Master's Thesis

석사 학위논문

Role of autophagy in differentiation of adult hippocampal neural stem cells.

Kyungrim Yi (이 경 림 李 京 林)

Department of Brain and Cognitive Sciences

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Advisor : Professor Seong-Woon Yu

Co-Advisor : Professor Hongsoo Choi

by

Kyungrim Yi

Department of Brain and Cognitive Sciences

DGIST

A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1)]

11. 29. 2016

Approved by

Professor Seong-Woon Yu _____(Signature)
(Advisor)
Professor Hongsoo Choi _____(Signature)
(Co-Advisor)

[1)] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

Role of autophagy in differentiation of adult hippocampal neural stem cells.

Kyungrim Yi

Accepted in partial fulfillment of the requirements for the degree of Master of Science

11. 29. 2016

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201525010

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Abstract

Neural stem cells (NSCs) in the brain have the ability to differentiate into neurons, oligodendrocytes and astrocytes. Autophagy is a major degradation pathway for cellular homeostasis and remodeling. However, little is known how autophagy is associated with differentiation of NSCs. This study was aimed to examine whether autophagy plays a critical role for self-renewal and differentiation of adult hippocampal neural stem (HCN) cells. Suppression of autophagy by knockdown of Atg7, a key autophagy gene, impaired differentiation of HCN cells into neuron, oligodendrocyte and astrocyte. After induction of differentiation of HCN cells to astrocytes by retinoic acid, considerable amount of autophagosomes was observed in the early time point, but later autophagy level returned to the basal state. Recent studies suggested that astrocytes are dynamic regulators of neuronal activity such as in neuronal development, activity and differentiation. Our findings suggest that autophagy plays a key role in regulation of NSC differentiation, and especially astrogenesis depends on autophagy. Our study will help to elucidate the importance of autophagy in regulation of HCN cell differentiation.

Keywords: neural stem cell, autophagy, differentiation

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1. Introduction (Background and significance)

Neural stem cells (NSCs) have two characteristics, self-renewal and differentiation to other neural cell types (Gage 2000). Neurogenesis is initiated by the proliferation of immature multipotent cells, the progeny of which migrate and differentiate into a variety of specialized neurons and glia. These functions of NSCs play an important role in regulating NSCs pool and retaining neural function (Figure 1).

In adult brain, NSCs are found in two regions. One is subgranular zone (SGZ) in dentate gyrus of hippocampus and the other is subventricular zone (SVZ) along the lateral ventricle. NSCs in the SGZ generate neurons contributing to processes involved in learning and memory, while neurogenesis in SVZ generated neurons destined for the olfactory bulb to function in fine olfaction discrimination (Zhao, Deng et al. 2008, Hsieh 2012).

The differentiation ability of NSCs can be induced by the presence of differentiation factors such as retinoic acid (RA) (Durston, Timmermans et al. 1989, Tan, Wang et al. 2015). Adult-derived hippocampal neural stem (HCN) cells can generate neurons, oligodendrocytes and astrocytes in vitro (Palmer, Takahashi et al. 1997). Astrocytes are the main class of neuroglia, serving a wide range of functions in the nervous system (Markiewicz and Lukomska 2006). Commonly, astrocytes are known to be playing passive roles such as supporting neurons, offering structure in the central nervous system. However, recent studies show that astrocytes play various roles quite actively contrary to a conventional perception. Astrocytes are known to play a role in neuronal development, activity, plasticity, differentiation and maturation (Haydon 2000, Ullian, Sapperstein et al. 2001, Villegas, Poletta et al. 2003). They also play a major role in brain pathology (Vincent, Tilders et al. 1997). Furthermore, astrocyte senescence is known to be a contributing factor for the pathogenesis of Alzheimer's disease (Itagaki,

McGeer et al. 1989, Minagar, Shapshak et al. 2002). In a recent study, morphological atrophy of astroglial cells are observed in dentate gyrus in Alzheimer's disease model (Rodríguez-Arellano, Parpura et al. 2016). Therefore, these studies implicate that the importance of studying astrocytes differentiation related to Alzheimer's disease.

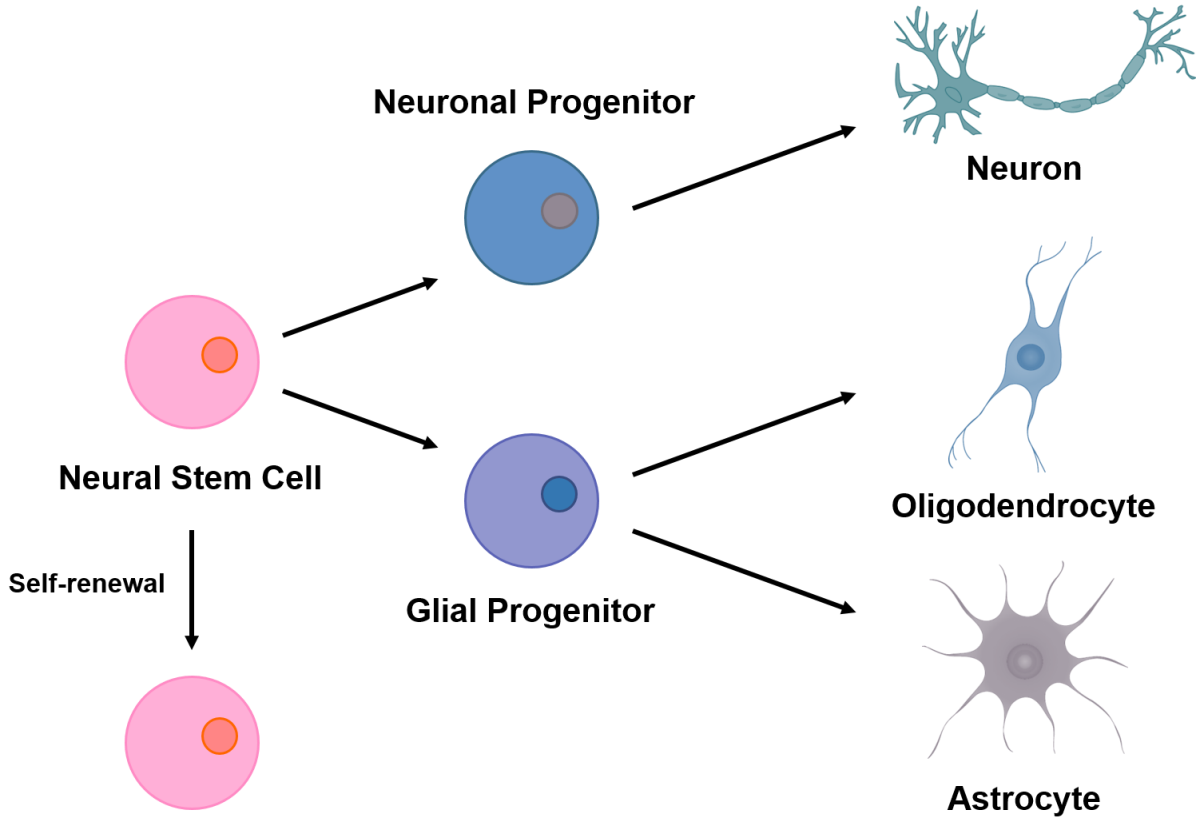


Figure 1. Schematic diagram of neural stem cells differentiation.

Neural stem cells have two characteristics, self-renewal and differentiation into other neural cell types. As they go through the glial progenitor stage, they differentiate into neuron from neural progenitor cells and differentiate into oligodendrocyte and astrocyte from glial progenitor cells. For these abilities neural stem cells can maintain neural stem cell pool and regenerate neural cells.

Autophagy is a major degradation pathway (Maria 2010). Autophagy is initiated by forming phagophore with autophagic precursors and isolated membrane. Upon completion of autophagosome formation, it fuses with the lysosome to form an autolysosome, where its contents are degraded by acidic proteases. Finally, all the contents are degraded and transported in the cytoplasm to be reused as a source of energy (Figure 2). The autophagy pathway is known to be highly activated during differentiation and development (Mizushima and Levine 2010).

