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# Inhibition of STAT3 Expression and Signaling in Resveratrol-Differentiated Medulloblastoma Cells[1]

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## Abstract

In this study, the potential influence of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) in signal transducer and activator of transcription 3 (STAT3) signaling of medulloblastoma cells was evaluated by checking the status of STAT3 signaling and its downstream gene expression in two medulloblastoma cell lines (UW228-2 and UW228-3) with and without resveratrol treatment. The results revealed that resveratrol induced neuronal differentiation of medulloblastoma cells. Signal transducer and activator of transcription 3 expression and phosphorylation were detected in normally cultured UW228-2 and UW228-3 cells that were apparently attenuated after resveratrol treatment. The expression of STAT3 downstream genes, *survivin*, *cyclin D1*, *Cox-2*, and *c-Myc*, was suppressed but Bcl-2 was enhanced by resveratrol. Meanwhile, the production and secretion of leukemia inhibitory factor, a STAT3 activator, became active in resveratrol-treated cells. To further ascertain the significance of STAT3 signaling for medulloblastoma cells, AG490, a selective inhibitor of STAT3 phosphorylation, was used to treat UW228-3 cells. Phosphorylation of STAT3 was inhibited by AG490 accompanied with growth suppression, differentiation-like changes, and down-regulation of *survivin*, *cyclin D1*, *Cox-2*, and *c-Myc*. Our data thus suggest the importance of STAT3 signaling in maintenance and survival of medulloblastoma cells. This signaling may be the major target of resveratrol. Enhanced leukemia inhibitory factor and Bcl-2 expressions in resveratrol-treated cells might reflect a compensatory response to the loss of STAT3 function.

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## Introduction

Medulloblastoma is the commonest malignant brain tumor in childhood. Despite the improved combination of surgery, radiotherapy, and chemotherapy, the outcome of medulloblastomas, especially those developed in early ages, remains poor due to the difficulty in removing the highly invasive intracranial tumor radically and the short- and long-term adverse effects of conventional postsurgical adjuvant therapies [1–3]. Consequently, exploration of reliable and less toxic chemotherapeutic approach is urgently required for better management of this sort of malignancy.

Resveratrol (3, 5, 4′-trihydroxy-*trans*-stilbene) is a plant polyphenol that is naturally occurring in grapes, red wine, and peanuts and has chemopreventive and anticancer properties [4,5]. This harmless compound exhibits anticancer activities in multiple human cancers with less-defined molecular mechanisms [4]. We found that resveratrol could induce differentiation and apoptosis of medulloblastoma cells through Fas-independent pathway [6], suggesting the involvement of other molecular factor(s) in the course of resveratrol action. *c-Myc* is a key element in controlling cell growth, differentiation, and survival [7], which is amplified and/or overexpressed in a great proportion of medulloblastomas [8]. Our previous study demonstrated that *c-Myc* down-regulation was closely associated with the antimedulloblastoma

Abbreviations: ICC, immunocytochemical; IF, immunofluorescent; LIF, leukemia inhibitory factor; Resveratrol, 3, 5, 4′-trihydroxy-*trans*-stilbene; STAT3, signal transducer and activator of transcription 3; p-STAT3, phosphorylated-STAT3; GFAP, glial fibrillary acidic protein

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effect of resveratrol [9]. Because *c-Myc* expression can be regulated by several signaling pathways including the one mediated by Janus kinase (JAK)/STAT3, resveratrol-induced *c-Myc* down-regulation implicates potential disorder(s) of certain signal pathways in the treated cells.

Signal transducer and activator of transcription 3 (STAT3) is a kind of transcription factor that is phosphorylated by JAK kinases in response to cytokine activation of a cell surface receptor tyrosine kinases [10]. Signal transducer and activator of transcription 3–mediated cell growth, differentiation, and survival depend on the ligand–receptor binding of IL-6 family members such as IL-6 and leukemia inhibitory factor (LIF) [11]. On activation, STAT3 molecules are translocalized to nucleus where they activate transcription of a series of target genes including *c-Myc*, *survivin*, *Cox-2*, and *cyclin D1* that are closely associated with the growth, survival, and progression of cancer cells [8,12–14]. The activation of STAT3 has been described in human medulloblastomas [15]. Inhibition of STAT3 signaling may commit medulloblastoma cells to growth arrest and apoptosis [16]. Negative regulation of STAT3 activation by resveratrol has been found in multiple myeloma cells [17] and prostate cancer cells [18] but remains unknown in medulloblastomas. Because LIF is constitutively expressed as an autocrinal growth factor in medulloblastoma cells *in vivo* and *in vitro* [19] and expressions of *c-Myc* and other STAT3 target genes are commonly observed in medulloblastoma cells [8,13], it would be possible that resveratrol conducts its antimedulloblastoma effects through altering STAT3 activation and/or production. This issue was addressed in the current study through multiple experimental approaches.

## Materials and Methods

### *Cell Culture and Treatments*

Established human medulloblastoma cell lines, UW228-2 and UW228-3, were derived from the surgical specimen of a 9-year-old female patient and were established by independent cultures of two cell aliquots from the tumor [20]. Both of them were cultured in Eagle's minimal essential medium containing 10% fetal bovine serum (FBS; Gibco Life Science, Grand Island, NY) under 37°C and 5% $CO_2$ condition. The cells ($5 \times 10^4$/ml) were plated to $\phi$60- and $\phi$100-mm dishes (Nunc A/S, Roskilde, Denmark) and incubated for 24 hours before further experiments. For morphologic evaluation and immunocytochemical (ICC) and immunofluorescent (IF) staining, the coverslips were put into the dishes before initial cell seeding and collected regularly during the experiments.

