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Inhibition of Neurogenesis of Subventricular Zone Neural Stem Cells by 5-ethynyl-2'-deoxyuridine (EdU)

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In the subventricular zone (SVZ) and the subgranular zone of the brain, neurogenesis occurs throughout one's lifespan. Neural stem cells (NSCs) in these regions divide to maintain their stem cell pools as well as differentiate into neurons and glial cells. To monitor cell division, a thymidine analogue such as 5-ethynyl-2'-deoxyuridine (EdU) has been used. In some cases, EdU was applied to label newly born neurons. Here, we report about the effects of EdU on the proliferation and differentiation of NSCs cultured from mouse SVZ. First, when NSCs were cultured in a proliferation medium containing EdU for 24 hr, they did not generate any neurons under the following differentiation conditions. When EdU was applied to the proliferating NSCs for 1 hr prior to differentiation, neurogenesis was still substantially reduced. Second, EdU decreased cell proliferation of NSCs in dose- and time-dependent manners. Finally, EdU inhibited differentiation into oligodendrocyte lineage, while the number of glial fibrillary acidic protein (GFAP)-positive astrocytes increased. To our knowledge, these findings are the first to show the effects of EdU on the differentiation of SVZ NSCs and suggest that cell division is necessary for differentiation into neurons and oligodendrocytes.

Key words : Cell proliferation, 5-ethynyl-2'-deoxyuridine (EdU), neural stem cells (NSCs), neuronal differentiation, subventricular zone (SVZ)

Introduction

Cell proliferation can be detected in many ways, among which detecting incorporation of nucleoside analogues into the DNA has been frequently used to examine cell division. Among nucleoside analogues, 5-bromo-2'-deoxyuridine (BrdU) has been mostly used as a thymidine analogue. However, detection of incorporated BrdU requires denaturation of the DNA in the cells so that antibodies against BrdU can get an access to the DNA [2]. A newly developed thymidine analogue, EdU, contains an alkyne group that can react with a fluorescent-tagged azide through so called 'click chemistry' [21]. This method does not require denaturation process, which preserves cell structures during EdU detection. Also, the intensity of fluorescence from the click chemistry is more linearly correlated with the level of EdU incorporated than antibody-based detection of BrdU. Thanks to these advantages, EdU has been used to label S-phase of the cell cycle in various biological experiments since its emergence [2]. Yet, cytotoxicity of EdU has been reported in many previous studies. Inhibition of cell proliferation by EdU has been shown in glioblastoma cells, human breast cancer cell lines and other cancer cell line [7, 13, 20, 24]. Especially, EdU incorporation induced cell cycle arrest in mouse embryonic stem cells, while BrdU did not [13]. EdU inhibited thymidylate synthetase by competing against 2'-deoxyuridylate [6]. Even though EdU has been used to label proliferating cells in SVZ and subgranular zone in hippocampus, [12, 17, 23], direct effects of EdU on NSCs have not been examined.

SVZ in the brain is the region where life-long neurogenesis occurs. It contains multiple types of cells such as ependymal cells, type B cells, transit-amplifying progenitor (TAP) type C cells and type A cells (neuroblasts) [8]. Type B cells can exist as both GFAP$^+$EGFR (Epidermal Grwoth Factor Receptor)$^-$Nestin$^-$ quiescent stem cells and GFAP$^+$EGFR$^+$Nestin+ activated stem cells [4]. Activated type B cells give rise to type C cells and type C cells undergo multiple cell divisions and finally commit to neuronal lineage by becoming type A neuroblasts [9]. Live imaging revealed slow-dividing NSCs and fast-dividing NSCs when they were cul-

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tured directly from SVZ and maintained in serum-and growth factor-free medium [5]. Half of the fast-dividing NSCs divided once before becoming post-mitotic neuroblasts. The other half of them divided 2 to 5 times before becoming neuroblasts. Consistently, antimitotic drug cytosine-beta-D-arabinofuranoside (Ara-C) infusion to the animals killed off proliferating type A and type C cells of SVZ [9, 11, 19]. However, whether or not cell division is necessary and essential for neuronal differentiation has not been directly tested *in vitro*.

