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Effect of doublecortin on self-renewal and differentiation in brain tumor stem cells

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Analysis of microarray probe data from glioma patient samples, in conjunction with patient Kaplan–Meier survival plots, indicates that expression of a glioma suppressor gene *doublecortin* (*DCX*) favors glioma patient survival. From neurosphere formation in culture, time-lapse microscopic video recording, and tumor xenograft, we show that DCX synthesis significantly reduces self-renewal of brain tumor stem cells (BTSC) in human primary glioma (YU-PG, HF66) cells from surgically removed human glioma specimens and U87 cells *in vitro* and *in vivo*. Time-lapse microscopic video recording revealed that double transfection of YU-PG, HF66, and U87 cells with *DCX* and *neurabin II* caused incomplete cell cycle with failure of cytokinesis, that is, endomitosis by dividing into three daughter cells from one mother BTSC. Activation of c-jun NH2-terminal kinase 1 (JNK1) after simvastatin (10 nM) treatment of DCX$^+$neurabin II$^+$ BTSC from YU-PG, HF66, and U87 cells induced terminal differentiation into neuron-like cells. dUTP nick end labeling data indicated that JNK1 activation also induced apoptosis only in double transfected BTSC with *DCX* and *neurabin II*, but not in single transfected BTSC from YU-PG, HF66, and U87 cells. Western blot analysis showed that procaspase-3 was induced after DCX transfection and activated after simvastatin treatment in YU-PG, HF66, and U87 BTSC. Sequential immunoprecipitation and Western blot data revealed that DCX synthesis blocked protein phosphatase-1 (PP1)/caspase-3 protein–protein interaction and increased PP1–DCX interaction. These data show that DCX synthesis induces apoptosis in BTSC through a novel JNK1/neurabin II/DCX/PP1/caspase-3 pathway. (*Cancer Sci* 2011; 102: 1350–1357)

Doublecortin (*DCX*) is a brain-specific gene and is only expressed in fetal neurons and migrating neuroprogenitor/neuroblasts.[1] Doublecortin synthesis is not detected in glioma cells.[2,3] From microarray analysis, *DCX* is absent among the differentially expressed genes in glioma cells from patients as well as glioma cell lines.[4–7] Ectopic DCX synthesis blocks glioma xenograft formation in immunocompromised hosts.[3] Single *DCX* gene therapy induces terminal differentiation in brain tumor stem cell (BTSC)-like cells, causes approximately 60% remission of xenograft 14 days after treatment in nude rats, and prolongs the survival of these animals.[8]

Cancer stem cells including BTSC are chemo-radiation therapy resistant.[9] Cancer stem cells have self-renewal ability and restore the transit-amplifying population, even if the proliferating cancer cells are completely inhibited.[9] Targeting self-renewal of BTSC is potentially an effective therapeutic approach for glioma treatment.[9] Doublecortin-mediated glioma suppression depends on phosphorylation by c-jun NH2-terminal kinase 1 (JNK1) and expression of another tumor suppressor gene, *neurabin II*.[2,3] We therefore hypothesize that ectopic DCX synthesis inhibits self-renewal and induces terminal differentiation of BTSC. Neurabin II synthesis and JNK1 activation augment the effect of DCX on BTSC differentiation. To test this hypothesis, we analyzed self-renewal of DCX$^+$/neurabin II$^+$ BTSC. We found that DCX synthesis inhibited BTSC self-renewal *in vitro* and *in vivo*. Double transfection of *DCX* and *neurabin II* induced incomplete cell cycle endomitosis in BTSC. Further activation of JNK1 by simvastatin treatment augmented the effect of DCX by inducing apoptosis in BTSC through the caspase-3 cascade pathway.

Materials and Methods

Cell culture, expression vector transfection, and lentivirus preparation. Human primary glioma (YU-PG,[10] HF66[2]) from Yale University (New Haven, CT, USA) and Henry Ford Health System (Detroit, MI, USA), and glioma U87 cells were maintained as previously described.[2,3,8,11–15] All experimental protocols were approved by the Institutional Review Board in Henry Ford Health System. Transfections of vectors were carried out as previously described.[2,3,8,11–15] Preparation and infection of lentivirus was carried out as previously described.[3,12,16] All experiments with human primary glioma YU-PG and HF66 cells were carried out between passage-2 and passage-5.

Quantitative real-time PCR (qrtPCR). The qrtPCRs were carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and analyzed by the comparative threshold cycle (ct) method ($2^{-\Delta\Delta ct}$ method) in five independent experiments, as previously described.[2,17] Sequences of primers are shown in Table 1.

Neurosphere initiation/formation assays. Brain tumor stem cells were prepared as previously described.[18–20] To evaluate BTSC self-renewal, neurosphere initiation assays were carried out in the single-cell suspensions from neurospheres of BTSC and mouse subventricular zone (SVZ) cells[11] as control for neuronal stem cells in 96-well plates according to Singh *et al.*[18–20] The number of spheres was quantified by manual counting. The number of spheres in SVZ cells was considered as normal self-renewal for neuronal stem cells.

Self-renewal assay by time-lapse microscopy. For self-renewal of BTSC, time-lapse microscopy for single-cell clonal expansion was carried out according to Shen *et al.* in a stage top chamber with 5% CO_2 at 37°C (live cell control unit), which was placed on the stage of a Nikon (Tokyo, Japan) TE2000-U inverted microscope equipped with a motorized z-stage.[21–23] Time-lapse video images of single cells were recorded for 3–4 days, then the cells were fixed with 4% paraformaldehyde in PBS for immunohistochemistry analysis.

