https://doi.org/10.5607/en.2019.28.2.229
Exp Neurobiol. 2019 Apr;28(2):229-246.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

Experimental Neurobiology

Autophagy Mediates Astrogenesis in Adult Hippocampal Neural Stem Cells

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Neural stem cells (NSCs) have the ability to self-renew and differentiate into neurons, oligodendrocytes, and astrocytes. Highly dynamic nature of NSC differentiation requires the intimate involvement of catabolic processes such as autophagy. Autophagy is a major intracellular degradation pathway necessary for cellular homeostasis and remodeling. Autophagy is important for mammalian development and its role in neurogenesis has recently drawn much attention. However, little is known about how autophagy is associated with differentiation of NSCs into other neural lineages. Here, we report that autophagy plays a critical role in differentiation of adult rat hippocampal neural stem (HCN) cells into astrocytes. During differentiation, autophagy flux peaked at early time points, and remained high. Pharmacological or genetic suppression of autophagy by stable knockdown of Atg7, LC3 or CRISPR-Cas9-mediated knockout (KO) of p62 impaired astrogenesis, while reintroduction of p62 recovered astrogenesis in p62 KO HCN cells. Taken together, our findings suggest that autophagy plays a key role in astrogenesis in adult NSCs.

Key words: Adult stem cells, Astrocytes, Autophagy, Autophagy-related protein 7, Cell differentiation, Neural stem cells, Sequestosome-1 protein

INTRODUCTION

Autophagy is an evolutionarily conserved, major intracellular degradation pathway [1]. Autophagy is initiated by phagophore formation and its expansion to generate autophagosomes [2, 3]. Upon completion of autophagosome formation with cargos inside, autophagosome fuses with a lysosome to become autolysosome, where its contents are degraded by acidic proteases [4]. Subsequently, degraded products are transported back to the cytoplasm to be reused as a source of energy [5]. There are several methods to monitor autophagy. Microtubule-associated protein light chain 3 (LC3, a mammalian homologue of yeast Atg8) is the most widely used autophagy-related protein used to measure autophagosome formation [6]. LC3 is a ubiquitin-like protein that is, upon synthesis, cleaved by Atg4 protease to generate the cytosolic form [7]. Then, at sites of autophagosome formation, LC3 is conjugated to phosphatidylethanolamine through the action of the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg3 [3, 7, 8]. The non-conjugated, cytosolic form is referred to as LC3-I and the phosphatidylethanolamine-conjugated, autophagic form as LC3-II [3]. An increase in LC3-II level as a marker of autophagosome formation can be measured by Western blotting for autophagy examination [9]. The appearance of puncta- or dot-

Received October 17, 2018, Revised March 6, 2019,
Accepted March 13, 2019

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like signals of a fluorescent-tagged LC3 protein is also indicative of autophagosome formation [6, 10]. p62 is an autophagy adaptor protein regulating the packaging and delivery of organelles and polyubiquitinated, misfolded proteins into autophagosomes for clearance [11]. During autophagic degradation, p62 is also degraded with the substrates, so a decrease in p62 protein amount is regarded as another marker of autophagy induction [11-13].

Increased autophagy flux includes not only more autophagosome formation, but also efficient fusion of autophagosomes and lysosomes for delivery of autophagic substrate cargos and their degradation [14]. Therefore, blocking the late autophagy steps, such as fusion between autophagosomes and lysosomes, with bafilomycin A1 (Baf.A1) can be used to distinguish whether an increase in autophagy markers is due to genuine up-regulation of autophagy flux (on-rate) or impaired autophagy flux (off-rate) [5, 15]. Blocking of autophagosome fusion under high autophagy flux conditions is expected to increase accumulation of LC3-II and p62, while an increase in autophagy markers such as LC3-II or LC3 puncta resulting from impaired autophagy will not be affected by additional blocking of autophagy with Baf.A1 [6, 15]. Since the pH inside autophagosomes differs from that inside autolysosomes, several assay methods based on pH have been developed to assess autophagy flux, including LC3 tagged with two fluorescent proteins, monomeric RFP and GFP, arranged in tandem (mRFP-GFP-LC3). Monitoring autophagy flux with mRFP-GFP-LC3 is based on different pH stability of mRFP and GFP proteins. The presence of GFP and RFP in autophagosomes gives yellow fluorescent signals; however, since GFP is easily quenched under acidic conditions, such as those in lysosomes, only RFP signals are retained after fusion of autophagosomes with lysosomes. Therefore, an increase in both the total number of puncta and red-to-yellow signal ratio indicates an increase in autophagy flux [16].

