Transcription of the SCL/TAL1 Interrupting Locus (Stil) Is Required for Cell Proliferation in Adult Zebrafish Retinas*

Received for publication, July 30, 2013, and in revised form, January 13, 2014 Published, JBC Papers in Press, January 27, 2014, DOI 10.1074/jbc.M113.506295

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Background: Stil is a human oncogene that is involved in cancer cell proliferation and survival.
Results: In adult zebrafish retinas, knockdown of Stil expression decreases the rate of cell proliferation after degeneration.
Conclusion: Through the Shh signal transduction pathway, Stil plays important roles in regulating cell proliferation.
Significance: The results uncover a novel function of Stil in adult tissues, namely neural regeneration.

The human oncogene SCL/TAL1 interrupting locus (Stil) is highly conserved in vertebrate species. Previously, we identified a homolog of the Stil gene in zebrafish mutant (night blindness b, nbb), which showed neural defects in the retina (e.g. dopaminergic cell degeneration and/or lack of regeneration). In this research, we examined the roles of Stil in cell proliferation after degeneration in adult zebrafish retinas. We demonstrated that knockdown of Stil gene expression or inhibition of Sonic hedgehog (Shh) signaling transduction decreases the rate of cell proliferation. In contrast, activation of Shh signal transduction promotes cell proliferation. In nbb +/− retinas, inhibition of SUFU (a repressor in the Shh pathway) rescues the defects in cell proliferation due to down-regulation of Stil gene expression. The latter data suggest that Stil play a role in cell proliferation through the Shh signal transduction pathway.

The SCL/TAL1 interrupting locus (Stil) was originally identified from leukemia-associated chromosomal translocation (1). Stil encodes a 143-kDa protein, which possesses no clear homology to other functional protein families or motifs (1–4). Stil is conserved in all vertebrate species examined thus far. In mice, functional expression of Stil is required for animal development. In homozygous Stil mutants, the embryos develop abnormally and die during embryonic stages (5, 6). In mammalian cell lines, the expression of Stil is required for the reproduction of centrosomes and primary cilia formation (7). Recent studies suggest that Stil also plays a role in tumor cell survival (8–11). In cancer cells, knockdown of Stil expression leads to cell apoptosis due to delayed mitotic entry between cell cycles (11).

Stil functions in the Sonic hedgehog (Shh) signal transduction pathway (5, 6, 12). The expression of Stil increases the Shh-targeted gene expression by inhibition of SUFU, which normally functions as a repressor in the Shh pathway. In neurons, SUFU depresses Shh signal transduction by suppressing transcription of the Shh-targeted gene Gli1. When Stil is expressed, SUFU binds SUFU, thereby relieving the inhibition of SUFU to Gli1 and resuming the transcription of Gli1 (12).

Several zebrafish mutations have been reported that are allelic of the Stil gene. These include Hi1262Tg, cassiopeia, and night blindness b (13–15). The essential roles of the expression of Stil in cell cycle and development in zebrafish have been carefully characterized (14). In a recent study, we demonstrated a novel function of Stil in adult animals, i.e. neural protection (16). In zebrafish, deficiency in the expression of Stil (i.e. in nbb +/+ mutants or wild-type fish treated with antisense Stil morpholinos) increased the susceptibility of dopaminergic (DA)3 cells to neurotoxin 6-hydroxydopamine (6-OHDA, a compound known to destroy DA neurons), thereby leading to cell apoptosis (16). The effect of Stil on DA cell drug susceptibility is likely mediated by the Shh signal transduction pathway. For example, inhibition of Shh signal transduction in wild-type fish (i.e. by treatment with Shh inhibitor cyclopamine) mimicked the defects in nbb +/− mutants. In contrast, activation of Shh signaling transduction (i.e. by knocking down SUFU expression with antisense morpholinos) decreased the susceptibility of DA cells to neurotoxins, thereby preventing DA cell death after drug treatment (16).