There are some methods for monitoring autophagy. LC3 is the most widely monitored autophagy-related protein to measure autophagy rate (Klionsky, Abdelmohsen et al. 2016). The LC3 protein is an ubiquitin-like protein that can be conjugated to phosphatidylethanolamine (PE). When forming autophagosome, LC3 conjugate to PE located in isolated membrane. The non-conjugated and PE-conjugated forms are usually referred to as LC3-I and LC3-II. Therefore, measurement LC3-II level in western blotting is commonly used for autophagy examination. In other way to monitor autophagy, we generated mRFP-GFP-LC3 stably expressing HCN cells using lentivirus. This tandem fluorescence tagged expressing LC3 vector is developed to overcome the weakness of monitoring autophagy using only GFP tagged LC3. GFP is relatively more sensitive to acidic condition so it is not expressed in autolysosome. However, mRFP is more resistant to lysosomal proteolytic degradation, so the early in autophagy both GFP and mRFP are detected and autophagosomes are observed as yellow puncta. When autophagosomes are maturated into autolysosome after fusion with lysosome, they only show the expression of mRFP as GFP can no longer express fluorescence (Figure 3) (Kimura, Noda et al. 2007).

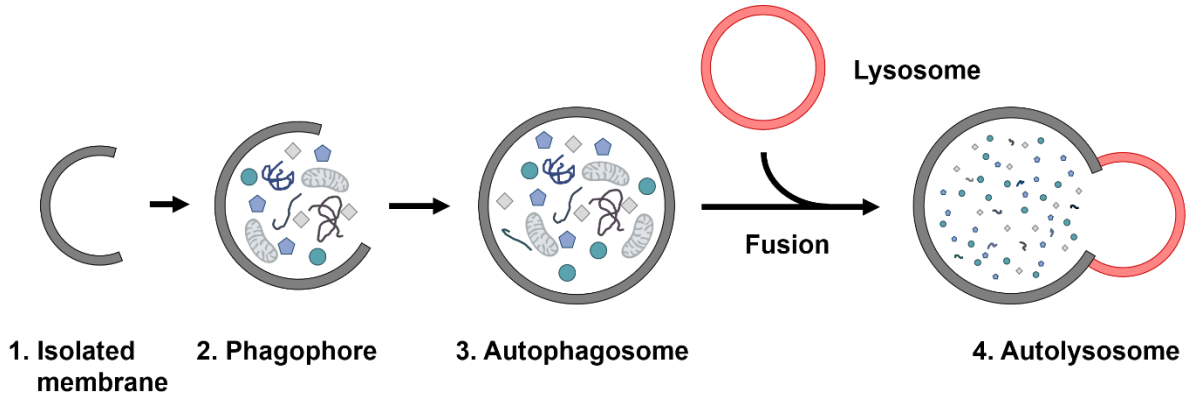


Figure 2. The process of autophagy.

Autophagy begins with an isolated membrane, which forms phagophore. This phagophore elongates to engulf cytoplasmic components, including cytosolic proteins and organelles which will be degraded. The enclosed autophagosome matures through fusion with the lysosome called autolysosome, promoting the degradation of the internal material in autophagosome by lysosomal acid proteases.

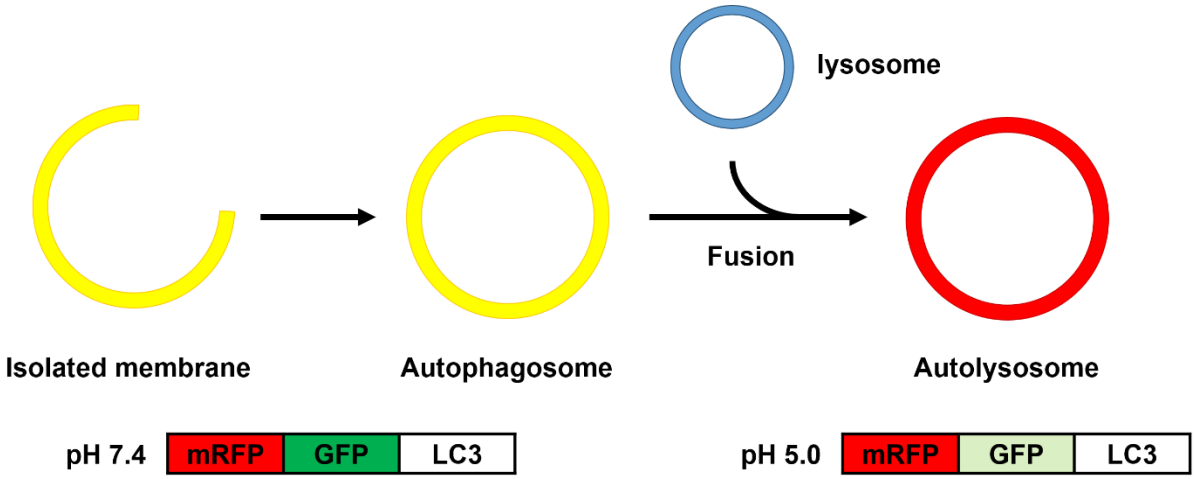


Figure 3. The concept of mRFP-GFP-LC3.

The tandem fluorescence tagged LC3 expressing HCN cells were used to monitor autophagy.

Recent studies have implicated autophagy in the regulation of self-renewal and maintenance of NSCs. Although it was recently shown that autophagy is required for the differentiation of stem cells (Zeng and Zhou 2008, Salemi, Yousefi et al. 2012, Pantovic, Krstic et al. 2013), it remained unclear whether HCN cells would also depend on intact autophagic pathways. Therefore, this study is aimed to elucidate how autophagy regulate the differentiation of HCN cells.

Autophagy plays an important role in neurodegeneration, as well as in normal brain function (Hong, Park et al. 2016). Recently, relations between autophagy and neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) has been discussed (Hara, Nakamura et al. 2006). Neurodegenerative disease indicates conditions which include the progressive loss of anatomically or physiologically related neuronal systems or functions (Lin and Beal 2006). Therefore, NSCs differentiation is catching on as a therapeutic way to treat incurable neurodegenerative diseases. However, mechanisms of differentiation of NSCs has not been elucidated yet. Therefore, understanding the role of autophagy in neuronal differentiation would contribute to a therapeutic design for the treatment of neurodegenerative diseases.

2. Materials and method

Primary culture of adult rat HCN cells

HCN cells were isolated as reported originally. 7 weeks old Sprague Dawley (SD) female rats were sacrificed for primary HCN cell culture. Rat dentate gyrus (DG) were collected and chopped using a scalpel blade until no large pieces remained. Chopped DG tissue was transferred into warmed PDD enzymes (2.85 U/ml Papain (Worthington), 0.8 U/ml Dispase 1 (Stem Cell), 120 U/ml DNase 1 (Stem Cell)) mixture and incubated for 20 min at 37℃, mixed well by tapping the tube every 3~5 min. Dissociation of the tissue mechanically was followed using a medium bore, fire polished, Pasteur pipette by pipetting gently. Pellets were collected by centrifugation at 150 × g for 5 min, followed by removal of the supernatant. Pellet was resuspended in 1 ml buffer solution (1× HBSS (Invitrogen), 30 mM glucose (Sigma-Aldrich), 2 mM HEPES (pH7.4) (Sigma-Aldrich), 26 mM $NaHCO_3$ (Sigma-Aldrich)) then filled up to 10 ml with buffer solution. After centrifugation at 150 × g for 5 min again, supernatant was removed and the pellet was resuspended in 5 ml of 20% Percoll (Sigma-Aldrich). Centrifugation at 450 × g for 15 min was followed and pellets were resuspended in 10 ml buffer. Once more centrifugation at 150 × g for 5 min, pellets were resuspended in 200 µl growth medium and plated into coated 96-well plate (Walker and Kempermann 2014).

