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Characterization of the human oncogene SCL/TAL1 interrupting locus (*Stil*) mediated Sonic hedgehog (*Shh*) signaling transduction in proliferating mammalian dopaminergic neurons

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ABSTRACT

The human oncogene SCL/TAL1 interrupting locus (*Stil*) is highly conserved in all vertebrate species. In humans, the expression of *Stil* is involved in cancer cell survival, apoptosis and proliferation. In this research, we investigated the roles of *Stil* expression in cell proliferation of mammalian dopaminergic (DA) PC12 cells. *Stil* functions through the Sonic hedgehog (*Shh*) signal transduction pathway. Co-immunoprecipitation tests revealed that STIL interacts with *Shh* downstream components, which include SUFU and GLI1. By examining the expression of *Stil*, *Gli1*, CyclinD2 (cell-cycle marker) and PCNA (proliferating cell nuclear antigen), we found that up-regulation of *Stil* expression (transfection with overexpression plasmids) increased *Shh* signaling transduction and PC12 cell proliferation, whereas down-regulation of *Stil* expression (by shRNA) inhibited *Shh* signaling transduction, and thereby decreased PC12 cell proliferation. Transient transfection of PC12 cells with *Stil* knockdown or overexpression plasmids did not affect PC12 cell neural differentiation, further indicating the specific roles of *Stil* in cell proliferation. The results from this research suggest that *Stil* may serve as a bio-marker for neurological diseases involved in DA neurons, such as Parkinson's disease.

1. Introduction

The human oncogene SCL/TAL1 interrupting locus (*Stil*) was isolated from a leukemia-associated chromosomal translocation in T-cells [1]. *Stil* encodes a protein of 1287 amino acids which was originally believed to have no significant homologies to other proteins and structural motifs [2]. Recent studies suggested that *Stil* shares partial sequence similarities to the C-terminus of TGF-β of mammal cells and the centriole duplication factor Ana2 in Drosophila [3,4]. In mammals, the expression of *Stil* is required for cell-cycle mitotic entry as well as centriole formation and duplication [5–9]. In humans, *Stil* plays a role in cancer cell survival and proliferation [10–12]. Inherited mutations in the *Stil* locus has also been suggested as candidates that lead to abnormal brain development, such as holoprosencephaly [13,14].

Stil is highly conserved in all vertebrate species and is involved in several signal transduction pathways, which include the Sonic hedgehog (*Shh*) pathway [15–17]. In human pancreatic cancer cells, for example, overexpression of *Stil* attenuated the tethering of *Shh* signaling suppressor SUFU, relieving its repression to GLI1, thereby increasing *Shh*-downstream gene expression and cancer cell proliferation. In contrast, down-regulation of *Stil* expression induced nuclear accumulation of SUFU, enhanced SUFU inhibition to GLI1, thereby leading to decreases in *Shh*-target gene transcription and cell proliferation [17]. In some *in vivo* models, the expression of *Stil* plays important roles in the regulation of organ development. In mice, for example, through the *Shh* pathway *Stil* played important roles for specification of body axial and development of the central nervous system (CNS). Knockdown of *Stil* or *Shh*-downstream gene expression resulted in animal lethality after embryonic day 10.5 [16]. In zebrafish *cassiopeia* mutants, down-regulation of *Stil* expression resulted in disorganized mitotic spindles and abnormal cell growth, and the embryos died between 7 and 10 days post-fertilization [18]. A recent study using the mutant zebrafish models suggested a novel function of *Stil* in the vertebrate CNS, namely neural protection [19]. In zebrafish *night blindness b* (*nbb*) mutants, for example, down-regulation of *Stil* expression or inactivation of *Stil*-mediated *Shh* signal transduction increased the susceptibility of dopaminergic (DA) cells to neurotoxins (such as 6-hydroxydopamine; 6-OHDA, which is known to

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selectively destroy DA cells), thereby leading to cell apoptosis. Inhibition of *Stil* expression by morpholinos in wild-type zebrafish mimicked the phenotypes in *nbb* mutant fish. In contrast, activation of the *Shh* pathway in mutant fish (i.e., by knockdown of the *Shh* repressor SUFU) decreased the susceptibility of DA cells to neurotoxic insult, thereby preventing DA cells from toxin-induced neural degeneration [19].