Resveratrol (Sigma Chemical, Inc, St. Louis, MO) was dissolved in dimethylsulfoxide (Sigma) to a stock concentration of 100 mM and diluted with culture medium to an optimal working concentration of 100 μM just before use [6]. The cells treated by the same concentration of DMSO were used as background control. The drug treatment lasted for 48 or 72 hours depending on the experimental purposes. The cells were checked in 12-hour intervals, followed by cell collection for protein and RNA extractions and *in situ* examination. Total cell numbers and cell viability of the cells without and with 100-μM resveratrol treatment were determined at 0-, 24-, and 48-hour time points by staining the single-cell suspensions with 0.25% trypan blue and counting the stained and unstained cells with the hemocytometers. Cell-bearing coverslips were harvested at each checking point and fixed properly for morphologic, ICC, and IF examinations. Each of experimental groups was set in triplicate, and the experiments were repeated at least three times to establish confidential conclusions.

### *Flow Cytometry Evaluation*

To determine the effects of resveratrol on cell cycle, UW228-2 and UW228-3 cells were plated at a density of $5 \times 10^4$/ml on 60-mm-diameter dishes. The cells treated by 100-μM resveratrol were collected in 12-hour intervals. For staining with DNA dye, the cells were resuspended in 0.5 to 1 ml of propidium iodide solution containing RNase and were incubated at 37°C for 30 minutes. Cell cycle profiles were obtained with a FACSvantage flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed with ModFit software (Verity Software House, Inc, Topsham, ME).

### *Immunocytochemical and Immunofluorescent Staining*

Immunocytochemical staining was performed on the coverslips obtained from each of the experimental groups. The antibodies against *STAT3*, phosphorylated-STAT3 (*p-STAT3*), *survivin*, *c-Myc*, *cyclin D1*, *Cox-2*, *LIF*, glial fibrillary acidic protein (*GFAP*), and *synaptophysin* were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and used according to the manufacturer's instruction. Briefly, the coverslips were washed with phosphate-buffered solution (PBS, pH 7.4), incubated for 10 minutes in 3% $H_2O_2$ and then with the appropriately diluted first antibody at 37°C for 60 minutes in a humid chamber, followed by the treatments with reagent A containing polymer enhancer for 20 minutes and with reagent B containing polymerized horseradish peroxidase (HRP) anti–mouse/rabbit IgG for 30 minutes (Zymed Lab, Inc, San Francisco, CA). Color reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride.

For IF staining, the coverslips were rinsed with PBS (pH 7.4), fixed for 20 minutes in 80% cold acetone and stored at −20°C until use. After being blocked with 10% goat serum in PBS for 20 minutes, the coverslips were incubated overnight with primary antibodies against target proteins in humid chamber at 4°C, followed by coincubation with fluorescence-labeled goat antirabbit or rabbit antimouse IgG (1:100; Santa Cruz Biotechnology, Inc) in a 37°C humid chamber for 60 minutes in darkness. The coverslips were sealed with 90% glycerol, observed, and photographed (DP70 Digital Camera; Olympus, Tokyo, Japan) under a fluorescence microscope (BX51; Olympus).

### *RNA Isolation and Reverse Transcription–Polymerase Chain Reaction*

Total cellular RNA were isolated from each of experimental groups using Trizol solution (Life Technologies, Grand Island, NY). Reverse transcription (RT) was performed on RNA samples, followed by polymerase chain reaction (PCR) amplification. For RT, 0.5 μg of the RNA sample was added to 20 μl of RT reaction mixture (Takara, Inc, Ltd, Dalian, China) containing 4 μl of $MgCl_2$, 2 μl of 10 × RNA PCR buffer, 9.5 μl of RNase-free distilled $H_2O$, 2 μl of deoxyribonucleotide triphosphate mixture, 0.5 μl of RNase inhibitor, 1 μl of AMV reverse transcriptase, and 1 μl of oligo dT-adaptor primer. The reaction was carried out by treating the samples at 55°C for 30 minutes, at 99°C for 5 minutes, and at 5°C for 5 minutes. Polymerase chain reaction was conducted using the primers specific for each of the target genes (Table 1). Briefly, 2.5 μl of RT products were mixed with 16 μl of PCR-grade water, then with 6.5 μl of PCR working solution containing 1 × PCR buffer, 1 μl of deoxyribonucleotide triphosphate, 2.5 units of Taq DNA polymerase, and 50 pM upstream and downstream primers for human *STAT3*, *survivin*, *Cox-2*, *cyclin D1*, *c-Myc*, *Bcl-2*, *LIF*, *GFAP*, and *synaptophysin*, respectively. Polymerase chain reactions for individual genes were performed according to the conditions reported elsewhere [21–29]. The PCR products