In this paper, we examined the role of Stil gene expression in cell proliferation in adult zebrafish retinas. Cell proliferation responses were induced by intraocular injections of 6-OHDA (16, 17). We examined the expression of Stil and the Shh-targeted gene Gli1 in regenerating retinas. We also correlated the gene expression of Stil and Gli1 to the expression of proliferating cell nuclear antigen (PCNA, which is expressed only in proliferating cells). The results provide the first evidence for the

*This work was supported, in whole or in part, by National Institutes of Health Grants R01-EY013147 and R01-EY018417, Chinese Natural Science Foundation Grant 81171066, Tianjin Science and Technology Grant 12JCZDJC24000, and the University of Notre Dame Center for Zebrafish Research.

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3 The abbreviations used are: DA, dopaminergic; Stil, SCL/TAL1 interrupting locus; nbb, night blindness b; PCNA, proliferating cell nuclear antigen; 6-OHDA, 6-hydroxydopamine; Shh, Sonic hedgehog; EdU, 5-ethyl-2′-deoxyuridine; MO, morpholino.
involvement of Stil and Stil-mediated Shh signal transduction in cell proliferation in adult neural tissues.

EXPERIMENTAL PROCEDURES

Fish Maintenance—Wild-type, mutant, and transgenic zebrafish were maintained in our zebrafish facility according to NIH animal care guidelines. Zebrafish were maintained in a 14:10 light-dark cycle in circulating water at 28.5 °C (18). The fish were fed twice a day with freshly hatched brine shrimps. All the experiments were conducted using adult zebrafish (between 8 and 20 months old).

Quantitative RT-PCR—Total RNA was isolated using TRIzol (Invitrogen). For each reaction, 4 retinas were used. For a 20-μl reaction, 1.0 μg of RNA was reverse transcribed by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For each experiment, 3 sets of quantitative RT-PCR were performed. Data were normalized by the 18 S rRNA quantity amount.

Western Blot—Retinas were treated with lysis buffer containing 50 mM NaCl, 10 mM Tris (pH 8.0), 2 mM MgCl2, 1 mM DTT, 1% Triton X-100, aprotin, leupeptin, and PMSF. For each blot, 4 adult zebrafish retinas were used. Proteins were electrophoresed by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST and incubated with antibodies: anti-SUFU (1:1000 anti-rabbit; Anaspec, Fremont, CA) and anti-ACTIN (1:3000 anti-rabbit; Sigma). After TBST washes, the membrane was incubated with peroxidase-conjugated secondary antibody (1:3,000 anti-rabbit; Sigma). After TBST washes, the membrane was incubated with peroxidase-conjugated secondary antibody (1:3,000 for SUFU, 1:30,000 for ACTIN; Jackson ImmunoResearch). The membrane was washed with TBST and incubated with antibodies: anti-SUFU (1:1000 anti-rabbit; Sigma). After TBST washes, the membrane was incubated with peroxidase-conjugated secondary antibody (1:3,000 for SUFU, 1:30,000 for ACTIN; Jackson ImmunoResearch).

Immunohistochemistry—Cryostat sections (10-μm thickness) were fixed in 4% formaldehyde at 4 °C. Following PBS washes, the sections were incubated in blocking solution (2% normal goat serum, 0.2% Triton X-100, 1% DMSO in PBS), and then with PCNA antibodies (1:200 anti-Mouse, Millipore). After PBS washes, the sections were incubated with secondary antibodies (1:500 anti-mouse Alexa Fluor 568 IgG; Invitrogen). The sections were viewed under a fluorescence microscope. PCNA-positive cells were counted from the sections adjacent to the optic nerve.

Intraocular Injections—Methods for zebrafish intraocular injections were similar as previously described (16, 17). Prior to injections, the fish were anesthetized with 0.016% 3-aminobenzoic acid ethyl ester. 6-OHDA (Sigma) and pargyline (Sigma) were dissolved in PBS at 5 μg/μl concentration. A 0.5-μl mixture of 1:1 6-OHDA and pargyline was injected into the vitreous of the eye. PBS was used for the control (sham) injection.

EdU (5-ethyl-2'-deoxyuridine; which incorporates into the replicating DNA and directly measures de novo DNA synthesis of proliferating cells) was used to identify proliferating cells. EdU was diluted in PBS to 1 mg/ml and injected (50 μl) at different times after 6-OHDA treatment. EdU detection was carried out using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen).