While the functions of *Stil* expression in cancer cells and the roles of *Stil*-mediated *Shh* signaling transduction in animal development and DA neural protection have been described, it is not known if the expression of *Stil* has a role (e.g., differentiation, growth, synthesis or release of neurotransmitter) in mammalian neurons. In this research, we examined the function of *Stil* in mammalian neural cells using the catecholaminergic PC12 cell line. The PC12 cell line was originally derived from a pheochromocytoma of the rat adrenal medulla [20]. PC12 cells proliferate rapidly when they are cultured in growth conditions. In low-serum culture medium, PC12 cells can be induced by nerve growth factor (NGF) to differentiate into sympathetic neuron-like DA cell types [21,22]. We transfected PC12 cells with *Stil* shRNA or overexpression plasmids, and then examined the roles of *Stil* gene expression in PC12 cell proliferation. In addition, we correlated the expression of *Stil* to subsequent *Shh* signaling transduction (such as *Shh*-target *Gli1* transcription) as well as to the expression of cell cycle marker CyclinD2 and proliferating cell nuclear antigen (PCNA). The results provide direct evidence for the involvement of *Stil* in mammalian dopaminergic cell proliferation. The results also offer insights for the development of new treatment strategies for neurological diseases caused by degeneration of dopaminergic neurons, such as Parkinson's disease.

2. Materials and methods

2.1. Cell culture

PC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 10% donor horse serum, penicillin (100 U/mL) and streptomycin (100 g/mL) at 37 °C and 5% CO_2. Medium was changed every 2 days, and cells were passaged every 3–5 days.

2.2. Plasmid construction and transfection

Pre-designed siRNA duplexes (based on the sequence of the rat *Stil* gene, XM_001069563.2) were used for *Stil* knockdown. The DNA fragment for shRNA was designed (5′-GGACUUU GUUCAAGA GAUACG AAAG-3′) and sub-cloned into the RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (Clontech). Full-length human *Stil* DNA (5′-TAGGGCCCCATGGAGCCTATATATCC-3′; 5′-GGGCGGCCGCGGTT AAAATAAT TTTGGT-3′) was sub-cloned into the pcDNA3.1 vector (Invitrogen) and used for *Stil* overexpression.

PC12 cells were transfected with plasmid DNA by Amaxa Nucleofector (Lonza). The efficiency of *Stil* knockdown or overexpression plasmid transfection was confirmed by Western blot of whole-cell lysate using rabbit polyclonal antibodies against STIL (Santa Cruz Biotechnology).

2.3. RT-PCR

Total RNA was extracted from PC12 cells using RNA pure cell kit (Tiangen Biotech) and reverse-transcribed by M-MLV reverse transcriptase (Promega). Real-time RT-PCR was performed using SYBR Green Master Mix reagent (Cowin Biosci). RT-PCR was performed according to standard protocols, and the melting curve analysis was performed using IQ5 software (Bio-Rad). Primer sequences: *Stil*, forward 5′-TATGGG CTTGCTGCTTGAGATAC-3′, reverse 5′-CAGGTT CCTTATGTGTCAATGAA-3′; *Gli1*, forward 5′-AGCTCCTGTGTAATTA CGTTCAGTC-3′, reverse 5′-GGCTCTGACTAACTTGAGAA CCTC-3′; *CyclinD2*, forward 5′-CATTGAGCACATCCTTCGCAA-3′, reverse 5′-CAT TCACTTCCTC GTCCTGCT-3′. *Gapdh*, forward 5′-GCACAGTCAAGGCC GAGAAT-3′, reverse 5′-GCCTTCTCCATG GTGGTGAA-3′.