Initially, we intended to map the fate of SVZ NSCs using EdU in our *in vitro* culture system. Instead, to our great surprise, 24-hr treatment of EdU during cell proliferation completely eliminated neurogenesis during the following differentiation period. This was accompanied by reduction of total cell numbers by EdU treatment. Short-term treatment such as 1-hr treatment of EdU also greatly inhibited neurogenesis during the following differentiation. We think these might not be due to nonspecific and general cytotoxic effects, because incubation of proliferating NSCs in EdU-containing medium did not kill off cells, but rather seemed to slow down overall cell proliferation. When numbers of cells were counted at different time points, EdU did not affect cell proliferation at 6 and 24 hr, but reduced the number of cells after 48 hr. Finally, no Olig2-positive cells were found after differentiation in EdU-treated samples, while the number of GFAP-positive astrocytes was increased. Our report showed that EdU inhibited both neurogenesis and oligodendrogenesis. All these results suggest that cell proliferation is indeed required for differentiation of NSCs to neurons and oligodendrocytes.

Materials and Methods

Mouse SVZ NSC culture

To obtain mouse SVZ NSCs, postnatal mice (5 to 7 day-old CD1 (ICR) mice from Orient Bio, Sungnam, Korea) were euthanized using carbon dioxide. Then, brains were taken out from the mice to cut out tissues of SVZ. Preparation of cells from the SVZ tissues was done in the same way as we reported previously [18]. This animal work was approved by Inje University Animal Care and Use Committee (approval ID number: 2016-011) and all the procedures were performed under the national guidelines for animal care and use of laboratory animals.

Cultured cells were grown in a proliferation medium, N5 medium, which contains DMEM/F12-GlutaMAX™ supplement (Gibco, ThermoFisher, Waltham, MA, USA), 5% fetal bovine serum (GenDEPOT, Texas, USA), N2 supplement (Gibco, ThermoFisher, Waltham, MA, USA), 35 μg/ml bovine pituitary extract (Gibco, ThermoFisher, Waltham, MA, USA), 20 ng/ml epidermal growth factor (EGF, Invitrogen, ThermoFisher, Waltham, MA, USA), 20 ng/ml basic fibroblast growth factor (bFGF, Gibco, ThermoFisher, Waltham, MA, USA), and antibiotic/antimycotic (Gibco, ThermoFisher, Waltham, MA, USA). NSCs were maintained in N5 medium at 37°C with 5% carbon dioxide. Cells were passaged every 2 or 3 days depending on confluency. All the SVZ NSCs used in this study were passaged 5 to 7 times before being used for actual experiments.

Neuronal differentiation of cultured SVZ NSCs

Proliferating NSCs were plated on a laminin-coated 8-well Lab-Tek CC2 chamber slide (Nunc, ThermoFisher, Waltham, MA, USA) at 1 day prior to differentiation. For laminin coating, 5 μg/ml laminin (Invitrogen, ThermoFisher, Waltham, MA, USA) dissolved in phosphate-buffered saline (PBS) was added to cover the surface of the chamber slide and incubated for 4 hr to overnight at 37°C. Before plating, the chamber slide was rinsed with PBS. To start differentiation, cells were briefly rinsed with N6 medium that is the same medium as N5, but lacking EGF, bFGF, and fetal bovine serum. Then, cells were incubated in fresh N6 medium for 5 days before fixation.

EdU incorporation and detection

For EdU incorporation and detection, all the procedures followed manufacture's instructions by using Click-iT Plus EdU Imaging Kit (Invitrogen, ThermoFisher, Waltham, MA, USA). Proliferating SVZ NSCs were incubated with EdU dissolved in N5 medium for 1 day and then, with fresh N6 medium to initiate differentiation. For the 1-hr pulse labeling, EdU-containing N5 medium was added to the proliferating NSCs at 1 hr prior to medium switch to N6. Cells were fixed in 4% paraform aldehyde (Sigma, St. Louis, MO, USA) for 15 min, rinsed with 3% bovine serum albumin (BSA) in PBS twice, incubated in blocking solution with 10% goat serum (Cell Signaling, Danvers, MA, USA) and 0.1~0.3% triton X-100 (Sigma, St. Louis, MO, USA) in PBS. Finally, EdU detection procedure was done following manufacturer's instructions. Alexa Fluor® 594 picolyl azide was added to make a reaction cocktail solution that was added

to the cells for 30 min in dark. For all the rinsing, 3% BSA in PBS was used.