**Table 1.** Sequences of RT-PCR Primers.

| Parameters | Primer Sequences | Annealing Temperature (°C) | Product Size (bp) | Reference |
|---|---|---|---|---|
| *STAT3* | F: 5′-gggtggagaaggacatcagcggtaa-3′<br>R: 5′-gccgacaatactttccgaatgc-3′ | 58 | 298 | [21] |
| *survivin* | F: 5′-ggcatgggtgccccgacgttg-3′<br>R: 5′-cagaggcctcaatccatggca-3′ | 58 | 439 | [22] |
| *Bcl-2* | F: 5′-tgtggccttctttgagttcg-3′<br>R: 5′-tcacttgtggctcagatagg-3′ | 56 | 280 | [23] |
| *LIF* | F: 5′-ctgttggttctgcactgga-3′<br>R: 5′-gggttgaggatcttctggt-3′ | 56 | 350 | [24] |
| *c-Myc* | F: 5′-tggtcttccctaccctctcaac-3′<br>R: 5′-gatccagactctgacctttttgcc-3′ | 56 | 265 | [25] |
| *GFAP* | F: 5′-tgagtcgctggaggaggagat-3′<br>R: 5′-tagtcgttggcttcgtgctt-3′ | 58 | 285 | [26] |
| *synaptophysin* | F: 5′-ccgccagacagggaacacat-3′<br>R: 5′-agggggcccactcaagactg-3′ | 61 | 621 | [27] |
| *Cox-2* | F: 5′-ttcaaatgagattgtgggaaaattgct-3′<br>R: 5′-agatcatctctgcctgagtatctt-3′ | 50 | 305 | [28] |
| *cyclin D1* | F: 5′-ctgtgctgcgaagtggaaaccat-3′<br>R: 5′-ttcatggccagcgggaagacctc-3′ | 57 | 257 | [29] |

were resolved on 1% agarose gel containing ethidium bromide (0.5 μg/ml). The bands were visualized and photographed using UVP Bio-spectrum Imaging System (UVP, Inc, Upland, CA). The PCR products generated from the same RT solution by a pair of β-actin primers were cited as internal quantitative controls.

### *Protein Preparation and Western Blot Analyses*

Total cellular proteins were prepared from the cells under different culture conditions by the method described previously [9]. For Western blot analyses, the sample proteins (50 μg/well) were separated by electrophoresis in 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Amersham, Buckinghamshire, UK). The membrane was blocked with 5% skimmed milk in TBS-T (10 mM Tris–Cl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) at 4°C overnight, rinsed three times (10 minutes each time) with TBS-T, followed by 3 hours of incubation at room temperature with the first antibodies in appropriate concentrations followed by 1 hour of incubation with HRP-conjugated antimouse or -rabbit IgG (Zymed Lab, Inc). The bound antibody was detected using the enhanced chemiluminescence system (Roche GmbH, Mannheim, Germany). After removing the labeling signal by incubation with stripping buffer (62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) at 55°C for 30 minutes, the membrane was reprobed with other antibodies one by one by the same experimental procedures until all of the parameters were examined.

### *Condition Medium Collection and LIF-Oriented Western Blot Analysis*

Cells cultured under normal condition and treated by 100-μM resveratrol for 36 hours were harvested for total protein preparation. Meanwhile, the cell-free culture media were collected from the experimental and normal culture groups, respectively. The medium containing 10% FBS was used as background control. The sample media were concentrated by centrifugal filtration through YM-10 filters (Millipore, Bedford, MA) and were adjusted to a working protein concentration of 50 μg/μl. A total of 3 μl of working solutions were prepared from each of the three samples; half of them were used for Western blot analysis to check secreted LIF by the method described previously. As quantitative control, the remaining half of the sample proteins were electrophoresed in an SDS-PAGE gel, stained by Coomassie brilliant blue (Bio-Rad Laboratory, Richmond, CA) and photographed under a UV transilluminator (Bio-spectrum Imaging System; UVP, Inc).

### *Leukemia Inhibitory Factor Immunoelectron Microscopic Examination*

Coverslips with UW228-3 cells were fixed for 30 minutes with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. They were washed with PBS (pH 7.4), incubated for 10 minutes in 3% $H_2O_2$ and then with an antihuman LIF antibody in a dilution rate of 1:80 at 37°C for 60 minutes, followed by the treatments with reagent A containing polymer enhancer for 20 minutes and with reagent B containing polymerized HRP antirabbit IgG for 30 minutes (Zymed Lab, Inc). Color reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride. After washing with PBS for three times (10 minutes each time), the coverslips were fixed in 2.5% glutaraldehyde (30 minutes, 50 mM cacodylate buffer, pH 7.2) and 2% $OsO_4$ (30 minutes, the same buffer). Ultrathin sections (0.1 μm) were prepared and examined under a Philips CM100 transmission electron microscope (FEI Company, Hillsboro, OR). Images were captured by charge-coupled device camera equipped with TCL-EM-Menu version 3 (Tietz Video and Image Processing Systems GmbH, Gaunting, Germany) as described elsewhere [30]. The samples without primary antibody incubation were used as negative controls.

### *Inhibition of STAT3 Activation with AG490*

AG490 is a $JAK_2$-specific inhibitor and therefore selectively inhibits STAT3 phosphorylation. This reagent (Sigma) was dissolved in DMSO to a stock concentration of 50 mM and was diluted to the final concentration of 60 μM with conventional culture medium just before use [31]. Three experimental groups were set as follows: group 1, normal culture; group 2, treatment with 1.2‰ DMSO as background control; group 3, treatment with 60 μM AG490. For morphologic evaluation and ICC staining for *p-STAT3*, *cyclin D1*, *survivin*, *Bcl-2*, *Cox-2*, *c-Myc*, *LIF*, and *synaptophysin*, the coverslips were put into the dishes before initial cell seeding and were collected after a 72-hour treatment during the experiments.

## Results

### *Resveratrol-Induced Neuron-Oriented Differentiation*

Under normal culture condition, UW228-2 and -3 cells were elliptical. They exhibited elongated phenotype after 100-μM resveratrol treatment for 24 hours and showed apoptosis at the 48-hour time point. Flow cytometry analyses (Figure 1*A*) revealed that the fractions of UW228-3 cells in $G_1$ and S phases were 41.7% and 58.3% in normally cultured cells, and turned to 82.9% and 17.1% on 100-μM resveratrol treatment for 24 hours and to 93.3% and 6.2% for 48 hours; similar phenomena were found in resveratrol-treated UW228-2 cells. Examination of neural differentiation biomarkers showed that both GFAP and synaptophysin were negative in normally cultured UW228-2 and UW228-3 cells. On resveratrol treatment, synaptophysin but not GFAP became expressed in both cell lines (Figure 2*B*).