2.4. Protein extraction and Western blot

Cells were washed in PBS and resuspended in lysis buffer: 50 mM NaCl, 10 mM Tris (pH 8.0), 1 mM DTT, 1% Triton X-100, Aprotinin, Leupeptin and PMSF, and incubated for 30 min on ice and centrifuged at 12,000*g* for 20 min. Supernatant was collected, and protein concentration was determined by using the BCA kit (Tiangen Biotech). Proteins were resuspended in loading buffer, denatured in 95 °C for 5 min and separated on SDS–PAGE gel. Proteins were transferred in Tris–glycine running buffer, and then electro-transferred onto nitrocellulose membranes and blocked with 5% skim milk in TBST. Membranes were incubated with primary antibodies (see below) overnight at 4 °C, and then washed with TBST, followed by incubation with HRP-conjugated secondary antibodies (1:3000, Abcam) for 1 h at room temperature. HRP was detected by exposure of the membrane to chemiluminescent substrate (Millipore).

Primary antibodies used in this study included the following: rabbit polyclonal antibodies against STIL (1:200, Santa Cruz Biotechnology), GLI1 (1:1000, Abcam), SUFU (1:500, Abcam), CyclinD2 (1:500, Boster Bioengin), TH (1:5000, Sigma), PCNA (1:3000, Sigma), and β-ACTIN (1:5000, Boster Bioengin).

2.5. Co-immunoprecipitation

PC12 cells were lysed with immunoprecipitation buffer: 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 1% Triton X-100, 0.5% NP-40 and protease inhibitors (Millipore) for 30 min at 4 °C. The crude cell lsyate was centrifuged at 12,000*g* for 20 min and assayed for protein concentration using the BCA Assay Kit. Concentration was adjusted to 1 mg/mL with immunoprecipitation buffer. The antibody–antigen complexes were isolated by magnetic separation using crude cell lysate, 2 μg of antibodies (polyclonal rabbit anti-STIL, Abcam; polyclonal rabbit anti-GLI1, Abcam), and Protein A magnetic beads according to protocol (New England Biosciences). The pellets were resuspended in 3× SDS loading buffer (187.5 mM Tris–HCl, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromophenol blue, 2% β-mercaptoethanol), subjected to a magnetic field and were analyzed by Western blot.

2.6. Cell proliferation assay

The rate of cell proliferation was assessed by using Cell Counting Assay Kit-8 (Cck-8) (Dojindo Labs). Cells were cultured in collagen-coated 96-well plates at an initial density of 50,000 cells per well. Cck-8 solution (10 μL) was applied to each well, and

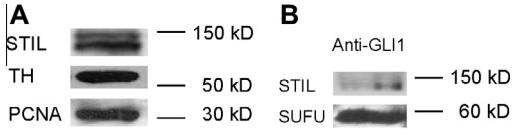

Fig. 1. Western blot and co-immunoprecipitation of STIL in PC12 cells. (A) STIL, TH and PCNA expression in proliferating PC12 cells (cultured in complete medium, with 15% serum). STIL were detected in two bands at approximately 143 kD. (B) Co-immunoprecipitation of STIL and *Shh*-downstream proteins. PC12 cell lysates were co-immunoprecipitated with anti-GLI1 antibodies, and then analyzed by Western blot with STIL and SUFU antibodies, respectively. A complex of GLI1, STIL and SUFU was detected by using anti-GLI1 pull-downs.