Media for maintenance and differentiation of HCN cells

HCN cells were maintained at 37℃, 5% CO_2 and grown on a poly-L-ornithine (Sigma-Aldrich) and laminin (Corning) coated dish in HCN cell medium. Medium composition was as follows: Dulbecco's modified Eagle's medium (DMEM)/F-12, 500 mg/l Insulin, 100 mg/l apo-

transfferrin, 100 U/l penicillin/streptomycin, 16 mg/l putrescin, 30 nM sodium selenite, 20 nM progesteron, 20 ng/ml basic fibroblast growth factor (bFGF) (Peprotech). Cells were passaged using 0.05% Trypsin EDTA (Hyclone).

For all differentiation studies, HCN cells were plated onto wells coated with poly-L-ornithine and laminin. Plated densities were as follows: 1.0×10^5 cells per well of a twelve-well plate (0.5×10^5 cells per well of a twenty-four-well plate, 0.75×10^6 cells per well of a six-well plate). Differentiation medium composition was as follows (Palmer, Takahashi et al. 1997):

Neuron	1 μM RA, 5 μM forskolin, 0.1% FBS in DMEM/F-12
Oligodendrocytes	1 μM RA, 2 ng/ml bFGF, 1% FBS in DMEM/F-12
Astrocytes	1 μM RA, 5% FBS in DMEM/F-12

Generation of stable cell line

For stable knockdown, the lentiviral shRNA clones targeting rat Atg7 (TRCN0000092164, TRCN0000369085) from the Mission library were purchased from Sigma-Aldrich, and for stable overexpression, HCN cells were infected with the lentiviral expressing clone pLjm1-mRFP-GFP-LC3. The lentiviruses were produced following the published protocols. For stable knockdown or overexpression, HCN cells were infected with the virus for 24 h and then the medium was replaced with fresh medium. After 72h, HCN cells were treated with puromycin (5 μg/ml) for 6 h and were then maintained in medium containing puromycin (1 μg/ml).

RNA Extraction and Quantitative real-time polymerase chain reaction (qRT-PCR)

Medium was removed from the dish then cells were rinsed with PBS and collected into the QIAzol Lysis Reagent (QIAZEN). Same volume of chloroform was added into the tube and shaked vigorously for 15 sec. Centrifugation at 12000 × g for 15 min at 4℃ was followed and the upper layer was transferred to a new tube. Same volume of isopropanol was added and mixed thoroughly by vortexing. After placing the tube at room temperature for 10 min, centrifugation at 12000 × g for 10 min at 4℃ was followed. The supernatant was discarded carefully and 1 ml of 75% ethanol was added, followed centrifugation at 7500 × g for 5 min at 4℃. The supernatant was completely removed and RNA pellet was air-dried for 5 min. RNA was redissolved in an appropriate volume of RNase-free water.

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using the ImProm-II Reverse Transcriptase kit (Promega) and cDNA was synthesized using oligo dT. PCR primers were commercially synthesized (Cosmogenetech). qRT-PCR was performed on the RT product using Taq Polymerase (Enzynomics) and primers specific for rat Sox2, NeuN (Neuronal nuclei), GalC (Galactosylceremidase), GFAP (Glial fibrillary acidic protein) and 18S (18S ribosomal RNA) cDNAs. 50-cycle amplification was applied for all primers using the CFX96 Real-Time System (Bio-Rad). 18S was used as the reference gene for normalization. Primers used for Sox2, NeuN, GalC, GFAP and 18S were listed in the table 1.

Gene	Primer sequence		Genebank number
Sox2	Forward Reverse	5'-ATAACATGATGGAGACGGAGC-3' 5'-CATTCATGGGCCTCTTGACG-5'	499593
NeuN	Forward Reverse	5'-CCCTACCATCATACCATCGGC-3' 5'-GTGAAGCGGCTGTACCCT-3’	287847
GalC	Forward Reverse	5'-GTGTCGCGGTGCCCTTGTTG-3' 5'-CTAGAAGCCGGGAGGTTGCC-3'	314360
GFAP	Forward Reverse	5'-GACCTGCGACCTTGAGTCCT-3' 5'-TCTCCTCCTTGAGGCTTTGG-3'	24387
18S	Forward Reverse	5'-GTAACCCGTTGAACCCCATTC-3' 5'-CCATCCAATCGGTAGTAGCGA-3'	100861533

Table 1. Primer sequence.

Primers used for Sox2, NeuN, GalC, GFAP and 18S. These primer sequences used for qRT-PCR were designed rat specifically.

Immunocytochemistry

Cells were rinsed in PBS and fixed in 4% paraformaldehyde for 10 min. Following twice rinses with PBS, cells were blocked for 5 min at room temperature in 0.2% Triton X-100 in antibody diluent reagent solution for permeabilization. Twice rinses and blocking in antibody diluent reagent solution for 10 min were followed. Primary antibody incubation was done overnight in blocking solution. Cells were rinsed the next day, followed by secondary antibody incubation for 1 h at room temperature at a 1:500 dilutions. Secondary antibodies conjugated to Alexa Fluor 488 (Jackson) or Cy3 (Jackson) were used to visualize primary antibodies. Following twice PBS rinses, all nuclei were stained for 10 min with Hoechst 33342 (Invitrogen) at a 1:1000 dilution. Fluorescence images were obtained from samples using LSM 700 confocal microscope (Carl Zeiss) and analyzed.

Western blotting

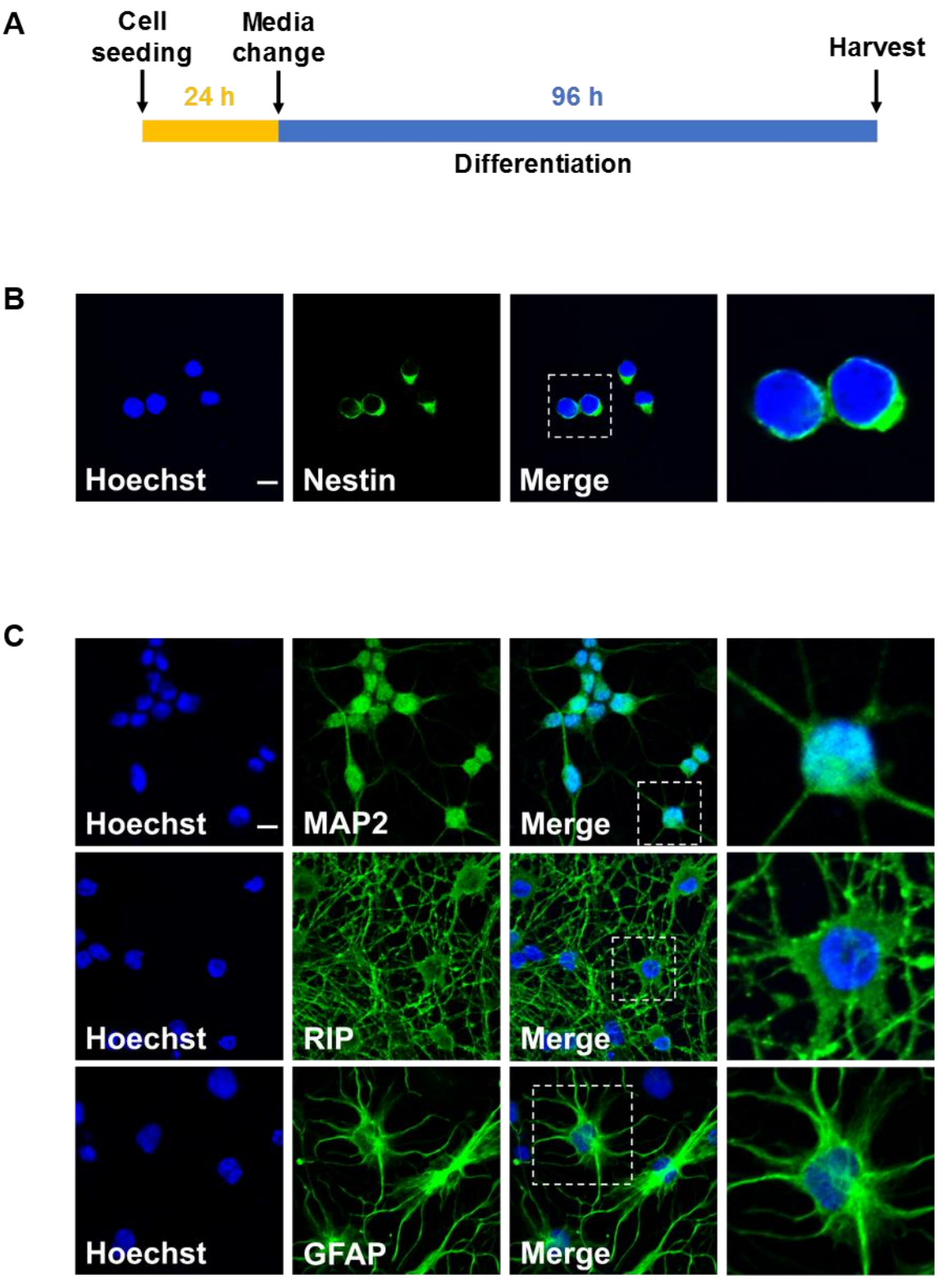
HCN cells and differentiated cells were harvested and lysed for 30 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific)). After centrifugation (13000 rpm 20 min), each sample's lysate was collected. BCA protein assay reagent (Thermo Fisher Scientific) were used to measure protein concentration in the lysate. Prepared samples were run on an SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane with a semi-dry electrophoretic transfer cell (Bio-Rad). Membranes were blocked with 5% nonfat dry milk powder dissolved in Tris-buffered saline