Brain tumor stem cell implantation. Control BTSC and DCX$^+$ BTSC were implanted into the striatum of male nude rats (250–300 g) (1000 cells/rat) on day 1 according to protocols approved by the Henry Ford Hospital Institution Animal Care and Use Committee, as previously described.[2,8,12] The rats were killed on day 28 after BTSC implantation. Paraffin-embedded 6 μm thick sections were made approximately every 0.5 mm from rat brain and stained with H&E, as previously described.[2,8,12]

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Table 1. Sequences of DNA primers used in quantitative real time PCR (qrtPCR)

Gene	Species	Sense	Anti-sense
DCX	Mouse	5′-ATGCAGTTGTCCCTCCATTC-3′	5′-ATGCCACCAAGTTGTCATCA-3′
DCX	Human	5′-TTGCCCTGTCTAATTTTGCC-3′	5′-AAAAGGGGCACTTGTGTTTG-3′
Neurabin II	Human	5′-AACTGGAAGGCTACTGGGGT-3′	5′-ACGCTGTGCAGTCTCCTTTT-3′
CD133	Human	5′-TTGCCCTGTCTAATTTTGCC-3′	5′-AAAAGGGGCACTTGTGTTTG-3′
Nanog	Human	5′-CTAAGAGGTGGCAGAAAAACA-3′	5′-CTGGTGGTAGGAAGAGTAAAGG-3′
SOX2	Human	5′-CCGTTCATCGACGAGGCTAA-3′	5′-TTATAATCCGGGTGCTCCTTCAT-3′
Oct4	Human	5′-CTGGAGAAGGAGAAGCTGGA-3′	5′-CAAATTGCTCGAGTTCTTTCTG-3′
β-actin	Mouse	5′-CATCATGAAGTGTGACGTTG-3′	5′-ATGATCTTGATCTTCATGGT-3′
β-actin	Human	5′-GATGAGATTGGCATGGCTTT-3′	5′-CACCTTCACCGTTCCAGTTT-3′

Immunohistochemistry. The stem cells were seeded in polylysine coated eight-well chamber slides (Lab-Tek; Nunc, Naperville, IL, USA), as previously described.[2,3,8] These slides were immunostained for DCX, CD133, nanog, microtubule associated protein-2 (MAP2), class III beta-tubulin (Tuj1) antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated form of neurofilaments (P-NF, 1:1000; Covance, Princeton, NJ, USA), glutamic acid decarboxylase-65/67 (GAD65/67, 1:1000; Sigma, St. Louis, MO, USA), von Willebrand factor (vWF, 1:300; Dako, Carpinteria, CA, USA), and CD31 (1:200; Millipore, Billerica, MA, USA) and counterstained with DAPI.[2,3,8,11–15] Secondary antibodies were labeled with either FITC or cyanine fluorophore for 1 h and examined under a fluorescent illumination microscope (IX71/IX51; Olympus, Tokyo, Japan). The slides were stained for terminal transferase TUNEL assay using the apoptosis detection kit, ApopTag Fluorescein Kits (Intergen, Purchase, NY, USA), according to the manufacturer's protocol.[8,12]

Immunoprecipitation and Western blot analysis. For treatment with specific inhibitors for JNK1, the cells were incubated for 3 h with JNK inhibitor II (1 μM; Calbiochem, San Diego, CA, USA).[3] The cells were then lysed and analyzed by sequential immunoprecipitation and Western blot, as previously described[11–15] with DCX, CD133, β-actin, JNK1(F-3), caspase-3 (1:1000; Santa Cruz Biotechnology), active JNK (1:1000; Promega, Madison, WI, USA), protein phosphatase-1 (PP1)α antibodies (Cell Signaling Technology, Danvers, MA, USA), and cleaved caspase-3 (Asp175) antibody (1:1000; Cell Signaling Technology) that detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175, and HRP (1:10 000; Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies. Each experiment was repeated at least three times.

Statistical analysis. One-way ANOVA followed by the Student–Newman–Keuls test were used. The values were the mean of 5–10 independent experiments for real-time PCR data and three independent experiments for Western blot analysis. The data are presented as the mean ± SD. $P < 0.05$ is considered significant.

Results

Doublecortin expression favors glioma patient survival. The most sensitive oligonucleotide microarray technology failed to detect *DCX* expression in RNA isolated by laser-captured microdissection of cryostat sections from human glioma biopsy tumor.[4–7] We therefore investigated the Rembrandt dataset (National Cancer Institute, http://rembrandt.nci.nih.gov) for differential expression of *DCX* in glioma patient samples analyzed by the Affymetrix probe-based microarray. These data did not reveal any significant differences between glioma and non-tumor brain cells in *DCX* expression and showed less *DCX* expression in glioblastoma than non-tumor brain cells (Fig. 1). Kaplan–Meier survival plot indicated that *DCX* expression significantly prolonged glioma patient survival compared to intermediate *DCX*-expressing glioma patients and to all glioma patients. In contrast, glioma patients lacking *DCX* survived the shortest among the glioma patients (Fig. 2). These data showed that *DCX* expression favors glioma patient survival and *DCX* deficiency is associated with glioma patient mortality.

Doublecortin synthesis inhibits BTSC self-renewal *in vitro* and *in vivo*. As DCX synthesis is associated with glioma patient survival and terminal differentiation of BTSC-like cells *in vivo*,[8] we investigated the effect of DCX synthesis on BTSC self-renewal and differentiation and their molecular mechanisms. All experiments were carried out in control and *DCX* lentivirus infected BTSC from primary glioma (YU-PG, HF66) and U87 cells with infection efficiency exceeding 80% (Fig. S1).