Immunocytochemistry and imaging

To stain cells using antibodies, the same method as previously reported [18] was performed. Primary and secondary antibodies and their dilutions were as followings: mouse anti-Tuj1 (Novex, ThermoFisher, Waltham, MA, USA) at 1:500 dilution, mouse anti-GFAP (Millipore, Billerica, MA, USA) at 1:500 dilution, rabbit anti-Olig2 (Millipore, Billerica, MA, USA) at 1:500 dilution (all primary antibodies were diluted in blocking solution), Alexa-488 - conjugated anti-mouse, and Alexa-594 - conjugated anti-rabbit secondary antibodies (1:500 in PBS; both from Jackson Immuno Research, West Grove, PA, USA). For nuclear staining, cells were incubated with DAPI (4′,6-diamidino-2-phenylindole, Sigma, St. Louis, MO, USA) at 1:1,000 dilution. In the case of EdU treatment, EdU detection preceded the incubation of cells with primary antibodies.

Images were taken using a fluorescence microscope (Olympus, Tokyo, Japan). To count cells, 4 to 5 non-overlapping images were taken per well. The number of DAPI was counted using the cell count macro in iSolution software (Olympus, Tokyo, Japan). Others such as Tuj1, GFAP, Olig2,

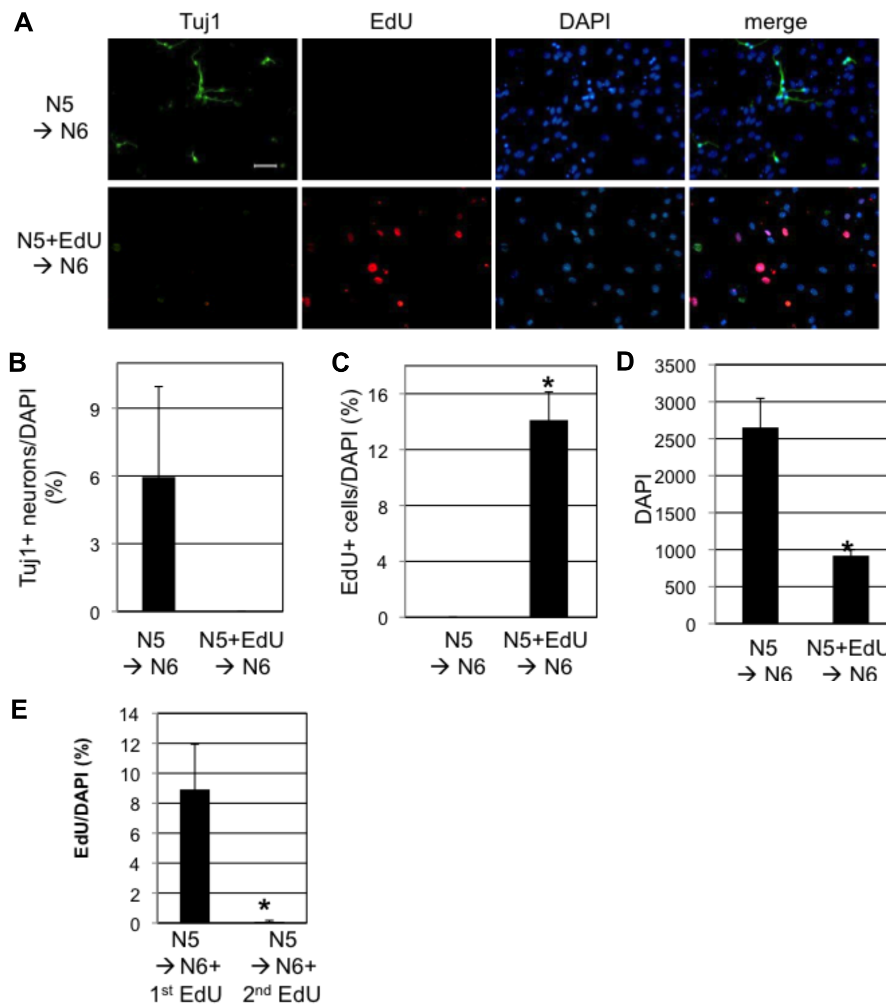

Fig. 1. Neurogenesis of SVZ NSCs was blocked by EdU. A. NSCs were maintained in proliferating medium (N5) in the absence of EdU (*upper lane*) or in the presence of 5 μM EdU (*lower lane*) for 1 day before differentiation. To initiate neurogenesis, NSCs were incubated in the differentiation medium (N6) for 5 days. Immunocytochemistry was done to detect neuronal specific marker (Tuj1) and DAPI for nucleus. Also, EdU incorporation into the cells was detected. Scale bar=20 μm. B. Tuj1-positive neurons were counted and divided by total number of DAPI. C. EdU-positive cells were counted and divided by total number of DAPI. D. Total number of DAPI counted is shown. E. Five μM EdU was added for 24 hr at the 1st day of differentiation (N5àN6+1st EdU) or 2nd day of differentiation (N5àN6+2nd EdU). B-E. In each condition, average and standard deviation from three different wells of cells are shown (*p<0.01, Student's T-test).

or EdU-positive cells were manually counted from 3 wells for each condition.