with 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were than incubated with primary antibodies for overnight with appropriate primary antibodies in 4℃ on a shaking incubator. The next day, membranes were washed with TBST for 3 times, 10 min each. Peroxidase-conjugated secondary antibodies diluted in blocking solution was applied for 1 h at room temperature. After washing, proteins of interest were detected using either chemiluminescence detection kit (Thermo Fisher Scientific) or WesternBright ECL (Advansta).

3. Results

HCN cells show intact differentiation ability into neurons, oligodendrocytes and astrocytes.

HCN cells have potential of self-renewal and differentiation into neurons, oligodendrocytes and astrocytes. HCN cells were isolated from rat hippocampus as described above. Before conducting differentiation assay, HCN cells were seeded on poly-L-ornithine and laminin coated plate and maintained in growth media. After 24 h for stabilization, HCN cells were exposed to differentiation media. HCN cells were then differentiated for 96 h and harvested to analyze (Figure 4A). Immunocytochemistry data shows that HCN cells have high expression of Nestin which is a NSC specific marker (Figure 4B). We also performed qRT-PCR to measure mRNA levels between undifferentiated and differentiated HCN cells. Sox2 mRNA levels were relatively high in undifferentiated HCN cells, but showed low levels in differentiated cells (Figure 4D). To confirm whether HCN cells have intact differentiation ability, we performed differentiation assay. Cells were differentiated by changing half medium freshly every 24 h and at 4 day of post differentiation, cells were then used for examination. To verify if HCN cells differentiated properly, differentiated cells were stained for markers for neurons (MAP2), oligodendrocytes (RIP), astrocytes (GFAP) and also with Hoechst. In immunocytochemistry, differentiated HCN cells into neurons, oligodendrocytes and astrocytes show high expression of their specific markers MAP2 (Microtubule associated protein 2), RIP and GFAP respectively (Figure 4C). As seen in Figure 1E, mRNA levels of each differentiated cell type markers are also increased when differentiated. These data show that HCN cells have intact differentiation ability into neuron and glial cells.


A
Cell seeding
Media change
Harvest
24 h
96 h
Differentiation
B
Hoechst
Nestin
Merge
C
Hoechst
MAP2
Merge
Hoechst
RIP
Merge
Hoechst
GFAP
Merge

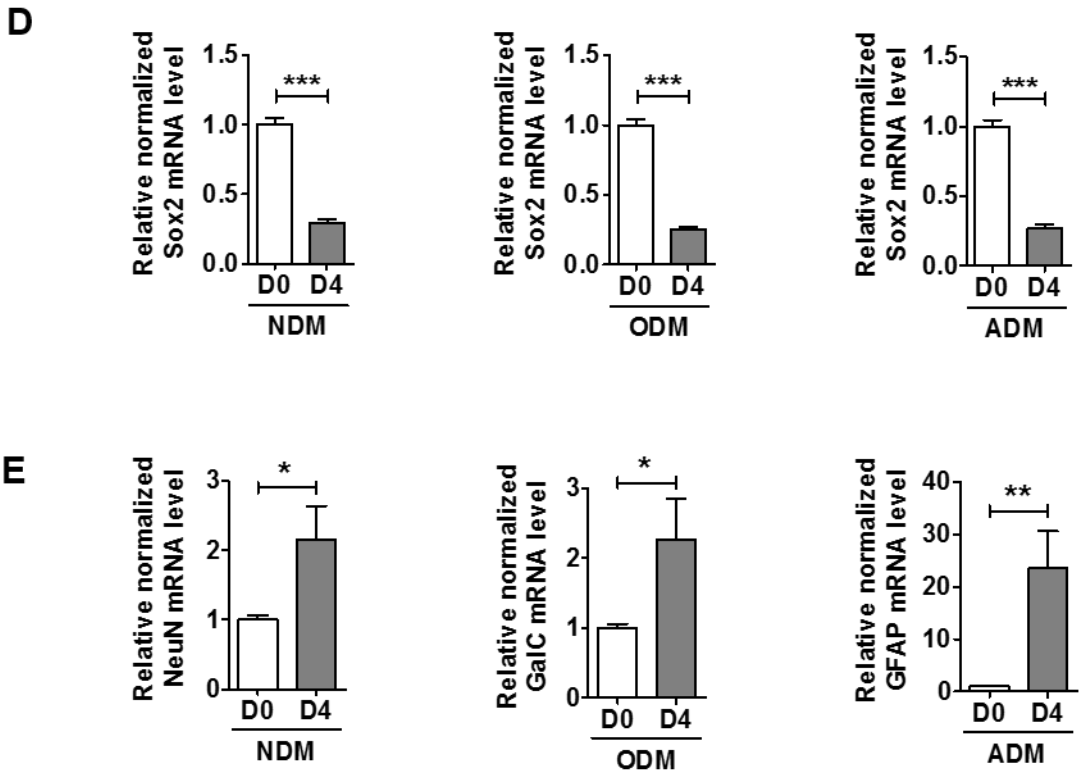


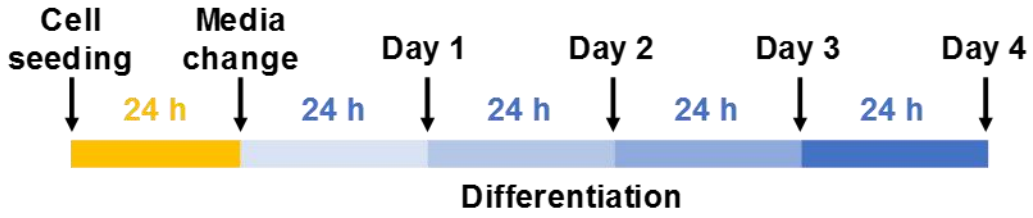
Figure 4. HCN cells show intact differentiation ability into neurons, oligodendrocytes and astrocytes.