To examine BTSC self-renewal, neurosphere formation assay was carried out. These data indicated that control BTSC produced a significantly higher number of neurospheres than control SVZ cells (Fig. 3). In contrast, all *DCX* lentivirus infected BTSC failed to generate conventional spheres (Fig. 3a). Doublecortin lentivirus infection had no effect on neurosphere formation in SVZ cells (Fig. 3). These data indicated that *DCX* infection significantly inhibited self-renewal of BTSC by reducing the number of spheres. The qrtPCR and Western blot data showed that *DCX* lentivirus infection significantly downregulated stem cell/stemness markers CD133, nanog, SOX2, and Oct4 in BTSC at the mRNA and protein levels (Fig. 4).

To determine the effect of DCX synthesis on self-renewal and differentiation in BTSC, a series of time-lapse microscopic

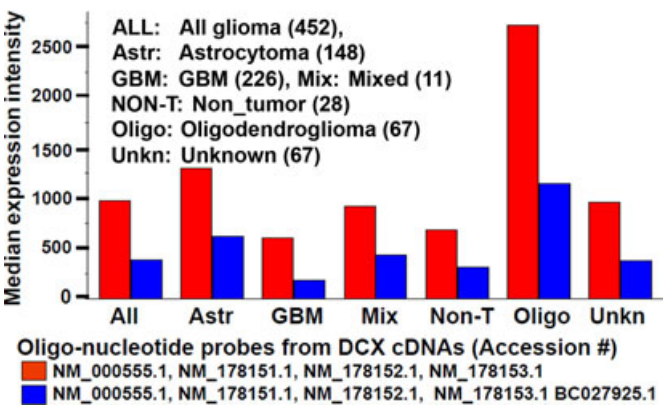

Fig. 1. Affymetrix probe-based microarray analysis for doublecortin (DCX) differential expression in glioma patient samples using the Rembrandt dataset. The bar graph indicates DCX expression. Samples types (number of samples) are shown inside the bar graph. Accession numbers of DCX cDNAs for oligo-nucleotide probes for two groups are shown at the bottom of the bar graph. GBM, glioblastoma multiforme.

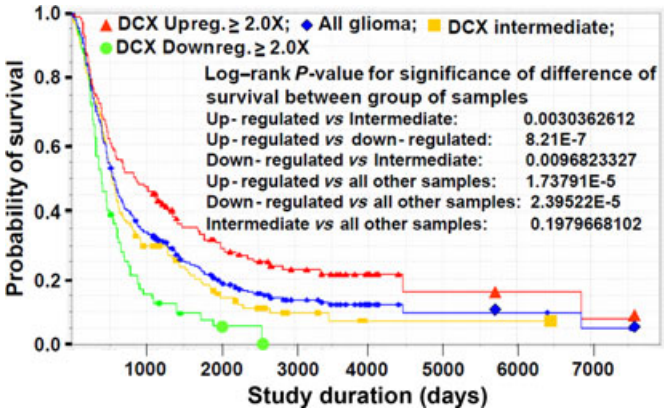

Fig. 2. *Doublecortin* (*DCX*) synthesis prolongs glioma patient survival. The Kaplan–Meier survival plot was downloaded from the Rembrandt dataset of Affymetrix gene chip microarray analyses for glioma patient samples with differential *DCX* gene expression. For example, *DCX* upregulated (upreg.), all glioma, intermediately *DCX* expressed (*DCX* intermediate), and *DCX* downregulated (downreg.) in relation to glioma patient survival.

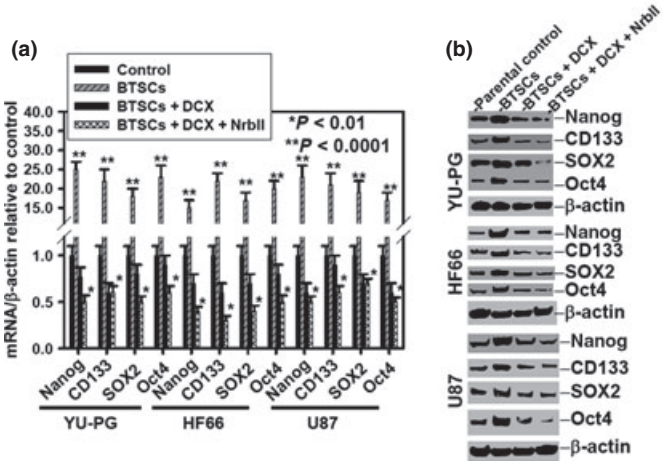

Fig. 4. Analysis of stem cell markers in brain tumor stem cells (BTSC). Stem cell markers CD133, nanog, SOX2, and Oct4 were analyzed by quantitative real-time PCR (a) and Western blot (b) in parental control and control, DCX^+, and DCX^+neurabin II^+ ($NrbII^+$) BTSC from YU-PG, HF66, and U87 cells.