Cell proliferation assay

The same numbers of NSCs were plated onto dish in N5 medium containing different concentrations of EdU, 0, 5, 15, or 45 μM. At 1 day of incubation in EdU, cells were trypsinized and harvested for cell counting manually using hematocytometer. For the time-point experiment, cells were kept in N5 containing 0, 5, or 15 μM of EdU. Then, at 6, 24, and 48 hr of EdU treatment, cells were harvested and the live cells were counted using EVE automatic cell counter (NanoEnTek, Seoul, Korea).

Results

Inhibition of neurogenesis by EdU

In general, proliferation and differentiation of stem cells have been regarded as separate processes. If cells are undergoing proliferation, they are not differentiating and vice versa. However, cells divide during neuronal differentiation of SVZ NSCs. In SVZ, NSCs produce transit amplifying cells which divide multiple rounds and later become migratory neuroblasts [15]. To examine relationship between proliferation and differentiation more closely, we used EdU, a thymindine analogue, which incorporates into DNA during S-phase. Cultured SVZ NSCs were maintained in N5 medium in the absence or presence of 5 μM EdU for 1 day and then, medium was switched to N6 medium to initiate neuronal differentiation. After 5 days of differentiation, cells were fixed for EdU detection and immunocytochemistry. To our great surprise, NSCs treated with EdU failed to generate any neurons, while about 6 % of cells were Tuj1+ neurons in NSCs without EdU treatement (Fig. 1A, Fig. 1B). Approximately 14 % of total cells were EdU+ in NSCs treated with EdU (Fig. 1A, Fig. 1C). Also, EdU treatment decreased total number of cells by 65%(Fig. 1D). Upon incorporation into the DNA, EdU probably blocked further process of cell division by holding cell cycle progression. This interruption might have been involved in complete loss of neurogenesis (Fig. 1A - Fig. 1D). When EdU was added as a pulse for the first or second day of N6 medium, approximately 9% or 0% of total cells were EdU-positive, respectively (Fig. 1E). This indicates that cells still divide during the first 24 hr of differentiation and then, stop dividing afterwards.

To further study about effect of EdU on neuronal differentiation, shorter incubation with EdU was performed. SVZ NSCs were incubated in N5 medium containing 5 μM EdU for 1 or 24 hr prior to differentiation. Consistently to the previous result, 24-hr EdU treatment abolished neurogenesis, while 53% of the total cells were Tuj1+ neurons in NSCs without EdU treatment (Fig. 2A). When NSCs were treated with EdU for 1 hr, 19% of the total cells were Tuj1+ neurons. These trends correlated with the total number of cells counted by DAPI staining (Fig. 2B). All these data suggest that uninterrupted cell division process upon the cue of differentiation might be required to produce neurons.

