaling pathways triggered by neurotrophins such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and retinoic acid (RA)³. Retinoic acid, a metabolic derivative of vitamin A, is important to both development and differentiation of the nervous system. RA-induced signaling (Figure 6) is initiated by binding to members of the nuclear hormone receptor families (such as RAR and RXR)⁴. The RA-RAR complexes are capable of binding to RA responsive elements (RARE) in the promoter regions of target genes and independently activating the Ras/PI3K/Rac1 pathway which results in cytoskeletal reorganization¹⁻².

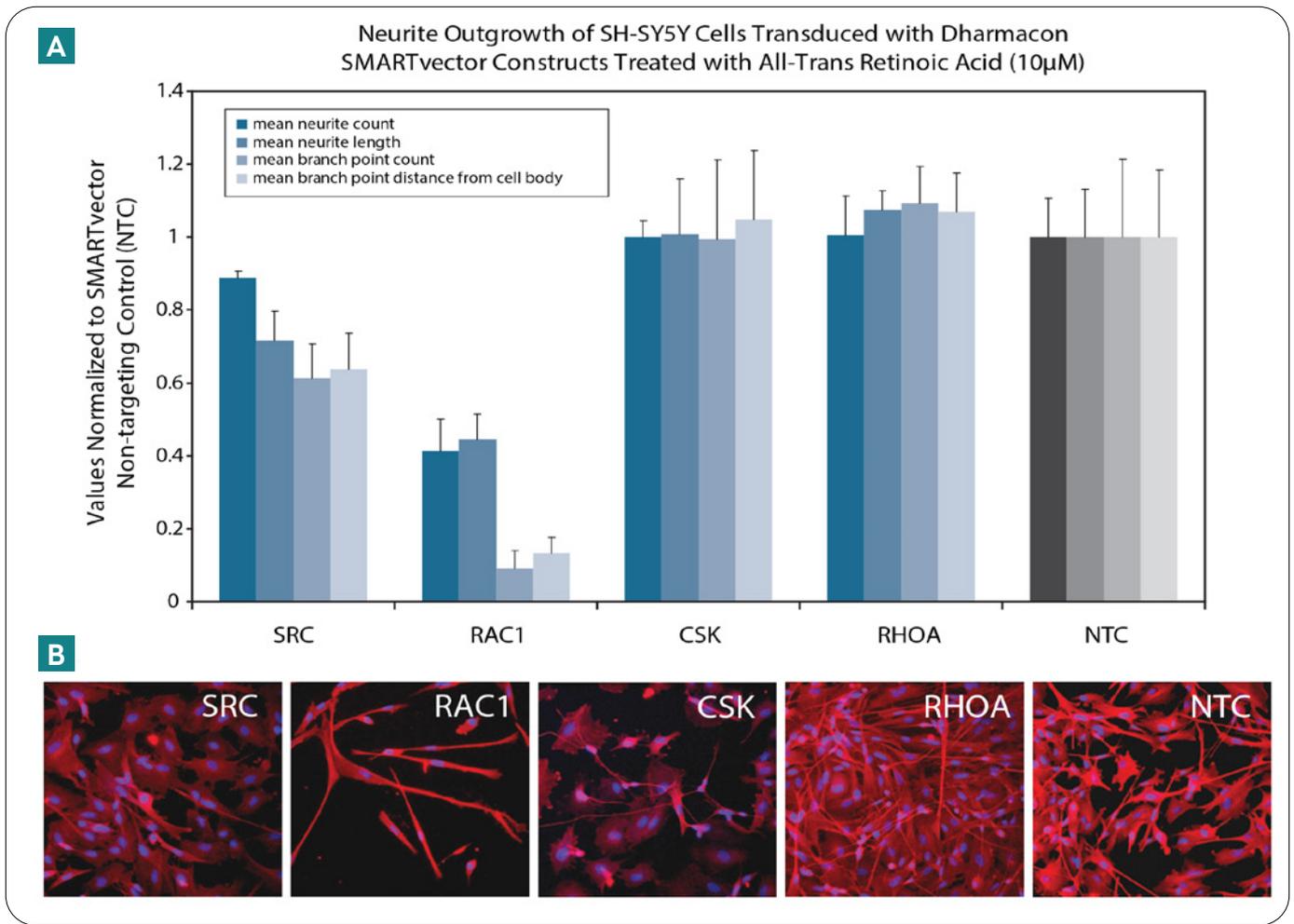


Figure 5: Neurite Outgrowth in Cells Treated with ATRA for Nine Days Post-Transduction. A. In ATRA-treated cells (10 µM), neurite outgrowth parameters measured with Cellomics Neuronal Profiling BioApplication (Mean Neurite Count, Mean Neurite Average Length, Mean Branchpoint Average Count and Mean Branchpoint Average Distance from the Cell Body) are shown for gene targets SRC, RAC1, CSK and RHOA. Data was normalized to SMARTvector Non-targeting Control treated under similar conditions. B. Representative micrographs of SH-SY5Y cells transduced with SMARTvector constructs. Cells were stained with Neurite Outgrowth HitKit (red) and Hoechst 33342 (blue).

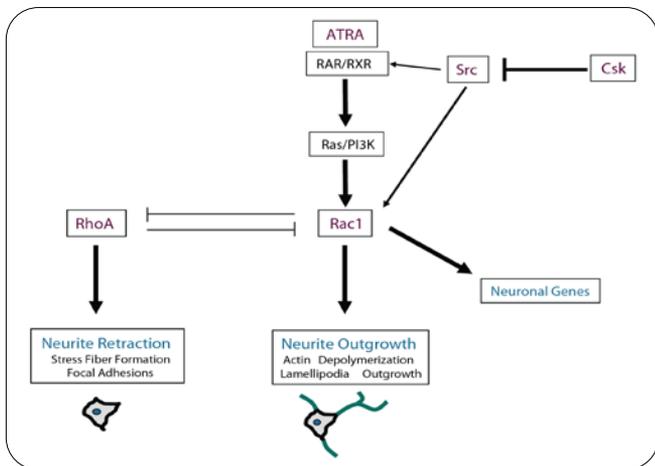


Figure 6: Retinoic Acid Signaling Pathway

Rho-GTPases are able to modulate both the cytoskeleton and transcriptional activation of target genes⁵. For example, Rac1 activity leads to actin depolymerization and lamellipodia outgrowth essential for neuritogenesis. In addition to cytoskeletal reorganization, interaction of Rac1 with Map3k1 activates the Mapk pathway leading to transcription of neuronal genes. In contrast, RhoA activity results in stress fiber formation and focal adhesions that hinder neuritogenesis. Long-term knockdown of RHOA and RAC1 in SH-SY5Y cells with the SMARTvector shRNA Lentiviral Particles resulted in measurable and quantifiable effects on neurite outgrowth. Silencing of RHOA enhanced neurite outgrowth, while depletion of RAC1 inhibited the ability of the cells to generate and maintain neurites even in the presence of ATRA.