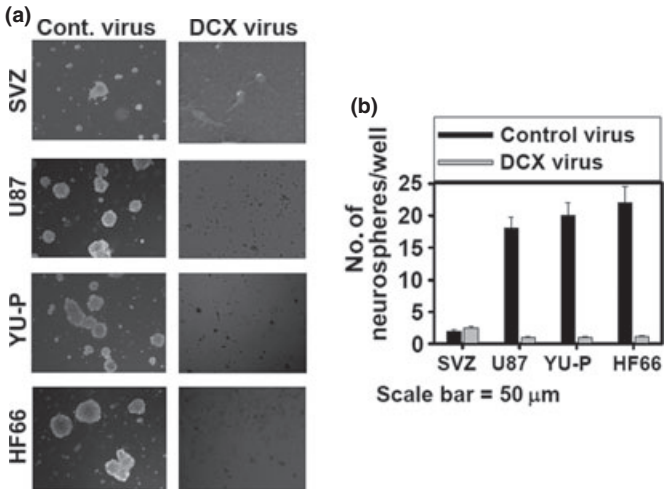

Fig. 3. Neurosphere initiation/formation was analyzed (a) and quantified (b) from single cell suspensions of control (Cont.) lentivirus and doublecortin (DCX) lentivirus infected mouse subventricular zone (SVZ), YU-PG, HF66, and U87 brain tumor stem cells.

video recording was carried out for 3 days, followed by double immunostaining with *DCX* and markers of stem cells and differentiation. These data showed that *DCX* lentivirus infection inhibited self-renewal and induced neuronal differentiation by reducing expression of stem cell markers CD133 and nanog, and by increasing expression of differentiation markers MAP2, Tuj1, P-NF, and GAD65/67 in BTSC (Figs S2–S5).[21–31] Western blot analysis indicated that *DCX* infection increased expression of P-NF and GAD65/67 in BTSC (Fig. S6). These data showed that DCX synthesis induced terminal differentiation of BTSC.

To further confirm the BTSC self-renewal data, we investigated the effect of *DCX* on BTSC xenograft formation. These data showed that DCX infection reversed the xenograft formation effect of BTSC after implantation of 10^3 cells/rat into the brain and inhibited BTSC self-renewal/tumorgenicity (Fig. S7). However, 10^4 or $>10^4$ parental glioma cells/rat were required to generate xenografts. Elongation and diffusion of BTSC xenograft with necrotic core, multiple xenograft islands, and necrosis at center of xenografts indicate the characteristics of dissemination and necrosis of BTSC xenografts (Fig. S7).[32,33]

Immunostaining of BTSC xenografts (after implantation of 5×10^3 cells/rat for 28 days) showed high expression and colocalization of vascular endothelial cell markers, for example, vWF and CD31 specifically within the BTSC xenografts, but not in non-tumor area (Fig. S8). In contrast, parental glioma xenografts rarely expressed vWF and CD31 (Fig. S8a). These data indicated that BTSC formed BTSC xenografts with typical characteristics of vasculogenesis in nude rat brains. Our data are also consistent with glioma stem cells isolated from surgical specimens of gliomas that give rise to tumor endothelium and tumor vascularization through endothelial differentiation of glioblastoma stem-like cells and that contribute to neovascularization through transdifferentiation.[34–36] Lentivirus-based *DCX* gene therapy significantly reduced vasculogenesis of BTSC xenografts in nude rat brains (data not shown).

Synthesis of DCX and neurabin II induces differentiation in BTSC through endomitosis. When both cell cycle progression and cytokinesis are genetically arrested during mitosis, stem cells (e.g. *Drosophila* abdominal neuroblasts) undergo differentiation.[37] Both cell cycle progression and cytokinesis are also arrested in double transfected U87 cells with *DCX* and *neurabin II*.[2,3] We therefore analyzed self-renewal in *DCX* infected BTSC from neurabin II transfected glioma cells (DCX^+neurabin II^+ BTSC) using time-lapse microscopy for single-cell clonal expansion followed by double immunostainings.[21–24] These data indicated that DCX^+neurabin II^+ BTSC underwent repeated incomplete cell cycle in which mitosis is aborted in late anaphase with failure of cytokinesis, that is, endomitosis (Fig. S9).[38–40] The mother cells with three nuclei were quantified by double immunostaining with DCX and neurabin II and counterstaining with DAPI in control and *DCX* lentivirus infected BTSC from *neurabin II* transfected UY-PG, HF66, and U87 cells. These data showed that the number of mother cells with three nuclei was markedly upregulated in *DCX* lentivirus infected BTSC from *neurabin II* transfected UY-PG, HF66, and U87 cells (Figs 5,S10). The triple nuclei mother cell was not detected in control YU-PG, HF66, or U87 BTSC (Fig. 5). These data showed that synthesis of both DCX and neurabin II induced differentiation through endomitosis in YU-PG, HF66, and U87 BTSC.

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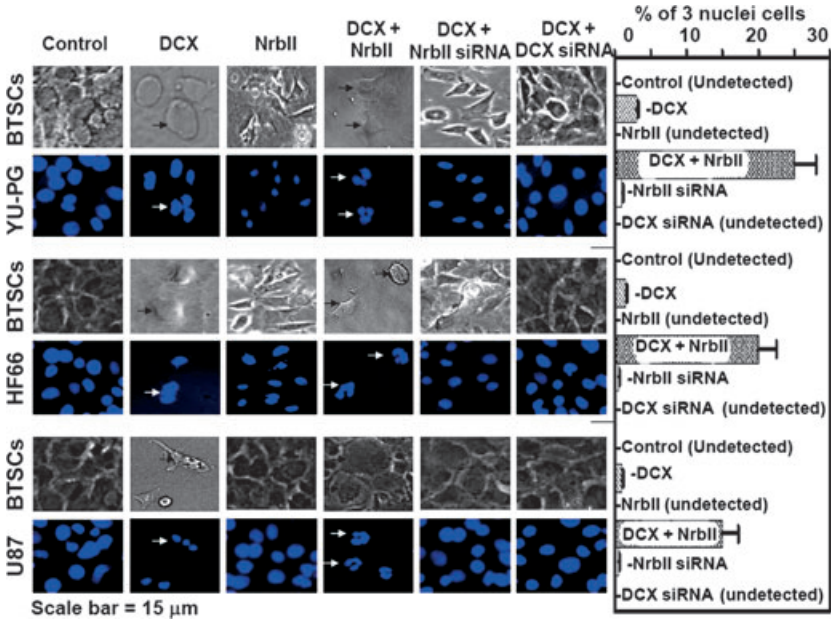