The chemosensitivities of UW228-2 and -3 cell lines to resveratrol were evaluated by measuring total cell numbers and cell viability in

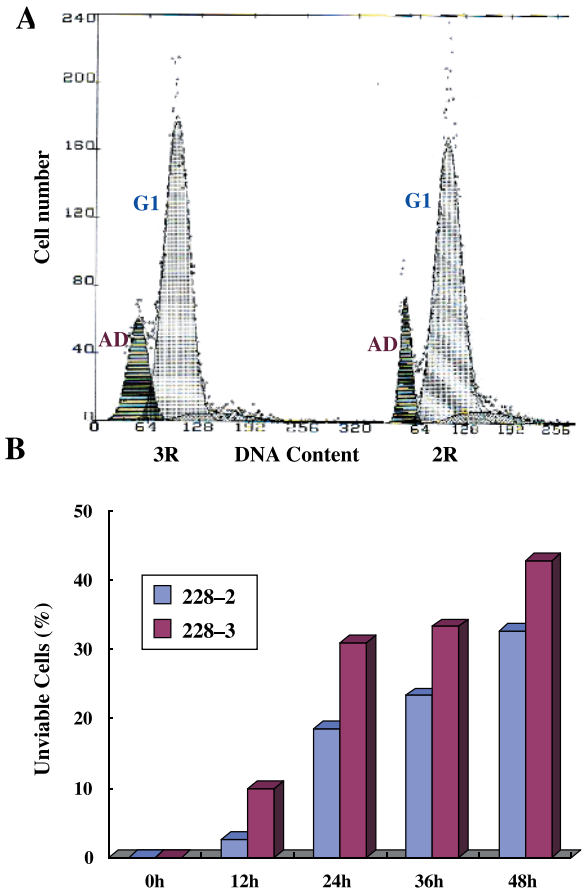


**Figure 1.** Evaluation of chemosensitivities of UW228-2 and UW228-3 cells to resveratrol treatment by flow cytometry analysis (A) and trypan blue discrimination of stained (unviable) and unstained (viable) cells (B). *3R* indicates resveratrol-treated UW228-3 cells; *2R*, resveratrol-treated UW228-2 cells; *AD*, cells died of apoptosis; *G1*, cells at $G_1$ phase.

12-hour intervals during the treatment by staining the suspended single cells with 0.25% trypan blue and counting the stained and unstained cells with the hemocytometers. The results revealed that after resveratrol treatment for 0, 12, 24, 36, and 48 hours, the percentage of dead cells was 0%, 10%, 31.03%, 33.33%, and 42.86% in the UW228-3 population and 0%, 2.7%, 18.6%, 16.6% and 22.7% in UW228-2 cells, respectively (Figure 1*B*), indicating that UW228-3 cells were more sensitive to resveratrol.

### *Resveratrol Inhibited STAT3 Transcription and Activation*

To elucidate the effects of resveratrol on STAT3 signaling, RT-PCR for STAT3 was performed on the RNA samples extracted from the cells without and with resveratrol treatment. As shown in Figure 3, STAT3 was expressed in normally cultured UW228-2 and -3 cells, which was down-regulated in both cell lines after resveratrol treatment. Immunofluorescent and ICC staining were performed on the same experimental groups to further check the location and relative abundance of STAT3 and p-STAT3 in UW228-2 and -3 cells. The result of IF staining showed that STAT3 (red fluorescence–labeled protein) was distributed in cytoplasm and nuclei of UW228-2 and -3 cells under the normal culture condition, which became apparently reduced in the nuclei after resveratrol treatment (Figure 2*A*). Immunocytochemical staining for p-STAT3 showed positive staining mainly in the nuclei of UW228-2 and -3 cells. After resveratrol treatment, p-STAT3 immunolabeling was diminished in the nuclei of the two cell lines (Figure 2*A*). In accordance with the RT-PCR, ICC, and IF results, Western blot analysis with the antibody against p-STAT3 showed distinct p-STAT3 reduction in resveratrol-treated UW228-2 and -3 cells (Figure 2*C*).

### *Resveratrol Altered STAT3 Downstream Gene Expressions*

To elucidate the effects of resveratrol on STAT3 downstream genes, the expressions of *c-Myc*, *survivin*, *Cox-2*, *cyclin D1*, and *Bcl-2* were examined by the methods of RT-PCR, ICC and IF staining, and Western blot analysis. As described previously [9], *c-Myc* was expressed in normally cultured UW228-2 and -3 cells and down-regulated in resveratrol-treated UW228-2 and, especially, UW228-3 cells (Figure 3). The same tendency of *survivin*, *Cox-2*, and *cyclin D1* expression was evidenced in the two cell lines (Figure 2). However, *Bcl-2* transcription and production was enhanced by resveratrol in both UW228-2 and -3 cells (Figure 2, *A*–*C*).