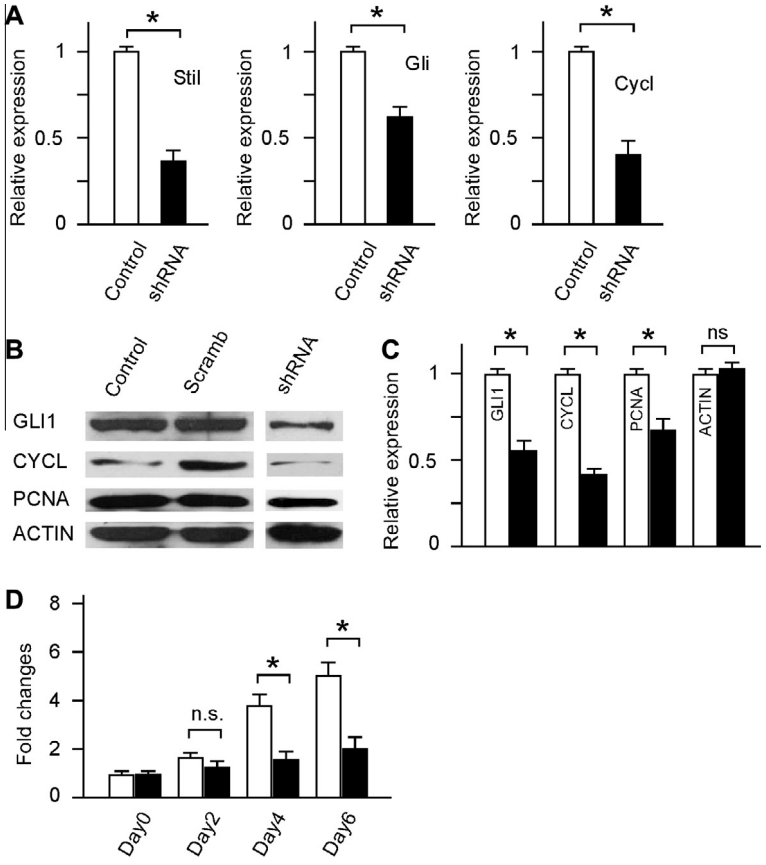

Fig. 2. Effects of *Stil* knockdown on *Shh*-target gene and protein expressions and cell proliferation. (A) RT-PCR analysis of *Stil*, *Gli1* and CyclinD2 mRNA expression after *Stil* knockdown (transfected by pSIREN-*Stil*-shRNA; black bars). The expression in control cells (transfected with scrambled plasmid; white bars) was normalized to 1, respectively. Note the decrease of *Stil*, *Gli1* and CyclinD2 expression in *Stil* knockdown cells. (B) Western blot of GLI1, CyclinD2 and PCNA in *Stil* knockdown and control cells. Note the decrease of protein expression after *Stil* knockdown. Actin was used as a loading control. (C) Quantitative analysis of protein expression in *Stil* knockdown (black bars) and control cells (white bars). Protein expression in control cells was normalized to 1, respectively. Note the decrease of GLI1, CyclinD2 and PCNA expression after *Stil* knockdown. (D) *Stil* knockdown decreased PC12 cell proliferation. Data was normalized by values collected on the first day of proliferation assays (designated day 0, set to 1). Knockdown of *Stil* expression (transfected with pSIREN-*Stil*-shRNA; black bars) decreased the proliferation rate of PC12 cells in comparison to controls (transfected with scramble plasmid, while bars). Data represent the Mean ± SEM; $^{*}p < 0.05$; ns, not significant.

the cells were incubated for 1 h at 37 °C in 5% CO_2. The absorbance at 490 nm was determined by using the automatic multi-wavelength spectrophotometer (Bio-Rad). For each experiment, the assays were performed in triplicate.

3. Results

3.1. STIL is expressed in proliferating PC12 cells and interacts with Shh-downstream components

STIL is expressed in PC12 cells cultured in growth conditions (in complete medium, with 15% serum). Western blot of the whole-cell lysate of PC12 cells with polyclonal anti-STIL antibodies revealed two bands of STIL proteins at expected sizes (~143 kD; Fig. 1A). The expression of dopaminergic cell marker tyrosine hydroxylase in PC12 cells and the proliferation of PC12 cells were confirmed by Western blot using anti-TH and anti-PCNA antibodies, respectively (Fig. 1A). In human cancer cells, STIL interacts with *Shh* downstream components, i.e., STIL binds to SUFU in the SUFU/GLI1 complex. This is essential to de-repress GLI1 from SUFU for transcription of *Shh*-target genes [15,16]. We performed co-immunoprecipitation experiments with anti-GLI1 antibodies using PC12 whole-cell lysates (Fig. 1B). The data suggests that STIL interacts with the SUFU/GLI1 complex in PC12 cells.