Since autophagy is readily inducible in response to various environmental cues and cellular stressors, autophagy is highly active during differentiation and development [17]. The critical role of autophagy in mammalian development has been well documented in mice lacking the Atg genes including *Atg5*, *Atg7*, and *Beclin1*, which show embryonic or perinatal lethality due to defective autophagy and nutrient depletion and starvation during embryonic development [18-20]. Autophagy is essential for the development of the nervous system and neurogenesis [21-25]. However, it is not known whether autophagy plays a role in differentiation of neural stem cells (NSCs) into other neural lineages, such as astrogenesis or oligodendrogenesis. Astrocytes far exceed neurons in cell number, and cellular diversity and functions [26]. Recently, astrocytes are gaining attention for their roles in regulation of synaptic plasticity and cognition, which have been regarded for a long time as functions specialized to neurons [27, 28]. Therefore, differentiation of NSC or progenitors to astrocytes plays an essential role in the normal structure and function of brain, and dysregulated astrocytes might underlie various neurodevelopmental and neurological diseases [28, 29].

To address the role of autophagy in astrocyte differentiation, we used murine adult NSCs, which were derived from the hippocampus of a 2-month-old male rat. The discovery of persistent generation of new neurons in the brain throughout adulthood has sparked interest in the role of adult neurogenesis in brain function and plasticity [30, 31]. Hippocampus is one of the two well-established regions of adult neurogenesis [32]. Since hippocampus is deeply involved in learning and memory, mood regulation, reward-seeking and other neurobehaviors, understanding the pathophysiological roles of adult hippocampal neurogenesis and its underlying neural mechanisms are of great interest [33, 34]. Various *in vitro* and *in vivo* methods have been developed to study hippocampal NSCs, including labeling of dividing cells with nucleotide analogs, infection and tracing of NSCs with retrovirus, and cell type–specific gene knockout [35, 36]. Isolation of murine NSCs from the adult hippocampus and subsequent monolayer or neurosphere culture have also been widely used for molecular and genetic studies [37]. We have previously used this cellular model to elucidate the role of autophagy in survival and death of adult NSCs, which we call hereafter adult hippocampal neural stem (HCN) cells [38-42]. Here, we report the dynamic regulation of autophagy flux during astrogenesis and requirement of autophagy genes for proper differentiation of HCN cells into astrocytes.

MATERIALS AND METHODS

Materials

Baf.A1 (BML-CM110, Enzo, USA), pepstatin A (PepA, P5318, Sigma-Aldrich, USA), E64d (E8640, Sigma-Aldrich), puromycin (NC9138068, Invitrogen, USA), hygromycin B (H0192, Duchefa, The Netherlands), fetal bovine serum (FBS, #101, Tissue Culture Biologicals, USA), forskolin (BML-CN100, Enzo), and retinoic acid (RA, #BML-GR100, Enzo) were purchased from the indicated companies. Horseradish peroxidase–conjugated β-actin (SC-47778, Santa Cruz Biotechnology, USA) and antibodies against ATG7 (#8558, Cell Signaling Technology, USA), LC3 (NB100-2220, Novus Biologicals, USA), p62 (P0067, Sigma-Aldrich, USA), sex-determining region Y-box 2 (Sox2, ab97959, Abcam, UK), microtubule-associated protein 2 (MAP2, ab5392, Abcam), receptor interaction protein (RIP, MAB1580, Merck, USA), glial fibrillary acidic protein (GFAP, NBP1-05198, Novus Biologicals), nestin (bs-0008R-A555, Bioss, USA), and GFP (SC-9996, Santa Cruz

https://doi.org/10.5607/en.2019.28.2.229

Biotechnology) were purchased from the indicated companies.