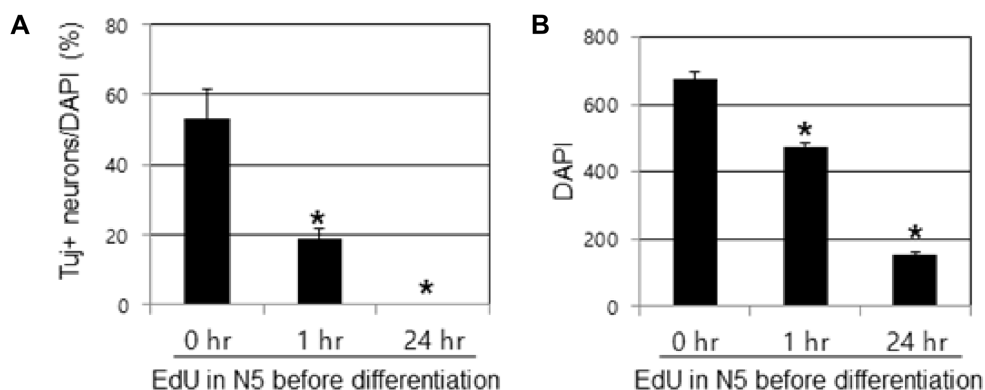

Fig. 2. The longer treatment of EdU during proliferation of SVZ NSCs resulted in the less neurogenesis. A. NSCs were incubated in N5 medium and 5 μM EdU was added to the N5 at 1 or 24 hr before starting differentiation using N6 medium. Negative control without EdU is 0 hr time-point condition. At 5 days of differentiation, cells were fixed for immunocytochemistry and EdU detection. Tuj1-positive neurons were counted and divided by total number of DAPI. B. Total number of DAPI counted for each condition is shown. A, B. Data are average and standard deviation from three different wells of cells for each condition (*p<0.01, Student's T-test).

Inhibition of cell proliferation by EdU

According to DAPI counting in figures 1 and 2, the number of cells decreased by EdU treatment. To examine the effect of EdU on cell proliferation, we applied different doses of EdU for various time periods. The same numbers of proliferating SVZ NSCs were treated with 0, 5, 15, and 45 µM of EdU for 1 day and numbers of live cells were counted. While 5 µM of EdU did not decrease the number of cell significantly, 15 or 45 µM of EdU decreased numbers of cells almost by half (Fig. 3A). Separate groups of proliferating NSCs were incubated in 0, 5, or 15 µM EdU-containing N5 medium and total numbers of cells were counted at 6, 24, and 48 hr post EdU treatment. After 6- or 24-hr treatment, numbers of cells were not significantly different (p>0.05) in all conditions (Fig. 3B). After 48-hr treatment, NSCs treated with 5 or 15 µM EdU were significantly less than control cells (**p<0.01). Since EdU did not kill off NSCs right after treatment, but rather it took time to reduce cell numbers, we think that EdU had held cell cycle progression in NSCs as previously reported in other cell types instead of having non-specific cytotoxic effects [13].

Effects of EdU on gliogenesis

Next, we investigated whether EdU has any effects on differentiation of glial cells such as astrocytes and oligodendrocytes. As in Fig. 1, SVZ NSCs were incubated in EdU-containing N5 medium for 1 day before the medium switch to N6 for differentiation. After 5 days of differentiation, cells were fixed for immunocytochemisty to stain GFAP, a marker for astrocytes, and Olig2, a maker for immature oligodendrocytes (Fig. 4A). GFAP+ cells were 6% of the total cells in no EdU condition, while GFAP+ cells were 17% in EdU-treated NSCs (Fig. 4B). More strikingly, Olig2+ cells were very few (0.2% of the total cells) in EdU-treated NSCs, while Olig2+ cells were 5% in no EdU condition (Fig. 4C). Total numbers of cells were significantly less in EdU-treated condition as expected (Fig. 4D). Therefore, perturbed cell cycle by EdU seemed to inhibit generation of oligodendrotye lineage cells from SVZ NSCs, but increase differentiation to astrocytes.

We think at least some of GFAP-positive cells in figure 4 might be quiescent NSCs, because (1) quiescent NSCs are GFAP-positive and resistant to antimitotic drug, EdU in our case [4, 10] and (2) addition of serum and growth factors in the form of N5 medium during differentiation increased neurogenesis (Fig. 5). As a control, cultured mouse SVZ NSCs were treated with differentiation medium (N6) for 10 days and Tuj1-positive neurons were detected in ~12% of the total cells (Fig. 5A, Fig. 5B, *upper panels*). As an experimental group, cells were treated with N6 medium for 5 days, then, incubated in N5 proliferation medium for 3 days before the final incubation in N6 for 2 days (Fig. 5A, Fig. 5B, *lower panels*). This medium switch with N5 in the middle of differentiation resulted in increased neurogenesis