Fig. 5. Quantification of number of brain tumor stem cells (BTSC) with three nuclei. Left panel: phase contrast (top) and its DAPI staining (bottom) of cells after infection with/without *doublecortin* (*DCX*) from YU-PG, HF66, and U87 cells subjected to *neurabin II* (*NrbII*) or *neurabin II* siRNA or *DCX* siRNA transfections. Right panel: bar graph indicates percentage of corresponding cells with three nuclei.

Simvastatin treatment markedly inhibits self-renewal in DCX$^+$neurabin II$^+$ U87 BTSC. Doublecortin phosphorylation by JNK1 is required for glioma suppression.[2] Our data of JNK1 activation in BTSC after simvastatin treatment are consistent with JNK1 activation in C6 glioma cells (Fig. S11).[41] We therefore investigated the effect of 10 nM simvastatin treatment on self-renewal in DCX lentivirus infected BTSC from neurabin II transfected glioma cells by time-lapse microscopy video recoding for 3 days. These data showed normal symmetrical self-renewal in control BTSC, as shown in Figures S2 and S3. In contrast, DCX$^+$neurabin II$^+$ BTSC from YU-PG, HF66, and U87 cells changed their morphologies into neuronal-like cells without cell division after 10 nM simvastatin treatment (Fig. 6) and eventually died in culture after 4 days. Treatment with JNK1 inhibitor or transfection with neurabin II siRNA or DCX siRNA reversed these effects into a proliferating stage (Fig. 6). These data indicated that simvastatin treatment induced neuronal differentiation in DCX$^+$neurabin II$^+$ BTSC in a JNK1/DCX/neurabin II-dependent pathway.

Simvastatin induces apoptosis in DCX$^+$neurabin II$^+$ BTSC. Simvastatin treatment induced neuronal diffentiation in DCX$^+$neurabin II$^+$ BTSC, which eventually died after 4 days. To confirm this cell death, TUNEL staining was carried out in BTSC after treatment with/without 10 nM simvastatin for 4 days or after infection with/without DCX lentivirus from control and *neurabin II* transfected YU-PG, HF66, and U87 glioma cells as well as after constitutively active *JNK1* transfection. These data showed that both simvastatin treatment and *JNK1* transfection induced apoptosis in *DCX* infected YU-PG, HF66, and U87 BTSC (Figs 7,8). These effects were markedly augmented after *neurabin II* transfection (Figs 7,8). Treatment with JNK1 inhibitor or transfection either with *neurabin II* siRNA or *DCX* siRNA reversed this apoptotic effect (Figs 7,8). These data indicate that simvastatin treatment induces apoptosis in BTSC through the JNK1/DCX/neurabin II pathway.

Simvastatin treatment induces caspase-3 activation in BTSC. Simvastatin treatment induces apoptosis in C6 glioma cells by upregulating caspase-3 activation.[41] To determine the

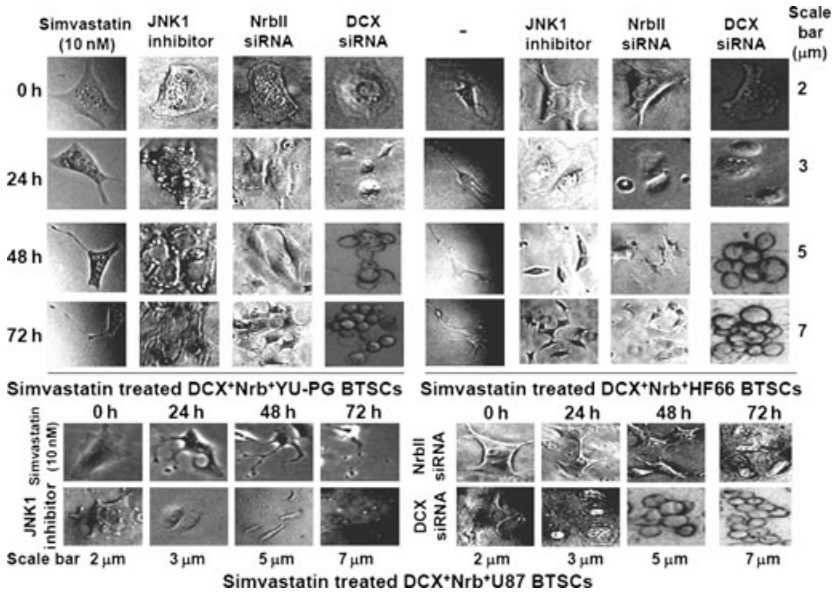

Fig. 6. Simvastatin treatment induces neuronal morphologies in doublecortin (DCX)$^+$ neurabin II (NrbII)$^+$ brain tumor stem cells (BTSC). DCX$^+$neurabin II$^+$ BTSC from YU-PG, HF66, and U87 cells were examined using time-lapse microscopic video recording for 3 days after treatment with 10 nM simvastatin followed by treatment with c-jun NH2-terminal kinase 1 (JNK1) inhibitor or transfection either with neurabin II siRNA or DCX siRNA.