3.2. Knockdown of Stil expression decreased Shh signaling transduction and PC12 cell proliferation

We examined *Shh* signaling in PC12 cells transfected with *Stil* knockdown plasmids (pSIREN-*Stil*-shRNA). The efficiency of *Stil* shRNA knockdown was confirmed by decreased *Stil* mRNA and protein expression, respectively. In response to *Stil* shRNA knockdown, the expression of *Stil* mRNA decreased to 37.1 ± 6.4% of the expression detected in control cells (transfected with pSIREN scrambled plasmid) (Fig. 2A). Because *Stil* functions in the *Shh* pathway, *Stil* knockdown resulted in decreases in *Shh* signaling transduction. In *Stil* shRNA knockdown cells, the expression of *Shh*-target gene *Gli1* mRNA decreased to 62.3 ± 5.5% of the control level (Fig. 2A). The expression of GLI1 protein also decreased to 55.8 ± 6.5% of the expression measured in control cells (Fig. 2B and C).

Stil knockdown resulted in decreased PC12 cell proliferation. In *Stil* shRNA knockdown cells, the expression of cell cycle marker CyclinD2 mRNA decreased to 41.2 ± 7.9% of the expression measured in control cells (Fig. 2A). The expression of CyclinD2 protein also decreased, i.e., to 42.4 ± 3.6% of the control level (Fig. 2B and C). Along with the decrease in ClyclinD2 expression, PCNA expression also decreased (to 66.8 ± 7.4% of the control level; Fig. 2B and C). Analysis with the Cck8 cell viability assay revealed that *Stil* shRNA knockdown significantly decreased the rate of PC12 cell proliferation (Fig. 2D). After 6 days of proliferation, for example, *Stil*