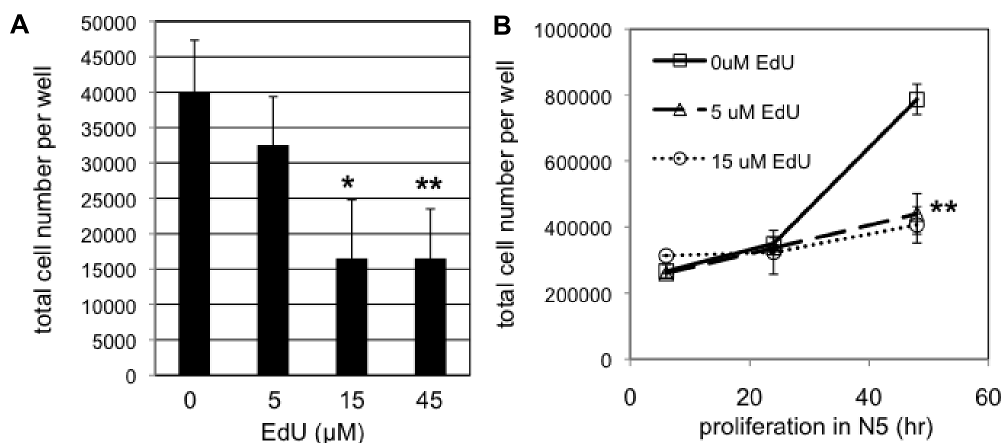

Fig. 3. Cell proliferation was inhibited by EdU in dose- and time-dependent manners. A. Same number of SVZ NSCs were plated onto culture dish. Then, they were incubated in N5 medium containing different concentration of EdU (0, 5, 15, or 45 µM). At 24 hr, number of cells was counted. Data are average and standard deviation from four different wells of cells. B. NSCs were incubated in N5 medium containing 5 or 15 µM EdU. As a negative control, NSCs were grown in N5 medium in the absence of EdU (0 µM EdU). At 6, 24, 48 hr post EdU treatment, number of cells were counted. Data are average and standard deviation from three different wells of cells. A, B. Statistical significance compared with the negative control (0 µM) is shown (*p<0.05, **p<0.01, Student's T-test).

by 44% (Fig. 5C). These results are consistent with the previous report that showed an increased level of neurogenic transcription factor *Dlx2* transcript by similar medium switch paradigm [17]. Since many neurons in this experimental group had shorter neuronal processes than in the control group, they might be immature neurons possibly differentiated from the final medium switch from N5 to N6 (Fig. 5A). Therefore, it is tempting to think that serum and growth factors activated quiescent cells to undergo neurogenesis, which is supported by previous report that showed re-entering of cell cycle of quiescent NSCs by EGF/FGF2 treatment [5].

Discussion

Since its development, EdU has been widely used to monitor cell proliferation as a final readout of the experiments. Also, EdU has been used to trace cell fate of NSCs *in vivo* [12, 17]. According to our results and others', EdU seems to inhibit cell proliferation possibly by arresting cell cycle at G2/M phase [7, 13]. Interestingly, another thymidine analogue and more widely used BrdU was also reported to induce cell cycle arrest in embryonic NSCs [22]. Therefore, careful analysis of lineage tracing and cell fate mapping using EdU and other nucleoside analogue is needed. Due to this inhibition of cell proliferation, the possibility of using EdU as an anti-cancer drug has been raised [16, 20, 24]. However, still other side effects including loss of neurogenesis as shown in this present study and disrupted DNA function and stability [1, 6] call for careful therapeutic applications of EdU.

In this present study, we showed that pre-treatment of EdU for 1 day prior to differentiation completely inhibited neurogenesis and oligodendrogenesis of SVZ NSCs. These suggest that cell division is necessary in differentiation to both neurons and oligodendrocytes, which have been speculated in the previous review publication [14]. According to the review, type B cells generate type C cells and the type C cells become neurogenic intermediate progenitor cells (nIPCs) or oligodendrocytic intermediate progenitor cells (oIPCs). The review focused more on neurogenesis, indicating cell division of nIPCs without mentioning of cell division of oIPCs. However, our present results suggest that oIPCs might undergo cell division as well. Still, it is not known why cell divisions are needed before fully committing to the specific cell fate. The most possible reason might be to in-