Media for maintenance and differentiation of HCN cells

HCN cells were maintained at 37℃, 5% CO_2 on dishes coated with poly-L-ornithine (#3655, Sigma-Aldrich) and laminin (#35432, Corning, USA) in chemically composed HCN cell medium, as previously described [41, 42]. For differentiation studies, HCN cells were plated onto coated dishes at a density of 1×10^5/cm^2; 24 h later, medium was changed to differentiation medium and the cells were cultured for 4 days (Fig. 1A). Differentiation medium composition was Dulbecco's modified Eagle's medium/F-12 with the following additions: for neurons, 1 μM RA, 5 μM forskolin, 0.1% FBS; for oligodendrocytes, 1 μM RA, 2 ng/ml basic fibroblast growth factor, 1% FBS; for astrocytes, 1 μM RA, 5% FBS.

Generation of stable cell lines

Lentiviral shRNA clones targeting rat Atg7 (TRCN0000092164, TRCN0000369085) were purchased from the Mission Library (Sigma-Aldrich). Lentiviruses were produced following published protocols and were used to infect HCN cells [43]. For stable expression of mRFP-GFP-LC3, HCN cells were infected with lentivirus expressing pLjm1-mRFP-GFP-LC3. 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 72 h, HCN cells were treated with puromycin (5 μg/ml) for 6 h and then maintained in medium containing puromycin (1 μg/ml). The rat p62 (AGCTGAAGCGGCGGATCTCGCGG) single guide (sg) RNA was designed by using an online program (http://crispr.mit.edu) and cloned into the plenti-CRISPR-v2 vector (#52961, Addgene, USA).

RNA extraction and qPCR

Cells were rinsed with phosphate-buffered saline (PBS), and cell lysis and RNA isolation were performed using the QIAzol Lysis Reagent (#79306, Qiagen, Germany) following the manufacturer's instructions. cDNA was synthesized using the ImProm-II Reverse Transcriptase kit (#A3800, Promega, USA) and oligo dT primers, and was used for qPCR with TOPreal qPCR 2X PreMIX (#RT500, Enzynomics, Korea) and the following primers: rat *Sox2* (F-ATAACATGATGGAGACGGAGC, R-CATTCATGGGCCTCTTGACG), *NeuN* (F-GAGGAGTGGCCCGTTCTG, R-AGGCGGAGGAGGGTACTG), *GalC* (F-GTGTCGCGGTGCCCTTGTTG, R-CTAGAAGCCGGGAGGTTGCC), *Gfap* (F-GACCTGCGACCTTGAGTCCT, R-TCTCCTCCTTGAGGCTTTGG), and 18S (F- GTAACCCGTTGAACCCCATTC, R-CCATCCAATCGGTAGTAGCGA). For all primers, 45 cycles of amplification were used in a CFX96 Real-Time System (Bio-Rad, USA).

Immunocytochemistry

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 10 min, rinsed twice with PBS, and blocked for 5 min at room temperature in 0.2% Triton X-100 in antibody diluent solution (#003218, Thermo Fisher Scientific, USA); Triton X-100 was added for permeabilization. Cells were incubated with primary antibodies diluted in antibody diluent solution overnight at 4℃ and then with secondary antibodies prepared in antibody dilution solution for 1 h at room temperature. Secondary antibodies conjugated to Alexa Fluor 488 (#A11034, Thermo Fisher Scientific) or 647 (#703-605-155, Jackson Immuno Research Laboratories, USA) were used to visualize primary antibodies. Following incubation with antibodies, cells were rinsed twice with PBS, and the nuclei were stained for 10 min with Hoechst 33342 (#H3570, Thermo Fisher Scientific) at a 1:1000 dilution. Fluorescence images were obtained under an LSM700 or 780 confocal microscope (Carl Zeiss, Germany) and analyzed in Zen software (Carl Zeiss).

Western blotting

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 (#78429, #1862495, Thermo Fisher Scientific). The samples were run on an SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane in 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 then incubated with appropriate primary antibodies overnight at 4℃ in a shaking incubator. Next day, membranes were washed with TBST 3 times, 10 min each and incubated with peroxidase-conjugated secondary antibodies diluted in blocking solution for 1 h at room temperature. After washing, proteins of interest were detected using a chemiluminescence detection kit (Thermo Fisher Scientific).