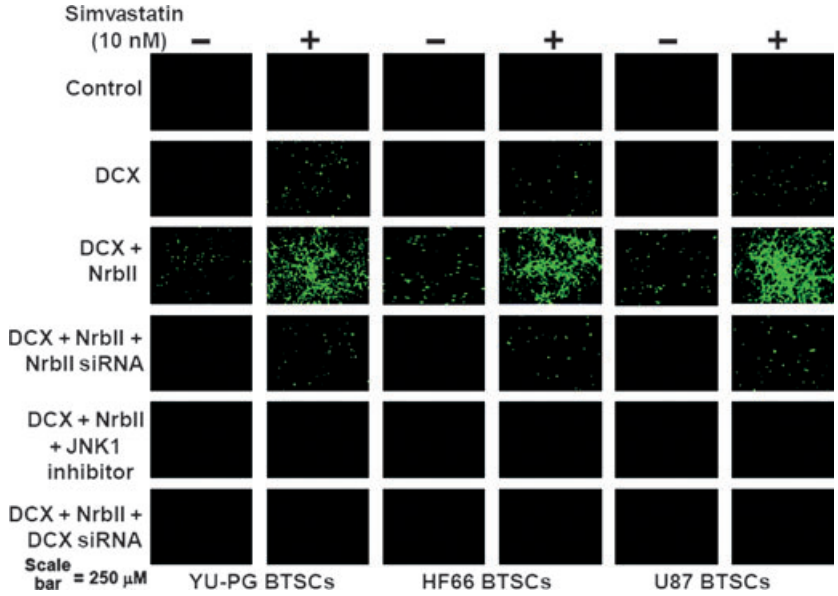

Fig. 7. Simvastatin treatment induces apoptosis in doublecortin (DCX)$^+$ brain tumor stem cells (BTSC). dUTP nick end labeling is shown in control, DCX$^+$ and DCX$^+$neurabin II (NrbII)$^+$ BTSC from YU-PG, HF66, and U87 glioma cells after treatment with (+) or without (–) 10 nM simvastatin. These DCX$^+$neurabin II$^+$ BTSC were subjected to c-jun NH2-terminal kinase 1 (JNK1) inhibitor or neurabin II siRNA or DCX siRNA transfection (three bottom rows).

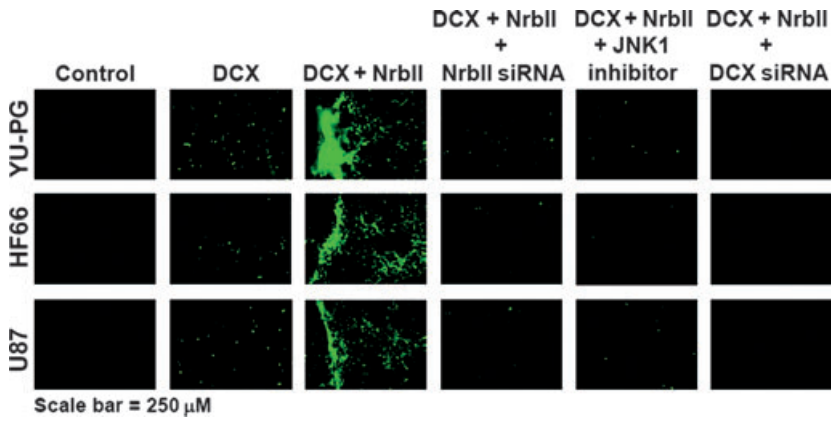

Fig. 8. Doublecortin (DCX)$^+$ brain tumor stem cells (BTSC) undergo apoptosis after constitutively active c-jun NH2-terminal kinase 1 (JNK1) transfection. Control, DCX$^+$ and DCX$^+$neurabin II (NrbII)$^+$ BTSC from YU-PG, HF66, and U87 glioma cells were stained for TUNEL after transfection with constitutively active JNK1. These DCX$^+$neurabin II$^+$ BTSC were subjected to JNK1 inhibitor or neurabin II siRNA or DCX siRNA transfection (three right columns).

mechanism of apoptosis in BTSC, we therefore examined caspase-3 activation in BTSC by Western blot analysis. *Doublecortin* lentivirus infection induced caspase-3 expression in YU-PG, HF66, and U87 BTSC (Fig. 9). However, the cleaved caspase-3 or the large fragment (17–19 kDa) of activated caspase-3, resulting from cleavage adjacent to Asp175, was not detected in *DCX* lentivirus infected YU-PG, HF66, and U87 BTSC (Fig. 9). In contrast, simvastatin treatment (10 nM) or transfection of constitutively active JNK1, increased activation of caspase-3 only in *DCX* lentivirus infected BTSC, but not in control BTSC from YU-PG, HF66, and U87 cells (Fig. 9). Treatment with JNK1 inhibitor, or neurabin II siRNA or DCX siRNA transfection, reversed caspase-3 activation in YU-PG, HF66, and U87 BTSC (Fig. 9). These data showed that JNK1, after activation by simvastatin, activated caspase-3 in DCX$^+$neurabin II$^+$ BTSC, which underwent apoptosis. The DCX$^+$neurabin II$^+$ BTSC underwent differentiation into neuron-like cells after simvastatin treatment (Fig. 6). These neuron-like cells differentiated for another day from the experiments shown in Figure 6, and underwent cell death *in vitro* (Figs 7–9).