### *Resveratrol Promoted LIF Expression and Secretion*

Leukemia inhibitory factor is constitutively expressed in medulloblastoma cells *in vivo* and *in vitro* as autocrinal growth factor [19]. RT-PCR and Western blot analyses demonstrated that LIF was obviously up-regulated in resveratrol-treated UW228-2 and -3 cells (Figure 3, *B* and *C*). Immunofluorescent staining was then conducted to check the pattern(s) of intracellular distribution and relative abundance of LIF in the two cell lines with and without resveratrol treatment (Figure 3*A*). Under normal culture condition, LIF protein was evenly distributed in the cytoplasm of UW228-2 and -3 cells, which became enriched in cytoplasm and accumulated in the form of multiple granules along the stretchy processes of resveratrol-treated cells. To further confirm the distribution pattern of LIF, immunoelectron microscopic examination was performed on UW228-3 cells, and LIF-oriented Western blot analysis was on the concentrated condition medium. As shown in Figure 3*D*, LIF proteins were labeled uniformly in the cytoplasmic space of normally cultured/nontreated cells. The LIF-labeling density became condensed in resveratrol-treated cells, and some outward membrane-bounded particles on the surface of cytoplasmic membrane were strongly positive in LIF. In parallel, Western blot analysis performed on concentrated condition media demonstrated that the level of LIF proteins was distinctly higher in the medium of resveratrol-treated cells than that of their normally cultured counterpart (Figure 3*C*), suggesting the enhanced LIF secretion by resveratrol. The LIF was undetectable in the concentrated medium containing 10% FBS.

### *Effects of AG490 on Medulloblastoma Cells*

To determine the correlation between the status of STAT3 activation and antimedulloblastoma effects of resveratrol, AG490, a selective inhibitor of STAT3 phosphorylation, was used to treat UW228-3 cells. Immunocytochemical staining demonstrated that STAT3 phosphorylation was inhibited in AG490-treated cells in terms of the reduction of p-STAT3 nuclear labeling (Figure 4). Meanwhile, the proliferation of UW228-3 cells was apparently suppressed by AG490 in a time-related fashion. After AG490 treatment for 48 hours, the cells became elongated with synaptophysin (Figure 4). The results of ICC staining for *c-Myc*, *Cox-2*, *cyclin D1*, and *survivin* revealed that the

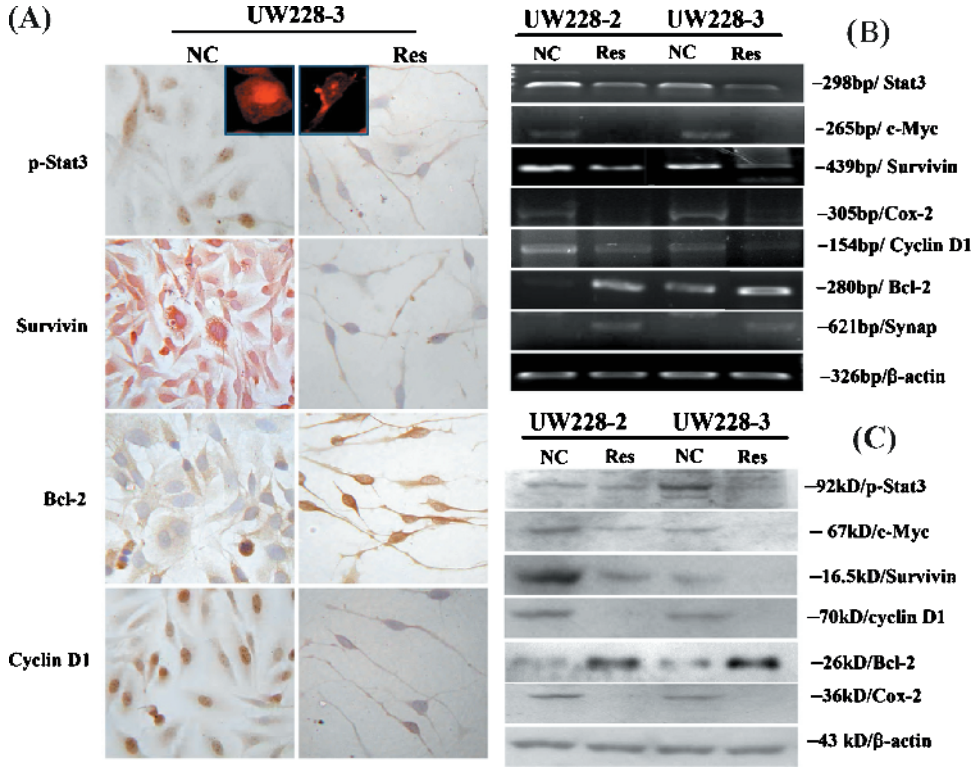


**Figure 2.** Evaluation of STAT3 phosphorylation and expression of *survivin*, *cyclin D1*, and *Bcl-2* as well as *Cox-2*, *c-Myc*, and *synaptophysin* in medulloblastoma cells by ICC and IF staining (A), RT-PCR (B), and Western blot analysis (C). *NC* indicates normally cultured/nontreated cells; *Res*, 100-μM resveratrol-treated cells. β-Actin was used as a quantitative control in RT-PCR and Western blot analysis. The negative data of GFAP examination were not shown.

expression levels of those genes were decreased in AG490-treated cells (Table 2). Similar with the findings from resveratrol-treated cells, the levels of *Bcl-2* and *LIF* expression were enhanced by AG490 treatment (Table 2). However, apoptotic cells were hardly observed throughout the course of AG490 treatment.