Cycloamine was used to inhibit Shh signaling transduction (16). Cycloamine (Sigma) was dissolved in ethanol at 10 μM. For each injection, a 0.5-μl drug solution was injected into the vitreous of the eye. Control (sham) injections included only ethanol. To increase Shh signaling transduction, antisense Sufu morpholinos (5’-GCTGCTAGGCCCATCTCATCCATC-3’; Gene Tool) were injected into the fish eye. For each treatment, a 0.5-μl solution that contained antisense Sufu morpholinos was injected. After the injection, electroporation was immediately performed using a CUY21 Square Wave Electroporator (Potech International) (19).

RESULTS

Cell Proliferation after 6-OHDA-induced Degeneration—Zebrafish retinas grow during the life of the animal. On a daily basis, a small number of retinal cells are produced from the progenitor cells in the germinal zone of the retina. Differentiated cells migrate toward the center of the retina, where they replace apoptotic cell types (20–28). In contrast, in response to massive cell losses due to injury (e.g. drug treatment, photobleaching or laser ablation), cell proliferation takes place mainly in the inner retina, i.e. by de-differentiation and proliferation of stem cell-like Müller glial cells and rod precursor cells. For example, intraocular applications of 6-OHDA destroy DA cells and trigger retinal cell proliferation and subsequent neural regeneration (16, 17). In transgenic zebrafish Tg(gfap::GFp), which express GFP in Müller glial cells (29), in response to 6-OHDA treatment (5 μg/μl), proliferation of retinal Müller glial cells and some of the outer nuclear layer cells, which are likely rod precursor cells, became evident. This was revealed by increases in the expression of PCNA (Fig. 1A). Proliferation of Müller glial cells and rod precursor cells was also evident by co-labeling 6-OHDA-treated retinas with PCNA and EdU (Fig. 1B). At 4 weeks post-6-OHDA treatment, EdU-positive cells persisted in the inner and outer nuclear layers (Fig. 1C), further demonstrating that cell proliferation had occurred in Müller glial cells and rod precursor cells. These cells will migrate, differentiate, and replace all the lost cell types (30–33).

We examined cell proliferation after 6-OHDA-induced degeneration in adult zebrafish retinas. 6-OHDA was delivered to zebrafish eyes via microinjections. At 2 days post-6-OHDA treatment, increases in the expression of PCNA were detected in Müller glial cells and rod precursor cells (Fig. 2A). Increase in the expression of PCNA was also observed in cells in the outer nuclear layer, which are either migrating Müller glial cells or differentiating rod precursor cells. In the next few days, the expression of PCNA continually increased. By 6 days post-6-OHDA injection, the expression of PCNA peaked, at which time clusters of PCNA-positive cells were seen in the inner and outer nuclear layers. At 6-days post-6-OHDA injection, 316 ± 32 PCNA-positive cells were counted per section (Fig. 2B). In control retinas, only a few PCNA-positive cells (less than 3 cells) were observed per retinal section (Fig. 2, A and B).

The increase of PCNA expression in 6-OHDA-injected retinas persisted a few days before gradually decreasing. At 7 days post-6-OHDA injection, the expression of PCNA decreased to ~50% of its peak expression levels. By 8 days, the expression of PCNA decreased further, at which time only 80 ± 12 PCNA-positive cells were counted per retinal section. By 14 days post-
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FIGURE 1. Proliferation of Müller glial cells and rod precursor cells in response to 6-OHDA treatment. A, retinal sections of transgenic zebrafish Tg (gfap::GFP) labeled with PCNA antibodies after 3 days of 6-OHDA treatment. The Tg (gfap::GFP) transgene is expressed in Müller glial cells, revealed by the expression of GFP (left panel, arrows). PCNA antibodies labeled proliferating cells (middle panel, arrows). Note that the GFP-positive cells included Müller glial cells that are either proliferating (PCNA positive) or quiescent (PCNA negative). Some of the PCNA-positive cells are likely Müller glial-derived neuronal progenitor cells. The merged image shows that GFP and PCNA co-localized in proliferating cells (right panel, outlined by ovals). B, double labeling of retinal sections with PCNA antibodies (left panel, arrows) and EdU (middle panel, arrows) after 3 days of 6-OHDA treatment. EdU was injected after 1 and 2 days of 6-OHDA injection. Clusters of cells were labeled by PCNA antibodies and EdU. The merged image shows that PCNA immunoreactivity and EdU were co-localized (outlined by ovals). C, EdU and DAPI labeling of retinal sections after 30 days of 6-OHDA treatment. EdU was injected after 4, 5, and 6 days of 6-OHDA injection. EdU-positive cells (green) were found in both the inner and outer nuclear layers. Abbreviations: RPE, retinal pigment epithelia; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm.