Mechanism of caspase-3 activation in simvastatin treated glioma cells. The PP1/PP2A inhibitors induce caspase-3 mediated apoptosis in several cell types.[42] Doublecortin is involved in DCX/PP1 protein–protein interaction and acts as a competitive inhibitor for PP1.[2,3] To determine whether DCX/PP1 interaction regulated caspase-3 activation, we analyzed sequential immunoprecipitation and Western blot analysis in *DCX* lentivirus infected YU-PG, HF66, and U87 BTSC after simvastatin (10 nM) treatment. These data showed caspase-3–PP1 interaction in YU-PG, HF66, and U87 BTSC after transfections with control, *neurabin II*, and *JNK1*, and after simvastatin treatment (Fig. 10). In contrast, *DCX* lentivirus infected BTSC either from *neurabin II* or *JNK1* transfected YU-PG, HF66, and U87 BTSC glioma cells, or after treatment with/without simvastatin, showed both caspase-3–PP1 and DCX–PP1 interactions (Fig. 10). Interaction between DCX and PP1 was found in DCX$^+$neurabin II$^+$ BTSC after simvastatin treatment, without caspase-3–PP1 interaction (Fig. 10). However, treatment with JNK1 inhibitor or transfection with either *neurabin II* siRNA or *DCX* siRNA reversed DCX–PP1 interaction into caspase-3–PP1 interaction (Fig. 10). These data suggest that JNK1 activation after simvastatin treatment induces DCX–PP1 interaction in DCX$^+$neurabin$^+$ BTSC and completely reduces caspase-3–PP1 interaction, which may inactivate caspase-3.

Discussion

The Kaplan–Meier survival plot from the Rembrandt dataset showed that DCX synthesis prolongs glioma patient survival. These data are also consistent with animal survival after

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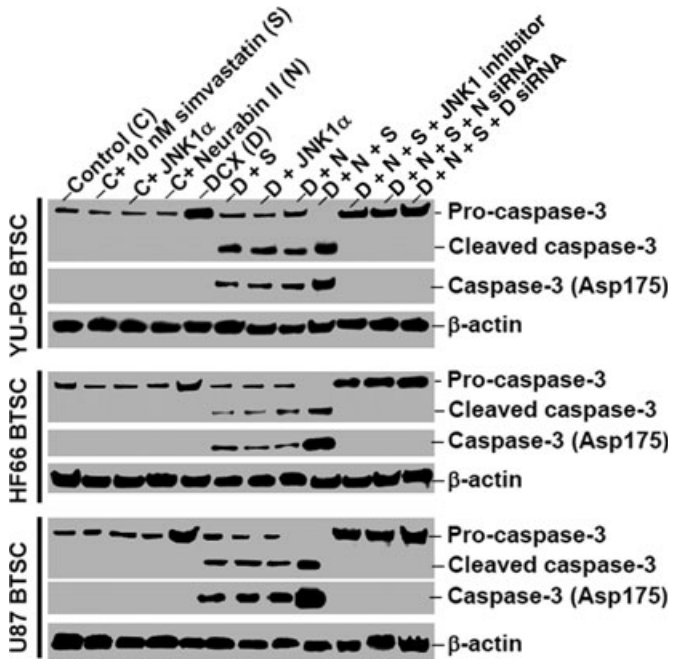

Fig. 9. Western blot analysis for caspase-3 activation. Different treatments and transfections of YU-PG, HF66, and U87 brain tumor stem cells (BTSC) are indicated at the top of the gel. The loading of the samples was normalized with β-actin. Migrations of proteins are marked at right. Caspase-3 (Asp175) indicates the large fragment (17–19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. DCX, doublecortin; JNK1, c-jun NH2-terminal kinase 1.

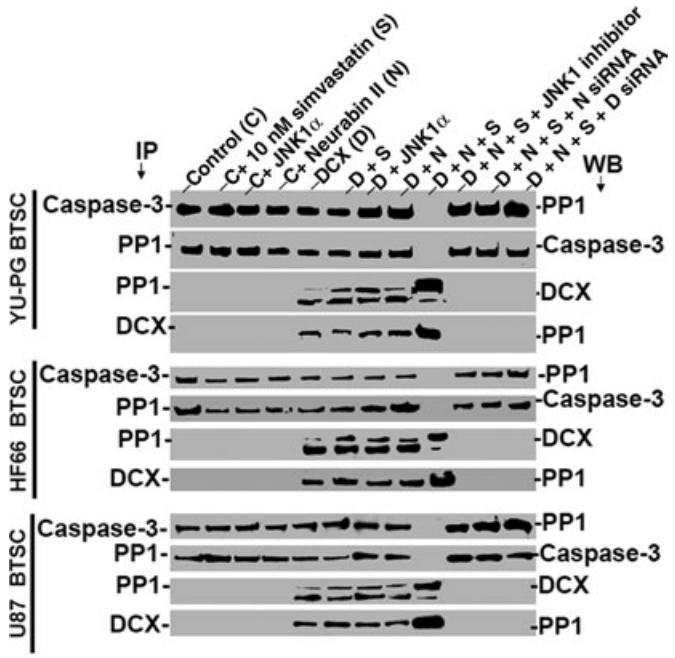

Fig. 10. Sequential immunoprecipitation (IP) and Western blot (WB) analysis of brain tumor stem cells (BTSC). Different treatments and infections/transfections of YU-PG, HF66, and U87 cells are indicated at the top of the gel. The antibodies for the sequential IP are shown at left, and those for WB shown at right. DCX, doublecortin; JNK1, c-jun NH2-terminal kinase 1; PP1, protein phosphatase-1.