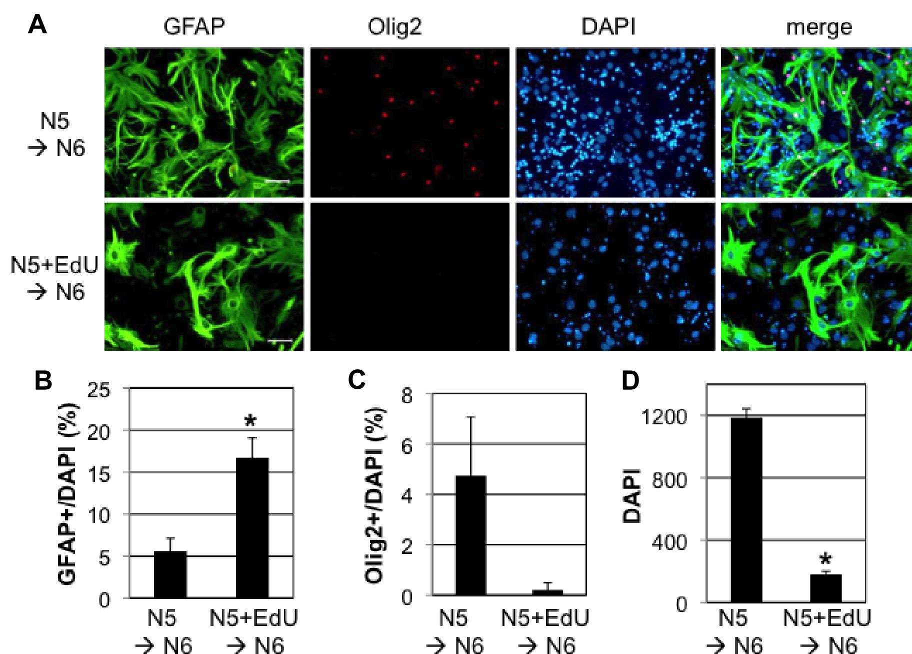

Fig. 4. Effects of EdU on glial differentiation of SVZ NSCs. A. Cultured NSCs were incubated in N5 without (*upper pannel*) or with 5 μM EdU (*lower pannel*) for 1 day. Then, medium was switched to N6 to start differentiation. After 5 days of differentiation, cells were fixed for immunocytochemistry to detect astrocytes (GFAP) and oligodendrocytes (Olig2). DAPI was used for nuclear staining. Scale bar=20 μm. B-D. GFAP-positive cells (B) and Olig2-positive cells (C) were counted and divided by total number of DAPI. Total number of DAPI is shown in D. Data are average and standard deviation from three different wells of cells (*p<0.01, Student's T-test).

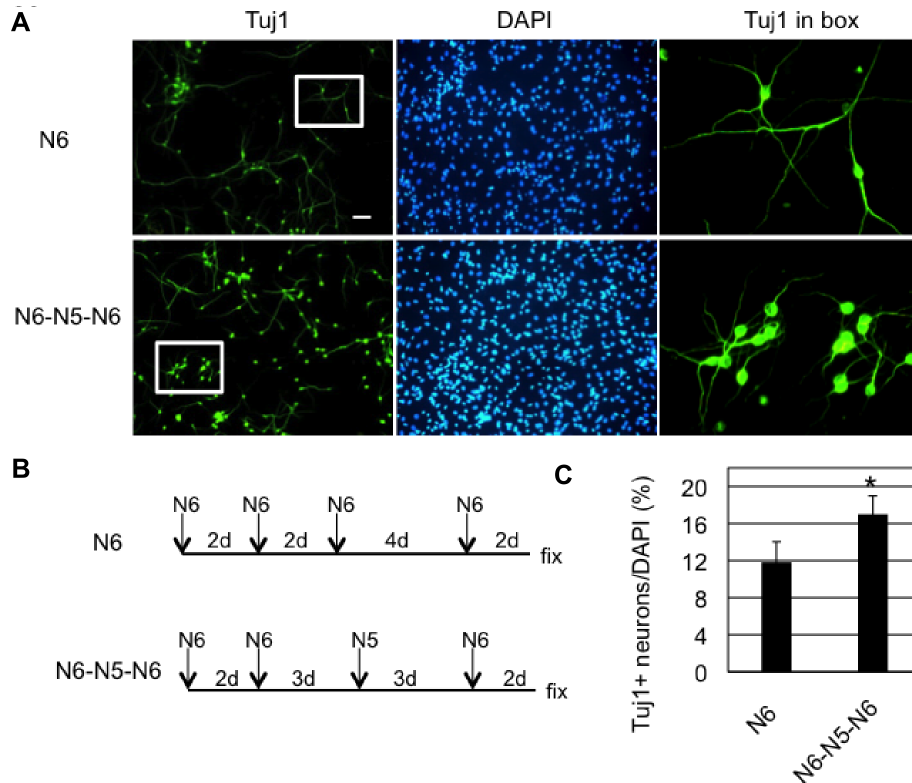

Fig. 5. Neurogenesis of SVZ NSCs was increased by proliferation medium during differentiation. A. In the case of N6 (*upper pannel*), NSCs were incubated in N6 medium for 10 days and medium was changed with fresh N6 medium every 2 or 4 days during the differentiation, as indicated in B (*upper pannel*). In the case of N6-N5-N6 (*lower pannel*), medium was changed as shown in the schematic in B (*lower pannel*). Scale bar=50 μm. The two images on the far right are zoomed ones of the boxed area. B. Experimental scheme is shown. At the end of the medium switch, cells were fixed for immunocytochemistry as in A. C. Tuj1+ neurons were counted and divided by total number of cells stained in DAPI. Average and standard deviation from three different wells of cells are shown (*p<0.05, Student's T-test).