6-OHDA injection, the expression of PCNA returned to similar levels seen in control retinas (Fig. 2, A and B).

Correlations between Stil Gene Expression and Shh Signaling Transduction during Cell Proliferation—During the course of subsequent cell proliferation, the expression of Stil mRNA in the retina fluctuated. At 4 days post-6-OHDA injection, the expression of Stil increased to levels ~8-fold higher than the expression in sham-injected (PBS) control retinas (Fig. 3A). At 5 days post-6-OHDA injection, the expression of Stil peaked to levels that were 32.3 ± 6.0-fold higher than the expression in sham-injected control retinas. A similar level of Stil gene expression was detected at 6 days post-6-OHDA injection. At 7 days post-6-OHDA injection, the expression of Stil began to decrease, yet at this time point the level of Stil expression remained significantly higher than the expression in control retinas. By 14 days post-6-OHDA injection, the expression of Stil returned to similar levels seen in controls (Fig. 3A).

During the same time period, cellular signaling transductions mediated by the Shh pathway also fluctuated. This was revealed by monitoring the expression of Shh-targeted Gli1 mRNA in different days post-6-OHDA injection. In the first few days post-6-OHDA injection, no obvious alterations in the expression of Gli1 mRNA were observed (Fig. 3B). At 5 days post-6-OHDA injection, however, significant increases in Gli1 expression were detected, at which time the expression of Gli1 mRNA was ~2-fold higher than the expression in control retinas. At 6 days post-6-OHDA injection, the expression of Gli1 slightly decreased from its peak expression, i.e. to levels ~1.7-fold higher than controls. By 7 days post-6-OHDA injection, the expression of Gli1 returned to levels seen in control retinas. No obvious changes were detected in Gli1 expression when examined at 14 days post-6-OHDA injection in comparison to its expression in control retinas (Fig. 3B).

Decrease of Stil Gene Expression Reduced the Rate of Cell Proliferation—In adult nbb+/- mutants, the number of DA cells in the retina is reduced in comparison to wild-type fish (15). To determine whether the decrease of DA cell number in nbb+/- mutants is related to cell proliferation, we examined cell proliferation in nbb+/- retinas after treatment with 6-OHDA. At 6 days post-6-OHDA injection, the retinas were harvested and examined for PCNA labeling (Fig. 4A). In wild-type retinas, more than 300 PCNA-positive cells per retinal section were counted. In nbb+/- retinas, the number of PCNA-labeled cells decreased; on average 179 ± 38 PCNA-positive cells were found per section (Fig. 4B).

The decrease in cell proliferation correlated with a decrease in Stil mRNA expression and Shh signaling transduction in nbb+/- mutants. At 6 days post-6-OHDA injection, for example, the expression of Stil mRNA in nbb+/- mutants was only 37.2 ± 8.6% of the expression detected in wild-type animals (Fig. 4C). At the same time point, the expression of Shh-targeted Gli1 mRNA in nbb+/- mutants was also decreased, to levels that were only 25.2 ± 7.1% of the expression detected in control animals (Fig. 4D).