Cell death assay

HCN cells were seeded in a 96-well plate with 1.0×10^5 cells per well and stained with propidium iodide (PI) (P4170, Sigma-Aldrich) and Hoechst 33342 (P3566, Invitrogen) at the indicated time points. The stock solutions of PI (10 mg/ml) and Hoechst (1 mg/ml) were diluted 1:1000 in PBS, and after incubation in the dark at 37℃ for 20 min, blue and red signal positive cells were counted under a fluorescence microscope (Axiovert 40 CFL, Carl

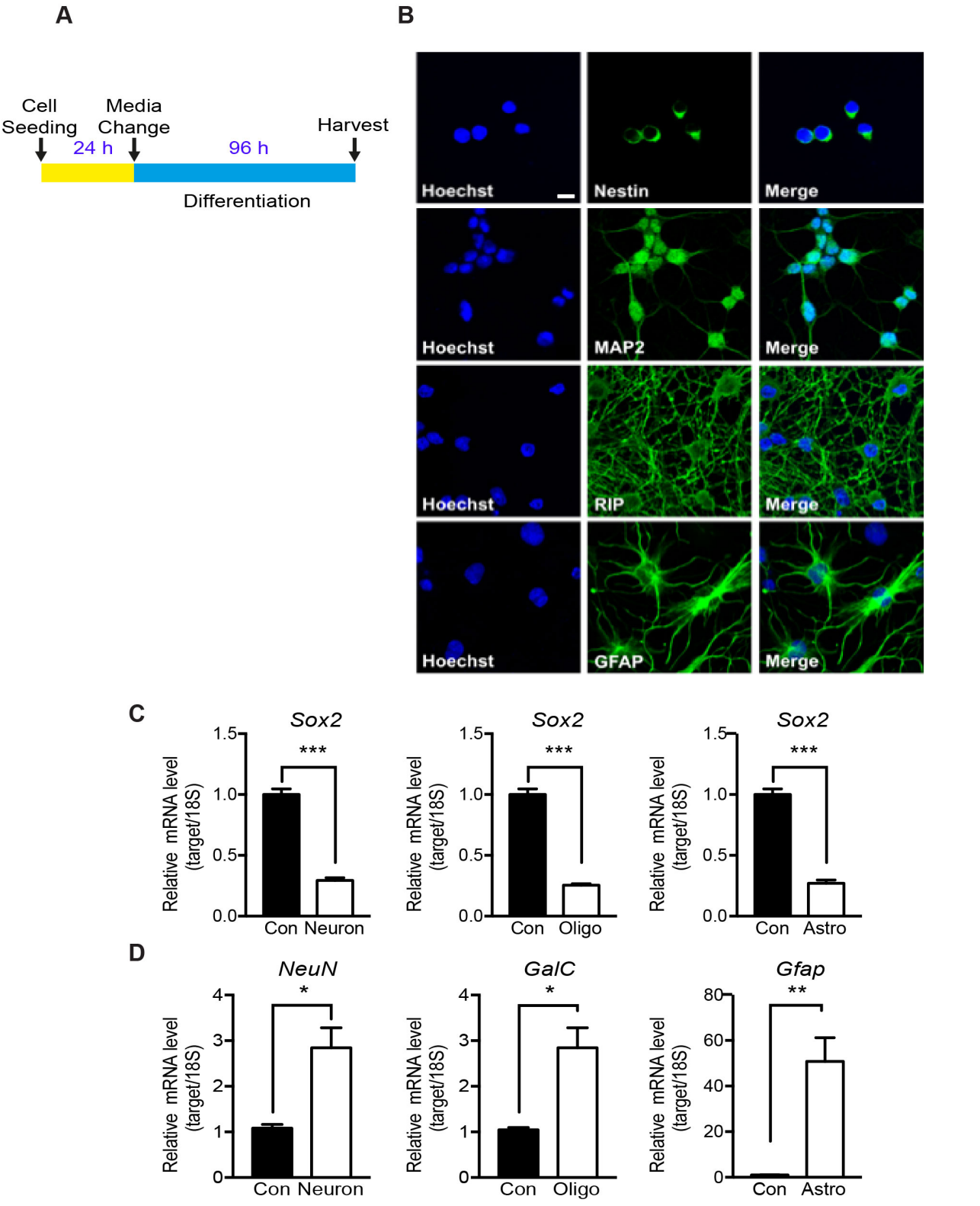

Fig. 1. Differentiation of HCN cells into neurons, oligodendrocytes, and astrocytes. (A) A schematic timeline for differentiation experiments. (B) Undifferentiated and differentiated HCN cells were stained for Nestin, MAP2, RIP, and GFAP (green) with Hoechst 33342 (blue), and imaged by confocal microscopy. Scale bar, 25 μm. (C) *Sox2* mRNA levels in undifferentiated HCN cells (Con) and HCN cells differentiated into neurons (Neuron), oligodendrocytes (Oligo), or astrocytes (Astro). (D) Changes in *NeuN*, *GalC* and *Gfap* mRNA levels after differentiation. *p<0.05, **p<0.01, and ***p<0.001. n≥3.

https://doi.org/10.5607/en.2019.28.2.229

Zeiss). Stained cells were quantified using automated image analysis software Matlab with CellC package. The percentage of cell death was calculated as follows:

Cell death (%)=[PI (red) positive cell number/ Hoechst (blue) positive cell number]×100.

Statistical analysis

All values are presented as mean±standard error of the mean (SEM) obtained by averaging the data from at least three independent experiments. Statistical significance was determined by a two-tailed unpaired Student's t-test using GraphPad Prism (GraphPad Software, USA).

RESULTS

HCN cells can differentiate into neurons, astrocytes, and oligodendrocytes

To confirm their potential to differentiate into neurons, astrocytes, and oligodendrocytes, HCN cells were exposed to different differentiation media (Fig. 1A). Immunocytochemistry data showed that undifferentiated HCN cells highly expressed nestin, an NSC-specific marker (Fig 1B). After differentiation into neurons, oligodendrocytes, and astrocytes, the expression of respective lineage-specific markers (MAP2, mature