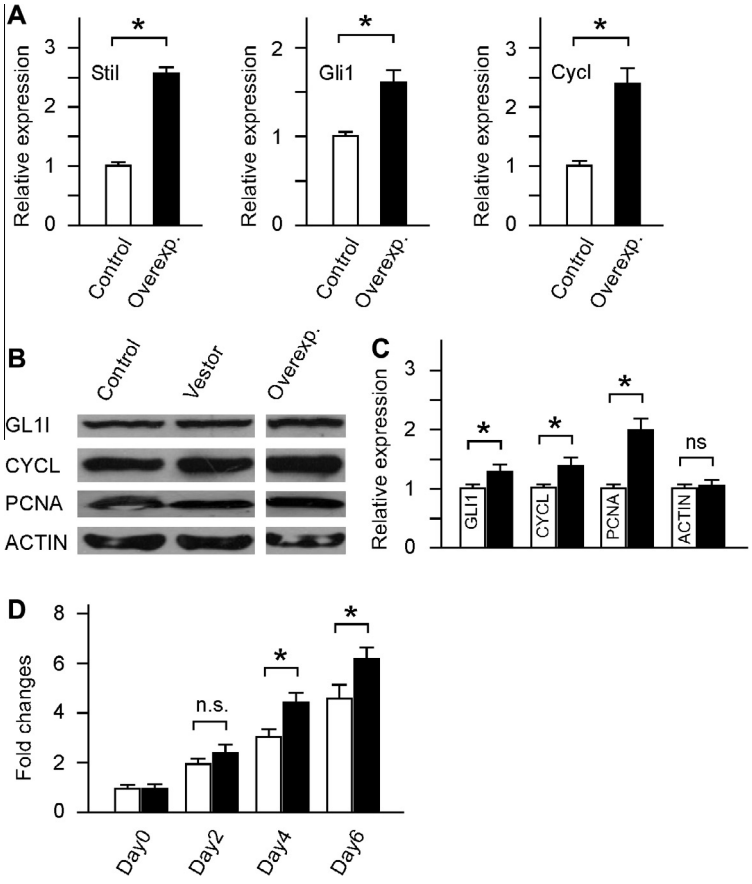

Fig. 3. Effects of *Stil* overexpression on *Shh*-target gene and protein expressions and cell proliferation. (A) RT-PCR analysis of *Stil*, *Gli1* and CyclinD2 mRNA expression in response to *Stil* overexpression (transfected by pcDNA3.1-Flh-*Stil* plasmids; black bars). The expression in control cells (transfected with empty vectors; white bars) was normalized to 1, respectively. Note the increase of *Stil*, *Gli1* and CyclinD2 expression after *Stil* overexpression. (B) Western blot of GLI1, CyclinD2 and PCNA in *Stil* overexpression and control cells. Note the increase of protein expression in *Stil* overexpression cells. Actin was used as a loading control. (C) Quantitative analysis of protein expression in *Stil* overexpression (black bars) and control cells (white bars). Protein expression in control cells was normalized to 1, respectively. Note the increase of GLI1, CyclinD2 and PCNA expression in *Stil* overexpression cells. (D) Overexpression of *Stil* increased PC12 cell proliferation. Data was normalized by values collected on the first day of proliferation assays (designated day 0, set to 1). Overexpression of *Stil* (transfected with pcDNA3.1-Flh-*Stil*; black bars) increased the proliferation rate of PC12 cells in comparison to controls (transfected with empty vectors, while bars). Data represent the Mean ± SEM; $^{*}p < 0.05$; ns, not significant.

shRNA knockdown cells increased only 2.0 ± 0.5-fold, whereas control cells increased 5.1 ± 0.6-fold ($p < 0.05$).

3.3. Overexpression of Stil increased Shh signaling transduction and PC12 cell proliferation

We examined the growth rate of PC12 cells transfected with *Stil* overexpression plasmids (pcDNA3.1-Flh-*Stil*). The efficiency of plasmid transfection was confirmed by RT-PCR and Western blot analysis of *Stil* mRNA and protein expression. In pcDNA3.1-Flh-*Stil* transfected cells, the expression of *Stil* mRNA increased 2.6 ± 0.1-fold in comparison to the expression in control cells (transfected with pcDNA3.1 empty vector) (Fig. 3A). Overexpression of *Stil* also resulted in increased *Gli1* mRNA and protein expression, i.e., by 1.6 ± 0.1-fold and 1.3 ± 0.1-fold respectively, in comparison to the control level (Fig. 3A–C).

Overexpression of *Stil* increased PC12 cell division and growth. In pcDNA3.1-Flh-*Stil* transfected cells, the expression of CyclinD2 mRNA and protein increased 2.4 ± 0.3 and 1.4 ± 0.1-fold, respectively, in comparison to the expression measured in control cells (Fig. 3A–C). Along with the increase in CyclinD2 expression, PCNA expression also increased, to levels approximately 2.0 ± 0.2-fold higher than the expression in control cells (Fig. 3B and C). Analysis with the Cck8 cell viability assay revealed that overexpression of *Stil* promoted PC12 cell proliferation (Fig. 3D). After 6 days of proliferation, for example, control cells increased 4.5 ± 0.5-fold, whereas *Stil*-overexpression cells increased 6.2 ± 0.5-fold ($p < 0.05$).

4. Discussion

Stil is a human oncogene that is highly conserved in vertebrate species [23]. The expression of STIL increases *Shh* signaling transduction. Normally, SUFU functions as a repressor which inhibits *Shh*-target *Gli1* transcription. The binding of STIL to SUFU lifts the suppression from SUFU to GLI1, releases GLI1 for nuclear translocation, thereby increasing *Shh*-target gene transcription [15–17]. Using co-immunoprecipitation assays, we demonstrated that in PC12 cells STIL interacts with GLI1 and SUFU. Considering the conserved roles of STIL in different species and cell types, it is conceivable to suggest that increases in STIL expression will result in increased binding of STIL to SUFU, thereby augmenting the level of non-repressed GLI1 for downstream transcription events that are needed for *Shh*-driven processes (e.g., gene transcription and cell proliferation). In contrast, the lack of STIL expression will result in constant suppression of GLI1 by SUFU and subsequent inhibition of *Shh* signaling downstream events, i.e., GLI1-mediated gene transcription and cell proliferation.