These results are consistent with recent reports that show similar effects with overexpression of dominant negative versions of Rac 1 and RhoA whereas constitutively active mutants expressed in other cell types demonstrated opposing effects⁶⁻⁷.

The RA-RAR complex also interacts with Src, a non-receptor tyrosine kinase, to initiate downstream signaling for neuritogenesis⁴. Chemical inhibitors of Src block neurite outgrowth suggesting a key role for this gene in neuritogenesis. As expected, when SRC expression was silenced in SH-SY5Y cells transduced with SMARTvector SRC shRNA Particles, ATRA-induced neurite outgrowth was blocked although to a lesser extent than with RAC1 silencing. The activity of SRC is regulated by CSK (C-terminal Src Kinase) which phosphorylates and inhibits SRC. CSK overexpression has been shown to block activation of SRC, thus inhibiting RA-induced neurite outgrowth⁴. In this study, we demonstrated that while expression of CSK can inhibit neurite outgrowth, silencing of CSK expression has no significant effect on promoting neuritogenesis indicating a non-essential role for this member of the pathway.

Summary

Potent and long-term knockdown of genes known to be involved in regulation of neurite outgrowth (RAC1, RHOA, SRC, CSK) was successfully achieved in the difficult-to-transfect SH-SY5Y cell line using the SMARTvector shRNA lentiviral technology. The Neuronal Profiling BioApplication software and Cellomics™ ArrayScan™ VTI HCS Reader further enabled detailed analysis that distinguished the phenotypes of neuroblast-like cells from the epithelial cells in a mixed population characteristic of SH-SY5Y cells. Moreover, our silencing experiments resulted in data consistent with the expected activities of genes known to be important to neurite outgrowth, further supporting the power of combining these biological and detection technologies.

References

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