neuronal marker; RIP, oligodendrocyte marker; GFAP, astrocyte marker) was readily detectable by immunofluorescent staining (Fig. 1B). We also performed qPCR to compare the mRNA levels of these markers between undifferentiated and differentiated HCN cells. First, we chose β-actin for normalization; however, its expression changed substantially during differentiation, possibly due to rapid remodeling of cytoskeleton (data not shown). Therefore, the gene for 18S ribosomal RNA (*18S*) was selected as a reference gene for normalization throughout this study. As expected, the mRNA level of *Sox2* (neural stem cell marker) was high in undifferentiated HCN cells, but declined dramatically during differentiation (Fig. 1C), with a concomitant increase in the expression of each neural cell type-specific marker, namely neuronal nuclei (*NeuN*) in mature neurons, *GalC* in oligodendrocytes, and *Gfap* in astrocytes (Fig. 1D). These data show that HCN cells have intact ability to differentiate into neurons, oligodendrocytes, and astrocytes under our experimental conditions.

Time course analyses of HCN cell differentiation into astrocytes

To study the role of autophagy during astrogenesis, we characterized the astrogenesis every 24 h (Fig. 2A). Before induction of differentiation, all HCN cells were nestin-positive, whereas GFAP was barely detectable. However, GFAP was detected in most cells while nestin was rarely detectable after the completion of astrogenesis (Fig. 2B). Time course analysis of GFAP immunoreactivity and protein levels showed a steady increase in the amount of GFAP during astrogenesis with a concurrent decrease in Sox2 (Fig. 2C and 2D). Cell morphology also changed from spherical stem cell shape to radial, which is typical for astrocytes (Fig. 2C). Consistent with the results of Western blotting analysis, mRNA levels of *Gfap* and tenascin C (*Tnc*), which is a marker of early stage of astrocyte differentiation [44, 45], also greatly increased during astrogenesis (Fig. 2E and 2F). On the other hand, mRNA levels of *NeuN* and *GalC* did not change, confirming the efficiency and specificity of the protocol used for astrogenesis induction (Fig. 2G).