Previous studies using pharmacological approaches targeting *Shh*-downstream Smoothened (Smo) receptor also suggested the role of *Stil* in the *Shh* pathway in PC12 cells [24]. For example, treatment with Smo receptor antagonist cyclopamine (which

blocks the *Shh* signal transduction pathway) decreased the rate of PC12 cell proliferation. Overexpression of *Stil* rescued the proliferation defects caused by *Shh* inhibition. Conversely, activation of the *Shh* signaling transduction pathway (by Smoothened receptor agonist purmorphamine) increased PC12 cell proliferation, but the effects of increased *Shh* signaling on PC12 cell proliferation diminished after *Stil* shRNA knock-down. In the current study, by monitoring *Gli1* mRNA and protein expression in response to *Stil* knockdown or overexpression, we provided direct evidence for the involvement of *Stil* in *Shh* signaling in PC12 cell proliferation. That is, overexpression of *Stil* increased *Shh* signaling transduction (i.e., *Gli1* expression), thereby increasing PC12 cell proliferation. Down-regulation of *Stil* expression inhibited *Shh* signal transduction and decreased the rate of PC12 cell proliferation.

It appeared that *Stil* knockdown had a greater effect than *Stil* overexpression on PC12 cell proliferation. For example, knockdown of *Stil* decreased cell proliferation by 2–3 folds in comparison to control cells, whereas overexpression only increased cell proliferation by 20–25%. This may be due to several reasons. First, because *Stil* shRNA knockdown blocks the *Shh* pathway, which is essential for gene transcription involved in cell proliferation, it is conceivable to hypothesize that knockdown of *Stil* expression may cause dramatic decreases in cell proliferation due to blockage of *Shh* signaling. In contrast, transfection with the overexpression plasmids may only increase cytoplasmic or nuclear STIL concentrations, but the increase will only be incremental. Thus, the exogenous STIL may only have minor impacts on *Shh* signaling evens (e.g., gene transcription and cell proliferation). Second, because STIL itself is involved in mitotic entry, it is possible that knockdown of STIL expression will block the initial cell-cycle events. In contrast, exogenous overexpression of STIL may enhance the initial cell-cycle events, but no dramatic increases in cell proliferation may occur.

The expression of *Stil* is not required for PC12 cell neural differentiation [24]. This is not unexpected, considering the primary roles of *Stil* in mitotic entry, spindle pole organization, centriole formation and duplication [7,9]. It has been reported that in mouse fibroblast cells, the expression of *Stil* is down-regulated during cell differentiation [23]. In PC12 cells, however, we observed constant expression of *Stil* during NGF induction. It is possible that constitutive expression of *Stil* in differentiated PC12 cells is critical for cellular functions, such as neurite out-growth, synthesis or release of neurotransmitters, or neural protection. This supports the role of *Stil* in the regulation of cell growth, such as microtubule elongation [25].

The finding that the mammalian dopaminergic PC12 cells are affected by *Stil* expression implies that *Stil* is required for the function of neural progenitor cells. To some extent, PC12 cells are "cancerous" in a sense that they are derived from a pheochromocytoma in the medulla of the adrenal gland (i.e., "tumor" of the neuroendocrine chromaffin cells in the sympathetic nervous system). However, they are also "precursor" cells that can be induced to differentiate into mature post-synaptic sympathetic-like neurons. Thus, decreases in proliferation of these neural precursor cells will yield a smaller population of available progenitors for subsequent neural proliferation and differentiation. This may hold true for *in vivo* neural stem cell populations that are *Stil* deficient, such as catecholaminergic (e.g., DA) cells in mutant or knock-out animals. Although the mechanisms that underlie *Stil*-mediated modulation of drug sensitivity remain to be further studied, the findings hint that *Stil* may play a major role in future development of therapeutic treatments for neurologic diseases involved in dopaminergic cells, such as Parkinson's disease.

Acknowledgments

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