Inhibition of Shh Signal Transduction Reduced the Rate of Cell Proliferation—To demonstrate the involvement of Shh signaling in DA cell proliferation, we examined cell proliferation responses in wild-type fish treated with cycloamine and...
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Treatment with cyclopamine resulted in decreased Shh signaling transduction. This was revealed by decreased expression of the Shhl-targeted gene Gli1 (Fig. 5A). In cyclopamine-treated retinas, the expression of Gli1 was only 76.2 ± 13.0% of the expression detected in control retinas. In cyclopamine-treated retinas, the rate of cell proliferation decreased. At 6 days post-6-OHDA injection, for example, 304 ± 37 and 118 ± 45 PCNA-positive cells were counted per section from control and cyclopamine-treated retinas (Fig. 5, B and C).

Activation of the Shh Pathway Increased the Rate of Cell Proliferation—To further demonstrate the roles of Shh signaling in DA cell proliferation, we tested a hypothesis that activation of the Shh signaling pathway increases DA cell proliferation. In response to Shh signaling, we tested a hypothesis that activation of the Shh signaling pathway increases DA cell proliferation. In response to Shh signaling, the expression of Gli1 increased to levels 1.55 ± 0.16-fold higher than the expression in untreated nbb−/− mutant retinas (Fig. 6B). This suggests that inhibition of Sufu relieved the repression of Gli1, thereby increasing Shh signal transduction.

The increase of Shh signal transduction in nbb−/− retinas (after Sufu-MO treatment) resulted in significant increases in cell proliferation. In control nbb−/− retinas (that received only PBS injections prior to 6-OHDA treatment), at 6 days post-6-OHDA injection, 144 ± 38 PCNA-positive cells per section were counted (Fig. 6, C and D). In nbb−/− retinas that received

6-OHDA, respectively. Cyclopamine is known to inhibit Shh signaling transduction (34–36). The fish received intracocular injections of cyclopamine prior to 6-OHDA treatment. After 6 days of cyclopamine and 6-OHDA treatments, the fish were sacrificed and the retinas were harvested and examined for Gli1 expression and PCNA labeling, respectively.

FIGURE 2. Cell proliferation in adult wild-type zebrafish retinas in response to 6-OHDA treatment. A, fluorescent images of retinal sections labeled with PCNA antibodies (red; proliferating Müller glial cells) at different days post-6-OHDA injection. Dashed lines outline the outer and inner nuclear layers of the retina (RPE is on the top of the image). Note the increase in PCNA cell number in the first 6 days post-6-OHDA injection. After 6 days, PCNA expression decreased. B, PCNA-positive cell count per retinal section at different days post-6-OHDA injection. Cryostat sections in the nasal retina adjacent to the optic nerve were used for cell counts. Note that PCNA expression peaked at 6 days post-6-OHDA injection. Data represent the mean ± S.E. (n = 5 in each group). dpi, days post-injection. Scale bar, 100 μm.

FIGURE 3. RT-PCR analyses of the transcription of Stil (A) and Gli1 (B) in wild-type retinas at different days post-6-OHDA injection. Control transcription of Stil and Gli1 measured in PBS-injected wild-type retinas was normalized to 1, respectively. Note the increase of Stil and Gli1 transcription at 5 and 6 days post-6-OHDA injection. Data represent the mean ± S.E. (n = 5 in each group). *, p < 0.01; ns, not significant.

FIGURE 4. Cell proliferation and gene expression in wild-type and nbb−/− retinas at 6 days post-6-OHDA injection. A, fluorescent images of retinal sections labeled with PCNA antibody (RPE is on the top of the image). Note the decrease in PCNA cell number in mutants. B, PCNA-positive cell count in wild-type and mutant retinas. Note the decrease in PCNA cell number in mutants. C and D, relative transcription of Stil and Gli1 mRNA in wild-type and nbb−/− retinas. The transcription of Stil and Gli1 in wild-type retinas were normalized to 1, respectively. Note the decrease in the transcription of Stil and Gli1 in mutants. Data represent the mean ± S.E. (n = 4 in each group). *, p < 0.01. Scale bar, 100 μm.
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**FIGURE 5. Effects of cyclopamine treatment on Shh signal transduction and cell proliferation in wild-type retinas.** A, RT-PCR analysis of Gli1 gene transcription in control and cyclopamine-treated retinas. The transcription of Gli1 in control retinas was normalized to 1. Note the decrease in transcription of Gli1 in cyclopamine-treated retinas. B, PCNA-positive cell count in control and cyclopamine-treated retinas. Note the decrease in PCNA cell number in cyclopamine-treated retinas. Data represent the mean ± S.E. (n = 4 in each group). *, p < 0.01. Scale bar, 100 μm.