HCN cells were differentiated for 4 days in each differentiation media and harvested at 4 day of differentiation (A). Undifferentiated normal status of HCN cells stained for Nestin (green) with Hoechst 33342 (blue) and imaged via fluorescence microscopy (scale bar : 10μm) (B). Differentiated HCN cells into neurons, oligodendrocytes and astrocytes stained with MAP2, RIP and GFAP respectively (scale bar : 10μm) (C). A comparison of Sox2 mRNA levels in HCN cells and differentiated HCN cells by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis (D). Changes in NeuN, GalC and GFAP mRNA levels after differentiation (E). NDM : Neuron Differentiation Media, ODM : Oligodendrocyte Differentiation Media, ADM : Astrocyte Differentiation Media. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with control cells.

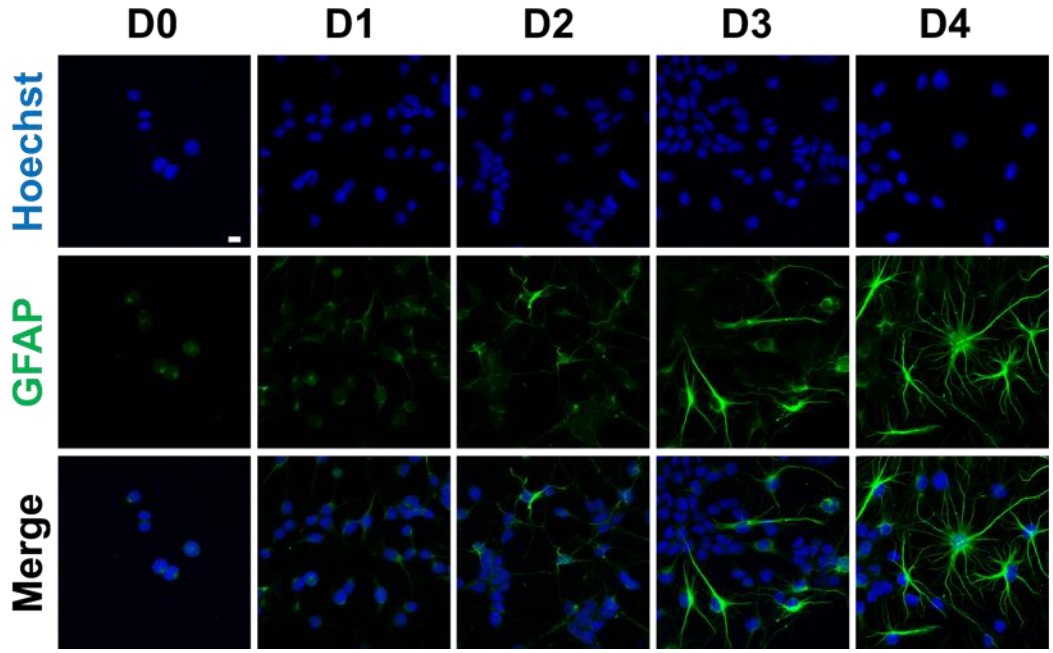
Characterization of HCN cells differentiation into astrocytes.

To examine whether the differentiation from HCN cells to astrocytes progresses properly, we observed the whole process of differentiation every 24 h (Figure 5A). As shown in previous result, undifferentiated normal status HCN cells have a spherical morphology and highly express NSC specific marker Nestin (Figure 4B). HCN cells do not show the expression of astrocyte specific marker GFAP, however, when exposed to differentiation media their GFAP expression steadily increased by day 4. Cell morphologies also change from spherical stem cell shape to radial which is typical astrocyte shape (Figure 5B). Western blotting also shows that the expression of astrocyte differentiation marker GFAP is gradually elevated in parallel with a decrease in NSC marker Sox2 (Figure 5C, D). We performed qRT-PCR to check mRNA levels by date of post differentiation and resulted that GFAP mRNA levels also increased co-relatively with protein levels (Figure 5E). These data show that HCN cells normally differentiate to astrocytes.

A


Cell seeding
Media change
Day 1
Day 2
Day 3
Day 4
24 h
24 h
24 h
24 h
24 h
Differentiation

B


D0
D1
D2
D3
D4
Hoechst
GFAP
Merge

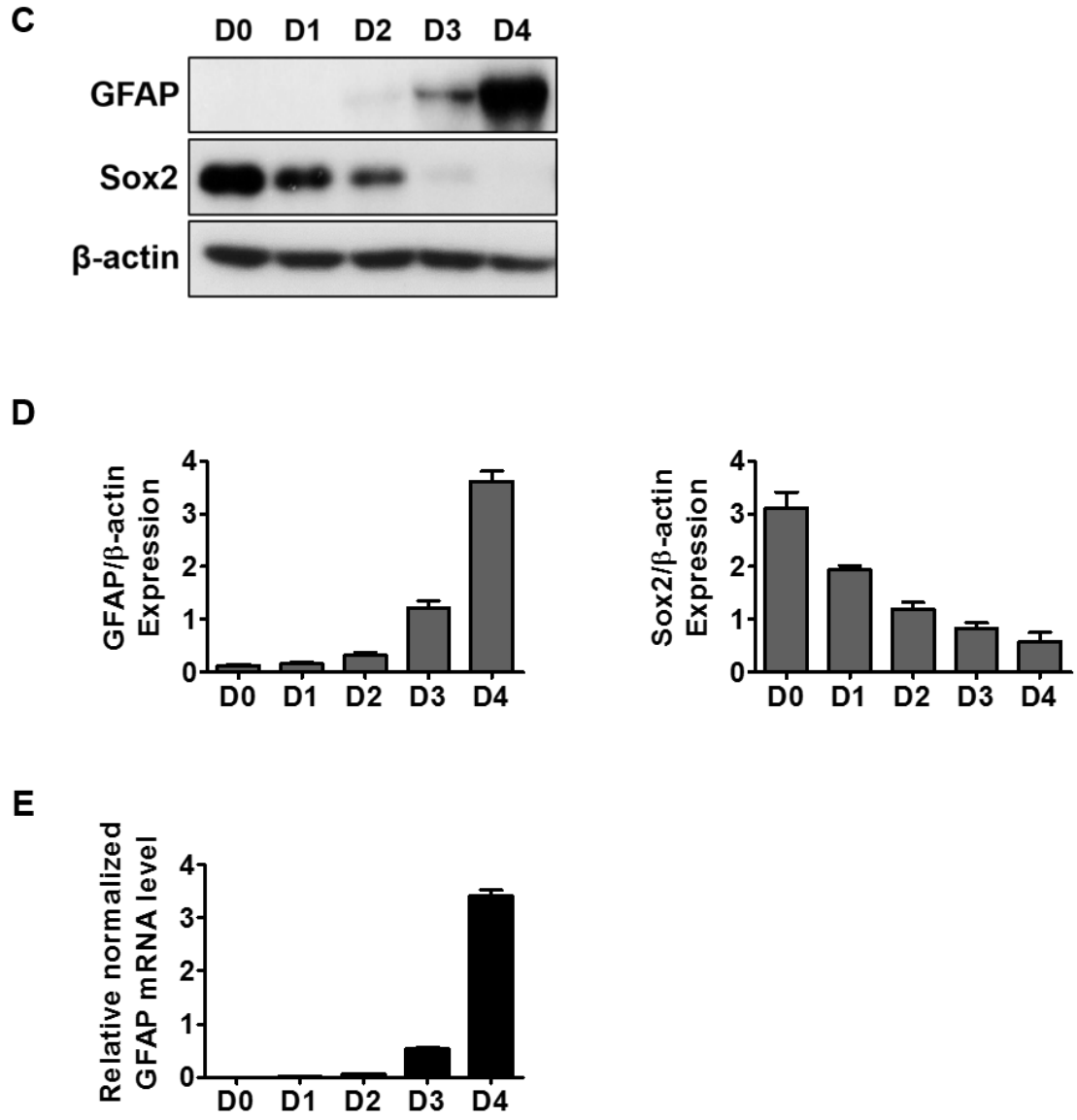


Figure 5. Characterization of HCN cells differentiation into astrocytes.