## Discussion

Signal transducer and activator of transcription 3 signaling is the important molecular machinery that regulates cell fate determination, renewal, differentiation, and apoptosis of various cell types, especially the ones at the embryonic developing stages [7,32,33]. When LIF- and ciliary neurotrophic factor–mediated phosphorylation occurs to STAT3 protein, the phosphorylated STAT3 shifts into nucleus and promotes self-renewal of early neural progenitor cells (NPCs) [34] or triggers GFAP expression in relatively late NPCs [35]. These phenomena suggest that the role of STAT3 in neurodevelopment largely depends on the stages where NPCs stay. Conversely, disordered STAT3 signaling may mislead stepwise neurogenic processes and result in primitive and unlimited proliferating statuses of NPCs [36–38]. Therefore, a link of STAT3 alteration to neuroectodermal tumor formation has been proposed [15]. Resveratrol is an effective differentiation and apoptosis inducer of medulloblastoma cells [6]. Although resveratrol could induce certain molecular changes of medulloblastoma cells [9], its detailed molecular effects on those cells have not been systemically clarified. Furthermore, despite the importance of STAT3 in tumor formation and the potential values of resveratrol in cancer prevention and treatment, their internal relation(s) in cancer cells was rarely mentioned [17]. The current study thus aimed to shed light on the above-mentioned issues.

Evaluation of the biologic effects of resveratrol on medulloblastoma cells revealed that this reagent arrested the cells at $G_1$ phase and induced differentiation and apoptosis of medulloblastoma cells. UW228-3 cells were more sensitive to the treatment. The appearance of synaptophysin but not GFAP expression in resveratrol-treated cells suggests that UW228-2 and -3 cells remained at a self-renewal and undifferentiated state, originally, and were promoted to the path of neuronal differentiation by resveratrol. Because of the importance of STAT3 signaling in the maintenance of medulloblastoma cells [15] and its unfavorable effect(s) on neuronal differentiation [35], the status of STAT3 expression and phosphorylation in the two medulloblastoma cell lines without and with resveratrol treatment was investigated by multiple approaches. Constitutive STAT3 expression with nuclear translocation could be found in the two cell lines under normal culture condition. On resveratrol treatment, STAT3 became down-regulated in the transcriptional level accompanied by the rarity of nuclear translocation. This finding demonstrated for the first time a link of the antimedulloblastoma effects of resveratrol with the suppressed STAT3 bioactivity. Signal transducer and activator of transcription 3 signaling regulates the expressions of multiple cancer–associated genes [8,13,14,39]. We therefore considered that its inactivation may alter the expression patterns of its downstream genes, of which some are crucial for cell growth and survival. Conversely, inhibition of STAT3 signaling might release the undifferentiated medulloblastoma cells for further differentiation, because diminished STAT3 may result in down-regulation of inhibitory bHLH factors and directly induce

neuronal differentiation of NPCs [40]. The appearance of neuronlike phenotype and synaptophysin expression in resveratrol-treated cell populations supports this notion.

Activated STAT3 signaling is commonly found in human cancers including medulloblastomas [15,16]. It plays oncogenic roles by promoting the expression of a panel of cancer-associated genes such as *cyclin D1*, *c-Myc*, *survivin*, *Cox-2*, and *Bcl-2* [8,13,14,41]. Given the evidence of *c-Myc* down-regulation in resveratrol-treated UW228-2 and -3 cells [9], we speculated that the expression patterns of other STAT3 target genes may also be altered in those cells on resveratrol treatment. Just as what we had expected, in parallel with *c-Myc* down-regulation, the levels of *cyclin D1*, *Cox-2*, and *survivin* were reduced in resveratrol-treated UW228-2 and, particularly, UW228-3 cells that were more sensitive to resveratrol. It is therefore reasonably speculated that one of major antimedulloblastoma effects of resveratrol would be the blockage of STAT3 signaling by directly suppressing STAT3 transcription, which may abate STAT3 signaling and, consequently, lead to the down-regulation of STAT3 target genes.

*Bcl-2* is famous in preventing apoptotic cell death, and its expression is closely related with STAT3 activation [10]. Because resveratrol committed UW228-2 and -3 cells to apoptosis [6], the influence of resveratrol in *Bcl-2* expression was speculated. Interestingly, *Bcl-2* expression was enhanced in resveratrol-treated UW228-3 and, especially, UW228-2 cells. Although *Bcl-2* down-regulation was usually accompanied with apoptosis [42], the exceptional cases could also be found in some cell types, e.g., enhanced *Bcl-2* expression in the apoptotic cardiac cells of chronic coronary heart disease [43,44], the hepatocyte apoptosis of alcoholic steatohepatitis due to alcohol-related stress [45], the treatment of multidrug-resistant cancer [46], and chemoresistant acute lymphoblastic leukemia cells [47]. One possible explanation to this phenomenon would be a feedback response to the increased apoptotic pressure or a compensatory approach to the loss of other cancer-associated gene expression, because in comparison with UW228-3 cells, resveratrol-treated UW228-2 cells expressed higher level of *Bcl-2* and were subjected to lesser apoptosis in the given times. In this context, the extent of *Bcl-2* enhancement might