Autophagy flux increased from the early stage of astrogenesis and remained high

Highly dynamic nature of neural development implies reconstitution of cellular components and remodeling of cellular structure during differentiation, requiring energy supply and participation of catabolic processes. To explore whether autophagy is activated in HCN cells during astrogenesis, we examined autophagosome formation in mRFP-GFP-LC3 stable cell lines. Before differentiation, the number of LC3 puncta was low in HCN cells. Interestingly, the number of LC3 puncta increased at early time points (D1 and D2), and decreased afterwards (D3 and D4) (Fig. 3A). Blocking autophagy flux with Baf.A1 should increase accumulation of LC3-II if autophagy flux rate is high [6]. Administration of Baf.A1 at D2 further increased autophagosome formation, suggesting that autophagic flux was very high at this time point (Fig. 3B). Western blotting analysis showed a similar increase in LC3-II and decrease in p62 at D1 and D2 of differentiation; their levels then returned to the basal state (Fig. 3C). To compare the autophagic flux between different time points, we treated the cells with Baf.A1 at D0, D2 and D4, and examined the LC3-II levels by Western blotting analysis. Interestingly, Baf.A1 treatment at D4 led to more significant accumulation of LC3-II than D2, although it did not reach statistical significance (Fig. 3D). These data suggest that although autophagy flux seems to return to the basal state after the peak at D2, the overall capacity of on-rate autophagy flux, which can be revealed after blocking of late stage of autophagy, still remains high until D4 during differentiation. In line with the assumption that autophagy was induced from early times points, Baf.A1 increased the levels of LC3-II at D2 (Fig. 3E). In addition to Baf.A1, we tested another autophagy blocker, pepstatin A and E64d (PepA/E64d), which inhibit lysosomal proteases [46]. Administration of PepA/E64d also increased LC3-II and p62 levels at D2 in a similar manner as Baf.A1 (Fig. 3E). These results suggest that autophagy flux