HCN cells were differentiated for 4 days in astrocyte differentiation media and harvested every day after inducing differentiation (A). Fluorescence microscopy images of differentiating HCN cells stained for GFAP (green) and Hoechst 33342 (blue) (scale bar : 10μm) (B). Western blot analysis of whole cell lysates from differentiating HCN cells (C). Quantification of western blots in panel C normalized to β-actin (D). A comparison of GFAP mRNA levels in differentiating HCN cells by qRT-PCR analysis (E).

Autophagy is highly activated in the early stage of differentiation and decreased later.

Previously reported, autophagy is necessary to collapse cellular structural components and other organelles during the course of differentiation (Sin, Andres et al. 2016). Additionally, autophagy is likely to have other roles during cellular remodeling such as differentiation (Mizushima and Levine 2010). Because differentiated cells are characteristically distinguished from stem cells, cellular components are reconstituted during the process of differentiation. Based on these studies, we expected that autophagy is also necessary in HCN cells differentiation. To explore whether autophagy is related in this process of reorganization of cellular components, we observed the expression of LC3 which is an autophagy marker during differentiation (Kimura, Noda et al. 2007). When measuring autophagy in immunocytochemistry, an elevated mRFP-GFP-LC3 puncta is often used to indicate the increase of autolysosome which is going through autophagy. Before differentiation, LC3 puncta were not observed in HCN cells, interestingly, early after the induction of differentiation, the number of mRFP and GFP co-expressing LC3 puncta were increased (Figure 6A). However, mRFP-GFP-LC3 puncta were downregulated and not observed later in differentiation. In western blots, LC3-II is highly increased after induced differentiation and decreased later by day4 (Figure 6B, C). p62 which is an ubiquitin binding protein is an additional autophagy marker. p62 attaches to LC3 and degraded by autophagy machinery (Bjørkøy, Lamark et al. 2009). Therefore, the decreased level of p62 can be used as an autophagy marker. As seen in Figure 6B, increased LC3 and decreased p62 in day1 and day2, suggest autophagy is highly activated. These data show that only during the early steps of differentiation, autophagy is robustly upregulated.

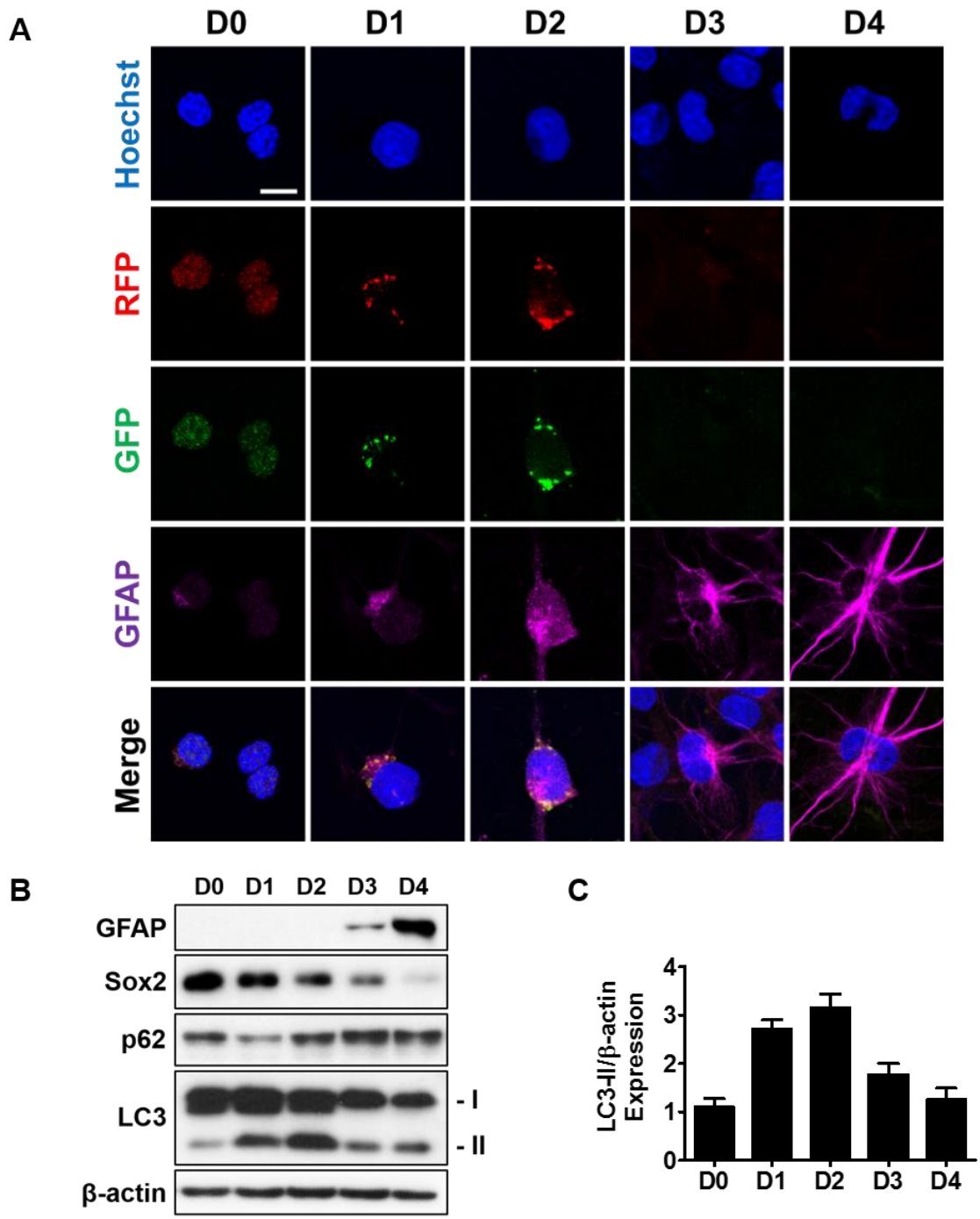


Figure 6. Autophagy is highly activated in the early stage of differentiation and decreased later.

Fluorescence images for mRFP-GFP-LC3 stably expressing HCN cells during the process of differentiation (scale bar : 10μm) (A). Western blot analysis of whole cell lysates from differentiating HCN cells (B). Quantification of western blots in B normalized to β-actin (C).

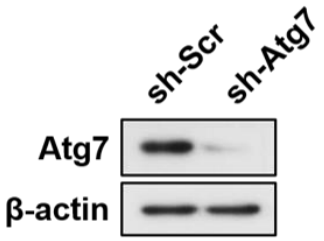
Atg7 stable knockdown impairs astrogenesis.

Then to investigate whether the process of autophagy is crucial for HCN cells differentiation into astrocytes, we repressed autophagy using Atg7 knockdown HCN cells. As Atg7 plays a role in forming autophagosome which is a critical step in autophagy (Yorimitsu and Klionsky 2005), knockdown of Atg7 resulted in downregulated autophagy. We adopted lentiviral shRNA to knockdown Atg7 for maintaining the decreased level of Atg7 during 4 days of differentiation. Atg7 stable knockdown was confirmed by western blotting (Figure 7A). Based on immunocytochemistry imaging, Atg7 knockdown HCN cells showed decreased differentiation rate and abnormal morphology compared to sh-Scr which is non-targeting shRNA (Figure 7B). Western blot results showed that an astrocyte marker GFAP is robustly expressed in differentiated astrocytes of sh-Scr HCN cells, but in Atg7 knockdown HCN cells show lower GFAP expression level after differentiation. Decreased level of Sox2 in sh-Scr after differentiation was reduced in sh-Atg7 HCN cells (Figure 7C). qRT-PCR results with mRNA also present that lower GFAP expression level in Atg7 knockdown cells (Figure 7D). These data illustrate that when autophagy is blocked, differentiation ability of HCN cells is impaired.