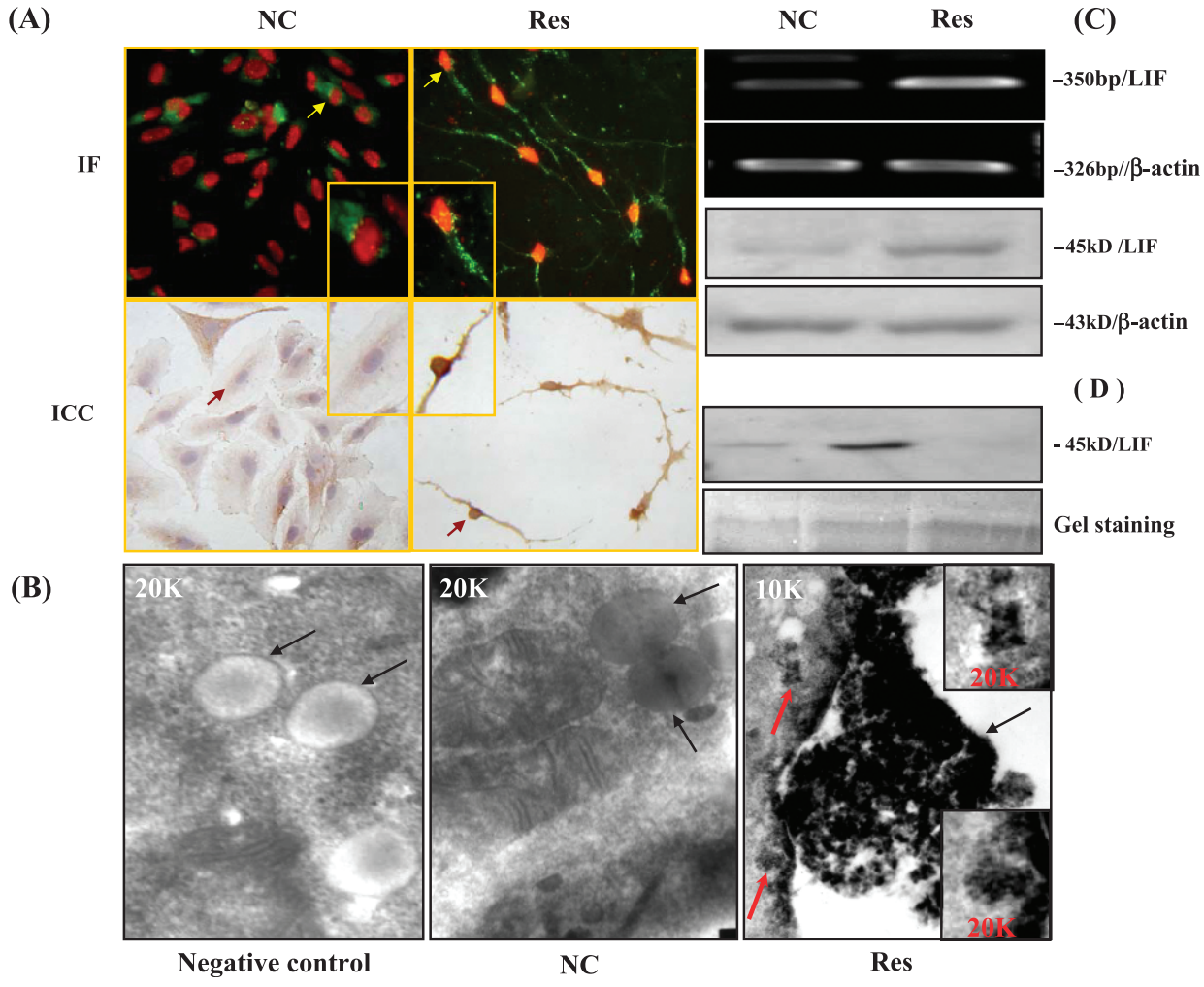


**Figure 3.** Analyses of LIF expression and intracellular distribution in UW228-3 cells without (NC) and with (Res) resveratrol treatment by ICC and IF staining (A), immunoelectron microscopic examination (B), and RT-PCR and Western blot analysis (C). LIF secretion activity was checked by Western blot analysis using concentrated condition media of UW228-3 cells cultured for 48 hours without and with resveratrol treatment. The medium containing 10% FBS was used as background control (D). In (A), the arrows indicate the cells shown in the inserts with high magnification. In (B), the magnification scales of electron microscopic illustrations are indicated as ×10K and ×20K (10,000 and 20,000 times); the black arrows indicate the vesicular structures and the red ones indicate the vesicles shown in the inserts.