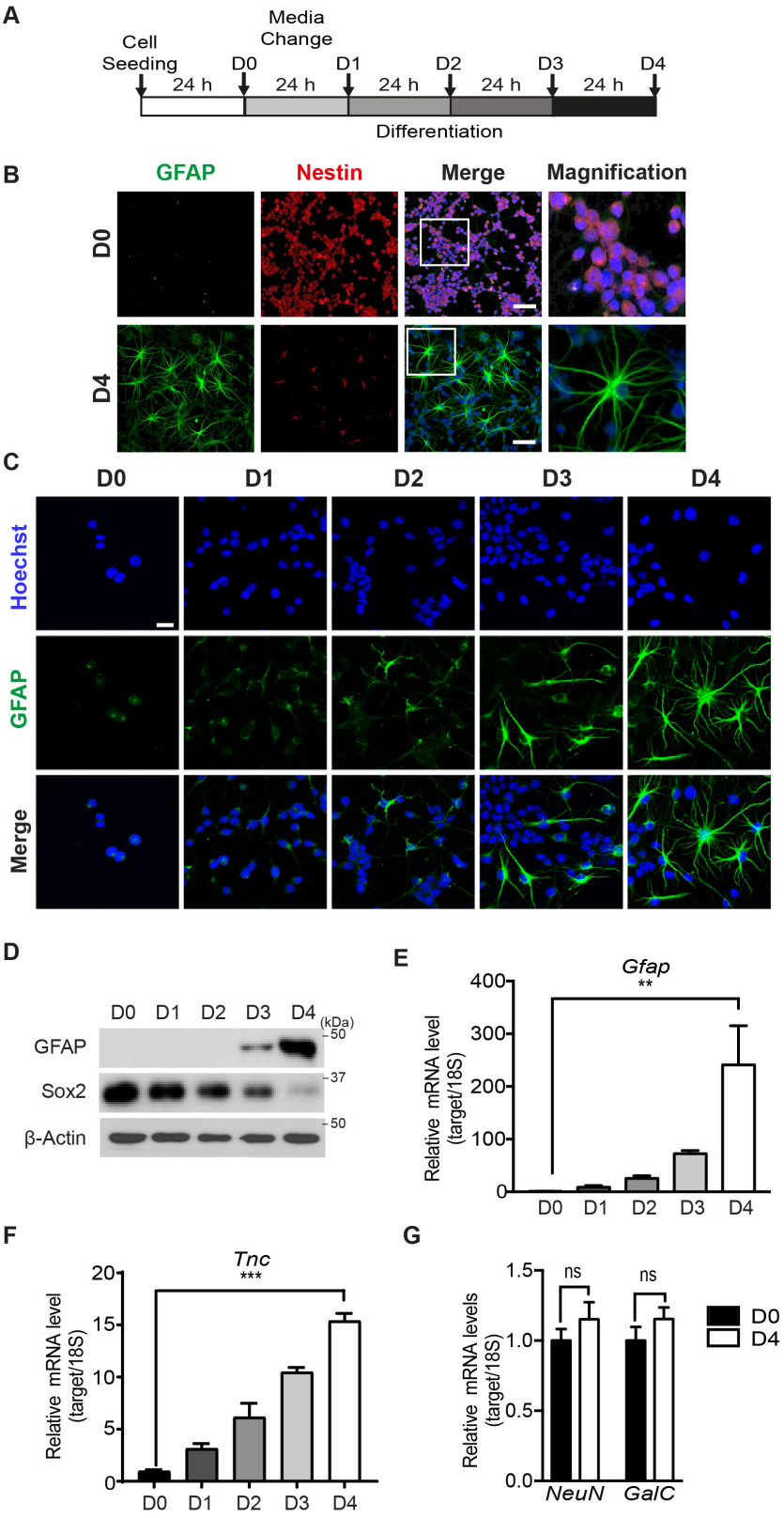

Fig. 2. Time course analyses of HCN cell differentiation into astrocytes. (A) A schematic timeline for astrogenesis experiments. D, day. (B) Images of HCN cells stained with nestin and GFAP antibodies at D0 and D4 during astrogenesis. Scale bar, 50 μm. (C) Astrocyte morphology examined with GFAP staining. Scale bar, 25 μm. (D) GFAP and Sox2 protein levels analyzed by Western blotting. (E) *Gfap* mRNA levels. (F) *Tnc* mRNA levels. (G) mRNA levels of other neural cell markers, *NeuN* and *GalC*. ns, not significant. **p<0.01, and ***p<0.001. n≥3.