**FIGURE 6. Effects of SUFU knockdown on Gli1 gene expression and cell proliferation in nbb+/− retinas.** A, Western blot of retinal lysates probed with anti-SUfu antibody. Note the decrease in SUfu expression in MO-treated retinas. B, relative Gli1 transcription in mutant retinas at 6 days post-6-OHDA injection. The transcription of Gli1 in untreated mutant retinas was normalized to 1. Note the increase in transcription of Gli1 after MO treatment. C, PCNA-positive cell count in untreated and MO-treated mutant retinas at 6 days post-6-OHDA injection. Note the increase in PCNA cell number in MO-treated retinas. D, fluorescent images of mutant retinas labeled with PCNA antibody (RPE is on the top of the image). Note the decrease of PCNA-positive cell count in control retinas was normalized to 1. Note the decrease in transcription of Gli1 in untreated mutant retinas was normalized to 1. Note the increase of PCNA-positive cell count in untreated and MO-treated mutant retinas at 6 days post-6-OHDA injection. Note the increase in PCNA cell number in MO-treated retinas. Data represent the mean ± S.E. (n = 4 in each group). *, p < 0.01. Scale bar, 100 μm.

Sufu-MO treatment, the expression of PCNA increased. At 6 days post-6-OHDA injection, 267 ± 54 PCNA-positive cells per section were detected (Fig. 6, C and D).

**DISCUSSION**

The human oncogene Stil is highly conserved in vertebrate species examined thus far. In mammals, the expression of Stil is required for the cell cycle and animal development (4–6, 37–40). In this research, we examined the roles of Stil gene expression in cell proliferation in adult animals. In zebrafish retinas treated with 6-OHDA, cell proliferation responses were observed. During the course of cell regeneration, the expression of PCNA in Müller glial cells (as well as a small number of rod precursor cells) increased. The expression of PCNA correlated with increases in Stil gene expression and Shh signaling transduction. Together, it provides evidence for the involvement of Stil gene expression as well as Stil-mediated Shh signal transduction in cell proliferation in adult neural tissues.

De-differentiation and proliferation of retinal Müller glial cells and rod precursor cells lead to a regeneration response. Ample evidence suggests that in fish retinas, treatment with 6-OHDA destroyed DA cells, and within a few months, DA neurons regenerated (41–45). Yet, evidence also exists that in fish retinas selective ablation of DA neurons is not sufficient for triggering DA cell regeneration, and that degeneration of other cell types must occur to initiate the proliferation response for regeneration of DA cells (46–49). In adult zebrafish retinas, there are only a small number of inner retinal DA neurons. Yet, in response to 6-OHDA (when applied at 5 μg/μl) treatment (intraocular injection, 5 μg/μl), we observed a large number of proliferating cells in both inner and outer nuclear layers (Fig. 1). This suggests that treatment with 6-OHDA (when applied at 5μg/μl) results in cell death of both DA and non-DA neural types. Labeling the retinas with PCNA and EdU supports this notion, e.g. PCNA- and EdU-positive cells were found in the inner and outer nuclear layers, which include Müller glial cells as well as rod precursor cells.

In adult nbb+/− retinas, the rate of cell proliferation was decreased likely due to down-regulation of Stil gene expression. In wild-type retinas, inhibition of Shh signaling transduction (i.e. by treatment with cyclopamine) decreased cell proliferation, which mimicked the defect in nbb+/− mutants. However, when the transduction of Shh was increased (i.e. by treatment with Sufu-MO), the defect in cell proliferation in nbb+/− mutants was rescued, in which case a dramatic increase in cell proliferation was detected. Together, the data suggest that Stil functions in the Shh signal transduction pathway, in which it plays important roles in regulating cell proliferation.