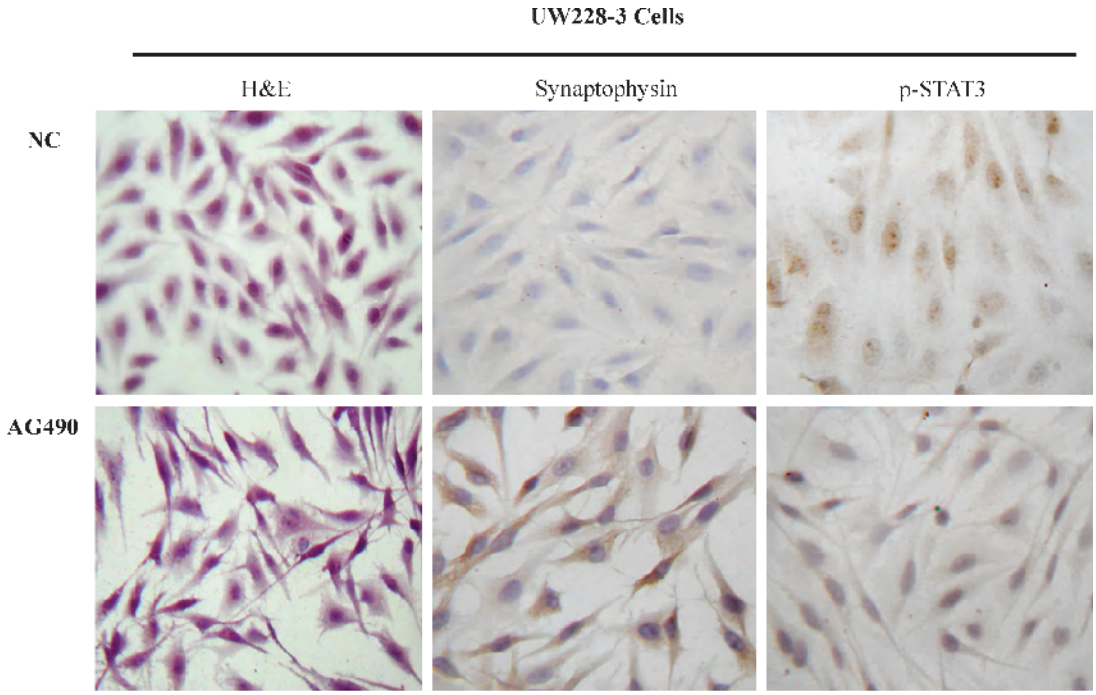


**Figure 4.** Morphologic changes, synaptophysin expression and the state of STAT3 phosphorylation of UW228-3 cells without (NC) and with AG490 treatment. The expression patterns of STAT3 downstream genes in those cells are summarized in Table 2.

reflect the apoptotic susceptibility of medulloblastoma cells to resveratrol. In addition to STAT3, *Bcl-2* can be regulated by other signaling pathways such as the ones mediated by NF-κB or Wnt signaling [17,48]. Therefore, more comprehensive investigation would be necessary to get new insight into the overall alterations of signaling network in resveratrol-treated medulloblastoma cells.

Leukemia inhibitory factor belongs to the member of IL-6 family, which acts as a key upstream stimulator of the JAK/STATs signaling [49]. For example, this protein could inhibit neuronal terminal differentiation through activating STAT3 signaling [50]. In the absence of IL-6 expression, LIF serves as a crucial autocrinal growth factor of medulloblastoma cells because blockage of its expression or bioactivity commits medulloblastoma cells to apoptosis [19]. Because of the close links of LIF with STAT3 signaling and medulloblastoma proliferation, its status in resveratrol-treated cells was examined by multiple approaches. Similar with *Bcl-2*, LIF was up-regulated in both UW228-2 and UW228-3 cells after resveratrol treatment. Moreover, IF staining and immunoelectron microscopic examination showed accumulated LIF-positive vesicular-like structures located beneath plasma membrane and on the membrane outward the extracellular space in resveratrol-treated cells, suggesting the increased activities of LIF expression and secretion. The results of Western blot analysis further confirmed this speculation by showing the higher LIF level in the condition medium of resveratrol-treated cells than that of their normally cultured counterpart and the undetectable LIF in the concentrated medium containing 10% FBS. The findings of elevated LIF expression and secretion in resveratrol-treated cells reflect again the presence of feedback or compensatory response to the inactivation of STAT3 signaling by strengthening the activation stimuli in resveratrol-treated cells. However, this response gives little help to the affected cells because of the distinct reduction of STAT3 transcription. Apparently, the inhibition of STAT3 expression and signaling would be a fundamental event in resveratrol-treated medulloblastoma cells, although the detail molecular mechanism of resveratrol-modulated STAT3 transcription awaits further investigation.

AG490, a JAK-specific inhibitor, can significantly suppress STAT3 signaling by inhibiting the phosphorylation of Tyr705 of STAT3 [38]. We therefore used it to block the JAK/STAT signaling in medulloblastoma cells. Together with the inhibition of STAT3 phosphorylation and *c-Myc*, *survivin*, and *cyclin D1* expressions, cell growth was suppressed efficiently, and the cell shape became elongated along with synaptophysin expression, a similar phenotype as we have observed in resveratrol-treated cells. These findings further support the roles of STAT3 signaling in promoting proliferation and maintaining the undifferentiated status of medulloblastoma cells. However, unlike resveratrol, AG490 did not lead to an apoptotic death of medulloblastoma cells. This phenomenon may have two implications: 1) other compensatory mechanism(s) may remain intact to sustain the survival of affected cells when only STAT3 signaling is inhibited by AG490; and 2) in addition to STAT3, other molecular elements in resveratrol-treated medulloblastoma cells may also be altered, resulting in more crucial events than that evoked by AG490.

**Table 2.** STAT3 Phosphorylation and Medulloblastoma-Associated Gene Expression in UW228-3 Cells Under Three Culture Conditions.

| | Normal Culture | Resveratrol | AG490 |
|---|---|---|---|
| *p-STAT3* | ++ | ± | – |
| *c-Myc* | ++ | + | + |
| *cyclin D1* | + | ± | ± |
| *survivin* | ++ | + | + |
| *Cox-2* | ++ | – | ± |
| *LIF* | + | ++ | ++ |
| *Bcl-2* | + | ++ | + |
| *synaptophysin* | – | + | + |

In summary, we found for the first time that resveratrol inhibited STAT3 signaling, by directly suppressing STAT3 transcription, and induced neuronal differentiation and apoptosis of medulloblastoma cells. Although some STAT3 downstream genes such as *survivin*, *cyclin D1*, *Cox-2*, and *c-Myc* were down-regulated, *Bcl-2* and *LIF* were overexpressed in resveratrol-treated cells, indicating the presence of feedback and/or compensatory response(s) to the apoptosis pressure and to the disordered STAT3 signaling. The limitation of AG490 in apoptosis induction suggests that additional survival machineries in medulloblastoma cells may also be altered by resveratrol. Our study thus provides strong evidence for the fundamental roles of STAT3 signaling in maintaining the undifferentiation, proliferation, and survival of medulloblastoma cells and the suppressive effects of resveratrol on the expressions of STAT3 and its so-called target genes. Further analysis will focus on the molecular mechanism of resveratrol-caused STAT3 gene silencing.

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