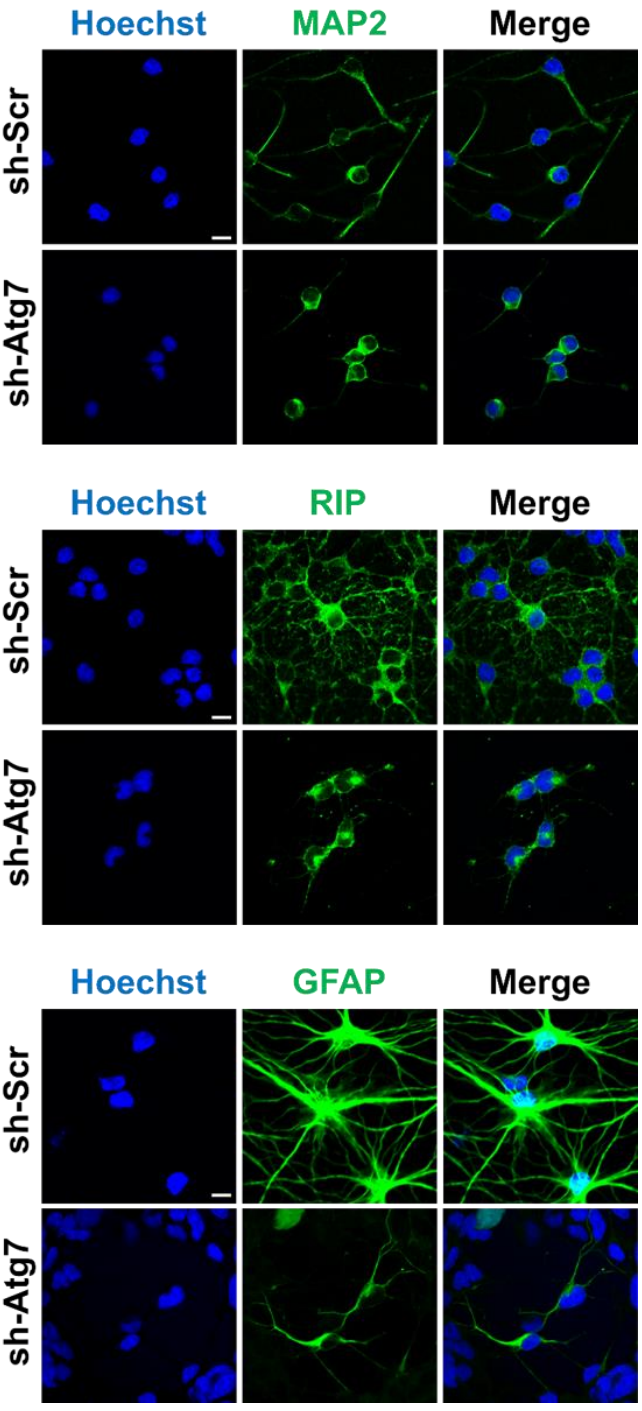
Previously we examined that autophagy is highly activated in the early when induced differentiation into astrocytes and decreased later. Then to investigate whether the reduced differentiation into astrocytes in Atg7 knockdown cells is for the reason that autophagy is not activated in the initiation step of starting differentiation, we compared LC3-II levels in scramble and autophagy impaired HCN cells by every post differentiation. As seen in Figure 7E, after inducing differentiation the expression of GFAP is delayed in Atg7 knockdown HCN cells compared to sh-Scr and shows lower GFAP expression at day 4 post differentiation. Also in contrast to rapidly decreasing Sox2 expression level in sh-Scr, in sh-Atg7 HCN cells, Sox2

expression level was slowly decreased along with differentiation. Additionally, LC3 turnover observed in the process of astrogenesis (Figure 6B) is rarely seen in sh-Atg7 HCN cells, which represents impaired autophagy process. These data illustrate that the progress of autophagy is essential for HCN cells differentiation into astrocytes.

A


sh-Scr
sh-Atg7
Atg7
β-actin

B


Hoechst
MAP2
Merge
sh-Scr
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RIP
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sh-Scr
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GFAP
Merge
sh-Scr
sh-Atg7

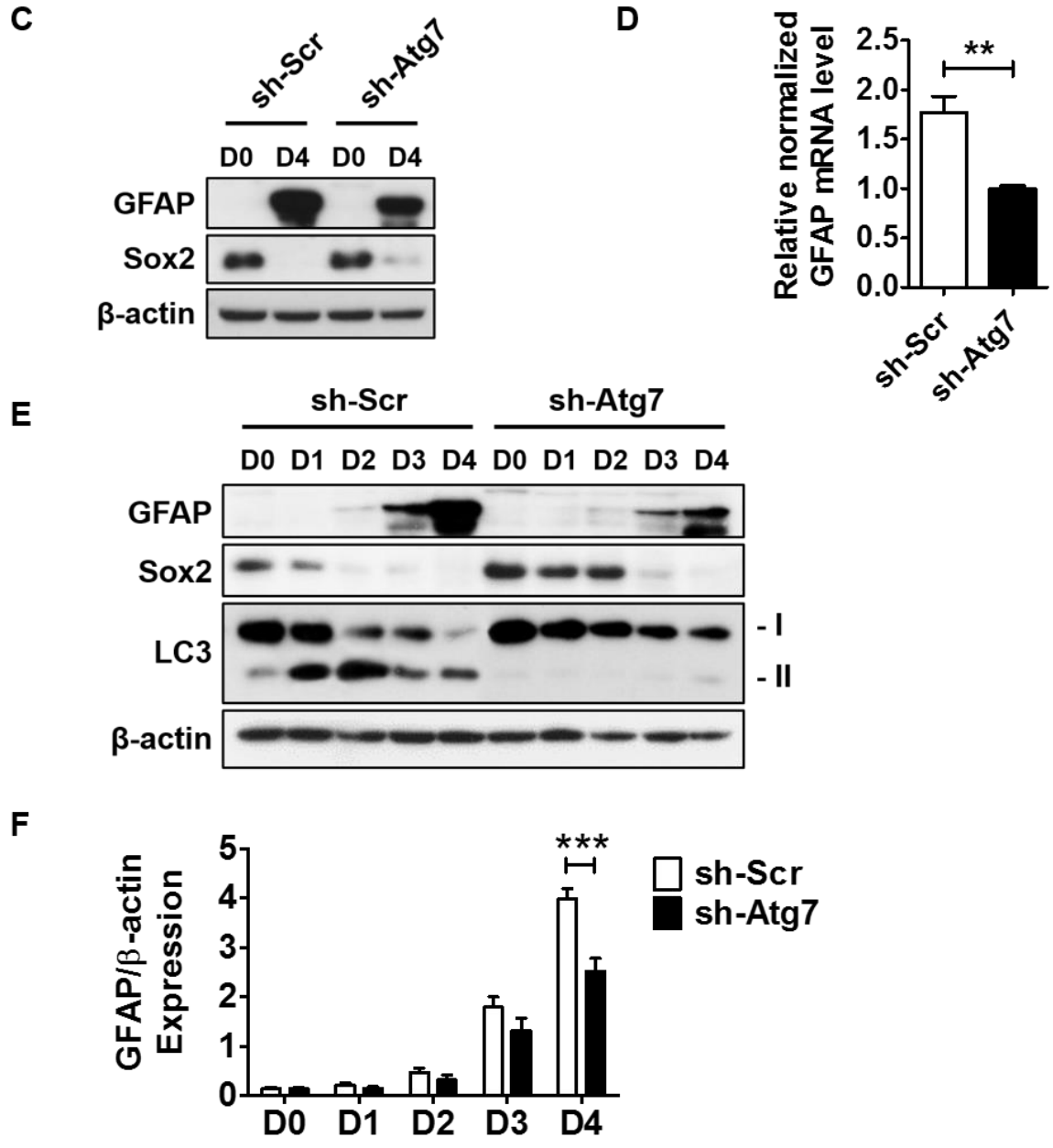

Figure 7. Atg7 stable knockdown impairs astrogenesis.