crease the number of finally differentiated cells. We speculate that an unknown process occurring during cell divisions might direct cell differentiation, because this hypothesis explains why the lack of cell division impairs cellular differentiation.

Type B NSCs exist in two flavors, activated (aNSCs) and quiescent (qNSCs) states [3]. Both aNSCs and qNSCs express astroctye marker, GFAP, but only aNSCs express EGFR that can transmit extrinsic mitogenic signals. In our study, EdU seemed to eliminate aNSCs leading to a loss of neurogenesis, but qNSCs might have survived EdU treatment. That might be the reason why we found more GFAP+ cells in EdU-treated samples in Fig. 4, even though it is still possible that more astrocytes (GFAP+) were generated at the cost of neurogenesis and oligodendrogenesis. The former speculation is supported by the increase of neurogenesis upon medium switch with N5 in Fig. 5. After 5 days in differentiation medium, most of aNSCs might have become type C cells and only qNSCs have remained in Fig. 5. These qNSCs might have been reactivated by growth factors in N5 medium and become type C cells, which are consistent to the previous report showing activation of qNSCs by growth factors [5]. Yet, it is puzzling that how EGFR-negative qNSCs can respond to EGF and FGF. One possibility is that growth factor lacking condition like N6 medium in our case might somehow stimulate qNSCs to escape their quiescence [3]. Finally, even though our speculation turns out to be right, still we have to investigate why qNSCs were not activated at the first place during proliferation period in the initial N5 medium, but activated by medium switch with N5 medium after 5 days of differentiation.

For the last several decades, there have been many publications about neural stem cells *in vivo* and *in vitro*. However, cellular behaviors and molecular components involved in maintenance and differentiation of neural stem cells still wait for much investigation. Here, we report that

cell division is necessary for neuronal differentiation of SVZ NSCs by using EdU. This opens a door to further study why and how cell division contributes cell fate decision. Also, our report implies that therapeutic approaches using EdU should be taken with much caution.

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초록 : 5-ethynyl-2′-deoxyuridine (EdU)에 의한 뇌실하 영역 신경줄기세포의 신경 세포로의 분화 억제

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뇌실하 영역과 subgranular zone은 뇌에서 평생 새로운 신경 세포를 만들어 내는 곳이다. 이 부위에 있는 신경줄기세포는 세포 분열을 통해서 줄기 세포군을 계속 유지할 뿐만 아니라, 신경 세포와 신경 교세포로 분화한다. 세포 분열을 측정하기 위해 thymidine 유사체인 5-ethynyl-2′-deoxyuridine (EdU)가 사용되어 왔다. 몇몇의 경우에서는 새롭게 만들어지는 신경 세포를 표지하려는 목적으로 사용되었다. 이번 연구에서는, EdU가 쥐의 뇌실 하 영역에서 분리해낸 신경줄기세포의 분열과 분화에 어떠한 영향을 미치는 지를 보여주었다. 첫째, 신경줄기세포가 EdU를 포함하는 세포 증식 배양액에서 24시간 동안 배양되었을 때, 추후에 분화를 유도하여도 신경세포로 분화가 전혀 일어나지 않았다. EdU를 1시간 동안 처리했을 때도 신경세포로의 분화가 상당부분 저해되었다. 둘째, EdU는 농도가 높을수록, 처리시간이 많을수록 신경줄기세포의 증식을 더욱 많이 저해하였다. 끝으로, EdU는 신경 교세포 중에서 oligodendrocyte으로의 분화는 억제하였지만, astrocyte로의 분화는 오히려 증가시켰다. 본 연구결과는 뇌실하 영역 신경줄기세포의 분화에 EdU가 어떠한 영향을 미치는 지를 처음으로 보여주었고, 이러한 결과들은 신경 세포와 oligodendrocyte로의 분화에 세포 분열이 반드시 필요하다는 것을 제안하고 있다.