Correlation between the expression of Stil mRNA and PCNA also reflects the significance of Stil gene expression in cell proliferation. In developing zebrafish embryos, the expression of Stil is maintained at high levels (16). For example, the expression of Stil is 9-fold higher in embryos than its expression in adult. After juvenile stages (when tissue development and growth are completed), the expression of Stil sharply decreases. In adult zebrafish, the highest expression of Stil is detected in fin tissues, which undergo active renewal and growth on a daily basis. In adult zebrafish retinas, the expression of Stil is maintained at a relatively low level (16). During cell proliferation (after 6-OHDA-induced cell degeneration), the expression of Stil increased, i.e. to levels ~30-fold higher than the expression in control animals. During the same time period, expression of the Shh-targeted gene Gli1 also increased. The increase of Stil and Gli1 mRNA closely correlates to the expression PCNA, suggesting that up-regulation of Stil and activation of the Shh
pathway increases cell proliferation. Worth noting is that the peak expression of PCNA (at 6 days post-6-OHDA injection) is somewhat delayed in comparison to the peak expression of Stil and Gli1 mRNA (detected at 5 days post-6-OHDA injection). This may suggest the following. First, an increase in cell proliferation is triggered by up-regulation of Stil gene expression and Shh signal transduction. Second, the delay of PCNA peak expression is due to PCNA protein synthesis. When the expression of STIL is down-regulated (in nbb+/− mutants or in wild-type fish treated with antisense Stil-MO), the rate of cell proliferation and neural regeneration are largely inhibited.

During the course of cell proliferation, the increase of Shh signal transduction is transient, occurring for a total of 48 h between 5 and 6 days post-6-OHDA injection. In the first 4 days post-6-OHDA injection, the expression of Gli1 was maintained at levels similar to the expression in control retinas. In contrast, the increase in Stil mRNA expression is long lasting; i.e. for 7 days. The data suggest that gene expression of Stil plays critical roles in initiating and maintaining the process of cell proliferation, whereas Shh signaling plays a role that maximizes the rate of cell proliferation (i.e. at 6 days post-6-OHDA injection).

The expression of Stil and Gli1 mRNA in nbb+/− retinas is lower than their expression in wild-type retinas, resulting in a lower rate of cell proliferation. The rescue experiment (by antisense Sufu MO in mutant retinas) provides additional evidence for the requirement of expression of the Stil gene and activation of Shh signaling transduction in cell proliferation. The involvement of Shh signaling in cell proliferation is also revealed in wild-type retinas after Shh signaling transduction was inhibited by treatment with cycloamine. In these cases, the rate of cell proliferation decreased.

Expression of the Stil gene is required for cancer cell growth and survival (8–11). In this paper, we provide evidence that an increase of Stil mRNA expression promotes zebrafish retinal cell proliferation after degeneration. However, whether the increase of Stil gene expression is causation or a result of cell proliferation remains to be further examined. Considering the conserved roles of Stil in different species and cell types, it is conceivable that an increase of Stil gene expression leads to an increase in cell proliferation.

Previously, we demonstrated that in zebrafish retinas the increase of Stil mRNA expression and Shh signal transduction protects DA cells (16). In nbb+/− mutants or in wild-type fish in which Shh signaling is inhibited by treatment with cycloamine, DA cells become more susceptible to treatment with neurotoxin 6-OHDA. In contrast, activation of Shh signal transduction (i.e. by SUFU knockdown) decreases the susceptibility of DA cells to 6-OHDA, thereby protecting DA cells after toxin insults. In the current study, we showed that the increase of Stil mRNA expression and Shh signaling transduction is also involved in cell proliferation. It is conceivable that the defective phenotypes observed in adult nbb+/− mutants (decreases in the number of DA cells in retinas) are due to a combination of increased cell degeneration and lack of proper cell proliferation. That is, when expression of the Stil gene is down-regulated (in heterozygous mutants), the rate of cell apoptosis increases (i.e. due to aging or in response to environmental toxins). At the same time, the rate of cell proliferation (regeneration) decreases.

Acknowledgments—We thank A. DeLaPaz for technical assistance and D. Smith at the Freimann Animal Facility for husbandry of zebrafish colonies.

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