Confirming Atg7 knockdown by western blot analysis (A). Fluorescence images for differentiated sh-Scr and sh-Atg7 HCN cells into neuron, oligodendrocyte and astrocyte, stained for MAP2, RIP and GFAP respectively (scale bar : 10μm) (B). Western blot analysis of differentiated astrocyte in sh-Scr, sh-Atg7 HCN cells (C). Relative normalized GFAP mRNA expression level (D). Western blot analysis of differentiating sh-Scr, sh-Atg7 HCN cells (E). Quantification of western blots in panel D normalized to β-actin (F). **$p < 0.01$ and ***$p < 0.001$ compared with control cells.

4. Discussion

In this study, we report the role of autophagy during differentiation of HCN cells into astrocytes. When autophagy is suppressed by stable knockdown of Atg7, differentiation ability of HCN cells into astrocytes was decreased. During the course of differentiation, the number of LC3 puncta was significantly increased, suggesting that autophagy is upregulated during differentiation. At the end of the course of differentiation, LC3-II level was decreased which shows that autophagy returned to the basal level when HCN cells differentiated into mature astrocytes. When cells go through the course of differentiation, cellular components which were adapted to assist the quiescent stem cell should be re-adjusted to support the improved or enhanced new functions of the differentiated cells. So we presumed that autophagy will be needed during differentiation to clear unnecessary cellular components and reuse those degraded elements. Our data illustrate that autophagy is robustly upregulated during the early steps of differentiation and it is needed for the intact differentiation process.

Atg7 is one of essential gene for controlling autophagosome formation. When Atg12 is conjugated to Atg5, it requires Atg7 and Atg10 for an ubiquitin-like reaction (Hanada, Noda et al. 2007). The Atg12-Atg5 conjugate then interacts with Atg16 to form a large complex (Levine and Klionsky 2004). LC3 (an autophagosomal ortholog of yeast Atg8) is cleaved at its C-terminal by Atg4 protease to generate the cytosolic LC3-I. LC3-I is conjugated to PE also in an ubiquitin-like reaction that requires Atg7 again and Atg3 (Tanida 2011). This lipidated form of LC3 is known as LC3-II, which is attached to the autophagosome membrane (Kabeya, Mizushima et al. 2000). Therefore, when Atg7 was knockdown by shRNA, it results the autophagy deficiency. During HCN cells differentiation into astrocytes, LC3 turnover was seen and it shows autophagy upregulation and downregulation. However, when Atg7 knockdown

HCN cells differentiate, LC3 turnover was not seen. Our results revealed that the disruption of autophagy with sh-Atg7 impairs HCN cells differentiation into astrocytes. This study reveals the importance of autophagy for stem cells differentiation.

This study reveals the importance of autophagy at the onset differentiation of HCN cells. Once differentiation is induced, the process of autophagy immediately promoted. Though, we still have many unsolved questions to understand the correlation between autophagy and HCN cells differentiation. First of all, resolve the possibility that autophagy could be involved in differentiation signaling. Previous study has shown that rapamycin, a serine/threonine kinase targeting mTOR (mammalian target of rapamycin), can induce the differentiation of various cell types such as glioma stem or progenitor cells and myeloid cells (Zeng and Zhou 2008, Pantovic, Krstic et al. 2013). Thus, it will be needed to explore if the activation of autophagy is associated with the AKT-mTOR pathway. Secondary, the specific role of the upregulated autophagy during differentiation is still not elucidated. Previous study has shown that autophagy is needed to breakdown and reassemble the mitochondrial networks during differentiation (Sin, Andres et al. 2016). Therefore, autophagy may have other roles during cellular remodeling when differentiating. It could include breakdown of cellular structural components and other organelles. And we need to find out molecular mechanisms over monitoring phenotypes. Because we only have shown that phenotypes in this thesis, finding specific autophagy machinery relating differentiation is required. Lastly, in this study, we examined the role of autophagy by only down-regulating of autophagy, but to better understanding, we have to consider the autophagy up-regulated conditions, too. It's reported that when autophagy related gene Atg5 or Beclin1 is overexpressed, it activated autophagy. Therefore, using these tools, we can make an autophagy up-regulated conditions.

The significance of studying autophagy has been raised in various field. Recently, research into the use of stem cells for the treatment of neurodegenerative diseases, such as AD, PD, ALS and multiple sclerosis (MS) have become interesting topics (Nixon, Wegiel et al. 2005, Pan, Kondo et al. 2008, Hetz, Thielen et al. 2009). Adult HCN cells differentiation can be a promising suggestion for treating these neurodegenerative diseases (Clarke, Johansson et al. 2000). As a clinical use of stem cells, using transplanted HCN cells which have the ability to differentiate into neural cells for neurodegeneration treatment is a fascinating tool. In this study, we suggest the interrelationship between autophagy and HCN cells differentiation. Although it has many questions should be resolved before the application of this technique for clinical use, we gave an advancement in these technologies.

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요 약 문

성체해마신경줄기세포의 분화 과정에서 자가포식현상의 역할

신경줄기세포는 스스로 자가증식하는 능력을 가지며 신경계를 구성하는 여러가지의 세포로 분화할 수 있는 분화능을 가진 세포이다. 우리 뇌에는 크게 두 군데에서 신경줄기세포가 발견되는데 그 중 한 곳이 뇌실하 영역이고, 다른 한 곳이 과립하 영역이다. 위의 두 영역에서 신경줄기세포는 스스로 분열하고, 신경세포 혹은 신경교세포로 분화하는 것으로 알려져 있다. 특히 과립하 영역의 해마에 위치한 신경줄기세포는 학습 및 기억력의 조절에 관여하는 뉴런으로 분화하는 것으로 알려져 있어 그 연구의 중요성이 주목받고있다.

자가포식작용이란 세포내 불필요한 물질들을 분해하는 과정으로 체내 항상성 유지에 관여하는 중요한 현상이다. 이 과정이 정상적으로 이루어지지 않을 경우 다양한 질병이 발병하는 원인이 된다고 알려져 있는데, 그 중 특히 퇴행성 뇌질환과의 관련성에 대한 연구가 다양하게 이루어지고 있다. 퇴행성 뇌질환은 다양한 이유로 신경세포들이 과다하게 죽어 생기는 질환으로, 알츠하이머나 파킨슨병과 같은 다양한 질병을 야기하게 된다. 신경줄기세포의 신경세포, 신경교세포로의 분화를 이용한 치료법은 퇴행성 뇌질환을 치료할 수 있는 훌륭한 대책으로 각광받고 있다. 하지만, 성체해마신경줄기세포의 분화 기전과 그 원리는 아직 명확히 밝혀지지 않고 있다.

본 연구는 성체해마신경줄기세포의 신경세포와 신경교세포로의 분화 과정에서 자가포식현상이 어떠한 역할을 하는지에 대해 다루었다. 정상적인 상태에서 성체해마신경줄기세포는 특정한 조건하에 신경세포, 희돌기교세포 그리고 성상세포로의 분화가 잘 이루어진다. 하지만 자가포식현상을 인위적으로 억제한 상태의 성체해마신경줄기세포에서는 신경세포와 신경교세포로의 분화가 잘 이루어지지 않았다. 본 연구에서는 특히, 성상세포로의 분화 과정에서 자가포식현상이 상당히 활성화 되어 있고 또 필요로 함을 다양한 방법으로 증명하였다. 본 연구는 성체해마신경줄기세포의 분화 과정에서 자가포식작용의 중요성을 확인하여 향후 퇴행성 뇌질환의 치료법으로서 신경줄기세포의 사용가능성을 보여주고 있다.

핵심어: 신경줄기세포, 자가포식작용, 신경세포 분화

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