lentivirus-based DCX gene therapy[8] as well as glioma patient survival.[43,44] From microarray expression profiling in high-grade glioma, proneural subclass displaying neuronal lineage markers shows longer survival, whereas proliferative and mesenchymal subclasses enriched for neuronal stem cell markers display equally short survival.[45] We showed that DCX synthesis significantly reduced self-renewal of BTSC and induced differentiation with the expression of neural marker MAP2.[18–22,24] Double transfection with *DCX* and *neurabin II* induces incomplete cell cycle endomitosis in BTSC, indicating a unique mechanism for differentiation. Further activation of JNK1 with simvastatin treatment not only increased the effect of DCX on terminal differentiation, but also induced apoptosis in DCX^+neurabin II^+ BTSC. After phosphorylation by JNK1, DCX induced DCX–PP1 protein–protein interaction and reduced caspase-3–PP1 interaction. Protein phosphatase-1 therefore failed to dephosphorylate caspase-3. Hyperphosphorylated caspase-3 was activated[46] and induced apoptosis in DCX^+neurabin II^+ BTSC in a novel JNK1/DCX/neurabin II/caspase-3 cascade pathway.

Normal stem cells maintain balance between self-renewal promoting genes, such as proto-oncogenes, and self-renewal limiting genes, such as tumor suppressors.[47] Mutations of tumor suppressors that inappropriately activate self-renewal programs cause cancers.[47] Ectopic expression of tumor suppressor neurabin II synergizes the DCX effect on glioma suppression by inducing apoptosis in U87 cells.[2] Our data showed that double transfection of *DCX* and *neurabin II* enhanced differentiation by inducing endomitosis in BTSC. These data are consistent with cytochalasin B mediated differentiation of megakaryocytes through endomitosis.[48] In genotoxic insult, p53 mutated tumor cells undergo mitotic catastrophe, leading to a switch from mitosis to endomitosis.[39] The essential difference in endomitosis from mitosis is that DNA synthesis is uncoupled from cell division, leading to the formation of endopolyploid cells.[39,49] The genomes (somatic reduction) of these endopolyploid cells are segregated into meiotic divisions in the tumor cell system.[39,49] The somatic reduction of polyploidy in eukaryotic cells is quite rare and the most polyploid cells terminally differentiate and degenerate.[39,49] In our data, three cells generated from one BTSC indicated the formation of endopolyploid BTSC that terminally differentiated and eventually died.

Pharmacological inhibitors of protein phosphatases, including PP1, block cell cycle progression at G_2/M phases and even induce apoptosis in cancer cells.[42,50] Doublecortin, neurabin II, and PP1 are also found in the same protein complex from mouse brain extracts and DCX transfected glioma cells.[3,51] Neurabin II belongs to this phospho/dephosphorylated class of regulators through protein–protein interactions, because it negatively regulates the PP1 catalytic subunit activity.[52] We found that JNK1 activation induced caspase-3 activation only in DCX^+neurabin II^+ BTSC, but not in DCX^+neurabin II^- or DCX^-neurabin II^+ BTSC. However, DCX synthesis induced procaspase-3 expression in BTSC. We found PP1/caspase-3 interaction in DCX^- BTSC. In contrast, PP1 interacted with DCX, but not with caspase-3 in DCX^+ BTSC. Doublecortin synthesis blocked PP1/caspase-3 interaction and influenced the hyperphosphorylation of caspase-3, which led to activation of caspase-3.[46] These data are also consistent with PP1/PP2A inhibitors, which induce apoptosis by activating caspase-3 in several cell types in culture.[42] Our data showed that DCX induced apoptosis in BTSC in a novel JNK1/neurabin II/PP1/caspase-3 cascade pathway.

In summary, DCX expression favors glioma patient survival. Doublecortin synthesis inhibited self-renewal of BTSC. Double transfection with *DCX* and *neurabin II* induced differentiation in BTSC through incomplete cell cycle endomitosis. Further activation of JNK1 after simvastatin treatment not only induced terminal neuronal differentiation, but also induced apoptosis in a novel JNK1/neurabin II/PP1/caspase-3 cascade pathway. Further investigation on the treatment of glioma with recombinant DCX and neurabin II along with simvastatin are warranted.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Determination of lentivirus infection efficiency in brain tumor stem cells (BTSC).

Fig. S2. Self-renewal analysis of brain tumor stem cells (BTSC) using time-lapse microscopic video recording.

Fig. S3. Effect of doublecortin (DCX) synthesis on brain tumor stem cell (BTSC) differentiation.

Fig. S4. Doublecortin (DCX) mediates Tuj1 expression.

Fig. S5. Effect of doublecortin (DCX) synthesis on mature neuronal markers.

Fig. S6. Western blot analysis for phosphorylated forms of neurofilaments and glutamic acid decarboxylase-65/67 (GAD65/67).

Fig. S7. Glioma xenograft formation.

Fig. S8. Determination of vasculogenesis of brain tumor stem cell (BTSC) xenografts.

Fig. S9. Time-lapse microscopic video recoding in doublecortin $(DCX)^+$neurabin II^+ brain tumor stem cells (BTSC).

Fig. S10. Doublecortin (DCX) and neurabin II synthesis induce endomitosis in brain tumor stem cells (BTSC).

Fig. S11. Activation of c-jun NH2-terminal kinase 1 (JNK1) and doublecortin (DCX) after simvastatin treatment.

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