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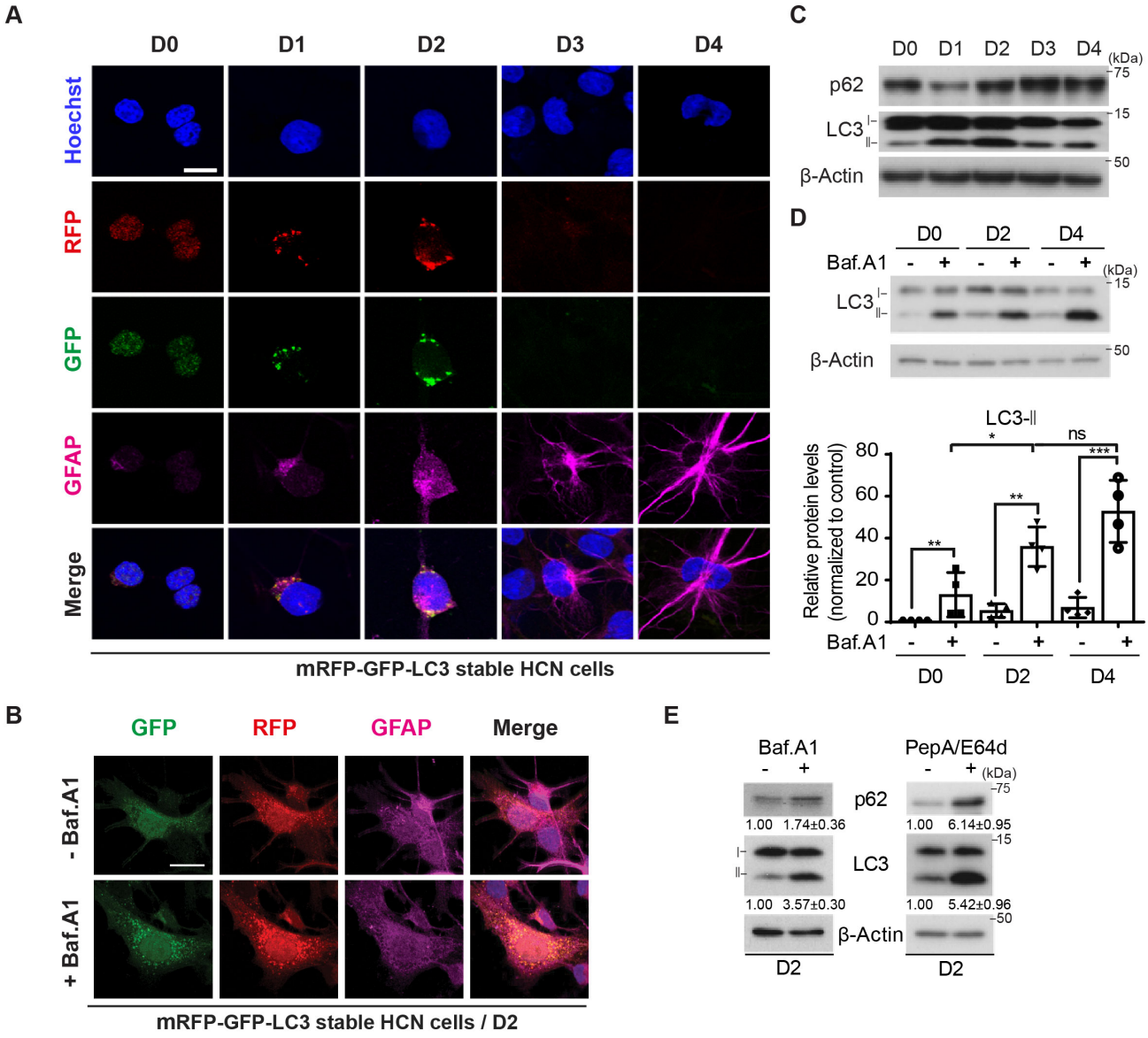

Fig. 3. An increase in autophagy flux from early time points during astrogenesis. (A) Analysis of autophagy flux using mRFP-GFP-LC3 stable HCN cells. Scale bar, 15 μm. (B) After Baf.A1 treatment, autophagy flux was measured in mRFP-GFP-LC3 stable HCN cells at D2. Baf.A1 (20 nM) was added 1 h before harvesting. Scale bar, 15 μm. (C) Time course analyses of LC3-II by Western blotting. (D) Time course analyses of autophagy flux by Western blotting of LC3-II after Baf.A1 treatment. Baf.A1 (20 nM) was added 1 h before harvesting. (E) Increased autophagy flux at D2. Baf.A1 (20 nM) or PepA/E64d (10 μg/ml for each) was treated 1 h before harvesting. *p<0.05, **p<0.01, and ***p<0.001. n≥3.

was dramatically increased from D1 and D2 upon the induction of astrogenesis and seemingly declined afterwards at basal state. However, overall capacity of autophagy remained high until D4 stage.

Suppression of autophagy alleviates astrogenesis in HCN cells

To examine whether the upregulated autophagy is crucial for HCN cell differentiation into astrocytes, we genetically repressed autophagy by stable knockdown of Atg7 in HCN cells (Fig. 4A). As Atg7 plays a key role in autophagosome formation [47], its knockdown prevents autophagy in HCN cells, as we previously demonstrated [38, 42, 48]. Western blotting analysis showed a substantial reduction in the GFAP level in HCN cells with stable knockdown of Atg7 (Sh-Atg7) in comparison with control shRNA–transduced HCN cells (Sh-Con) (Fig. 4B). qPCR results also indicated a decrease in the *Gfap* mRNA level in Sh-Atg7 HCN cells (Fig. 4C). In addition, Sh-Atg7 HCN cells displayed abnormal morphology

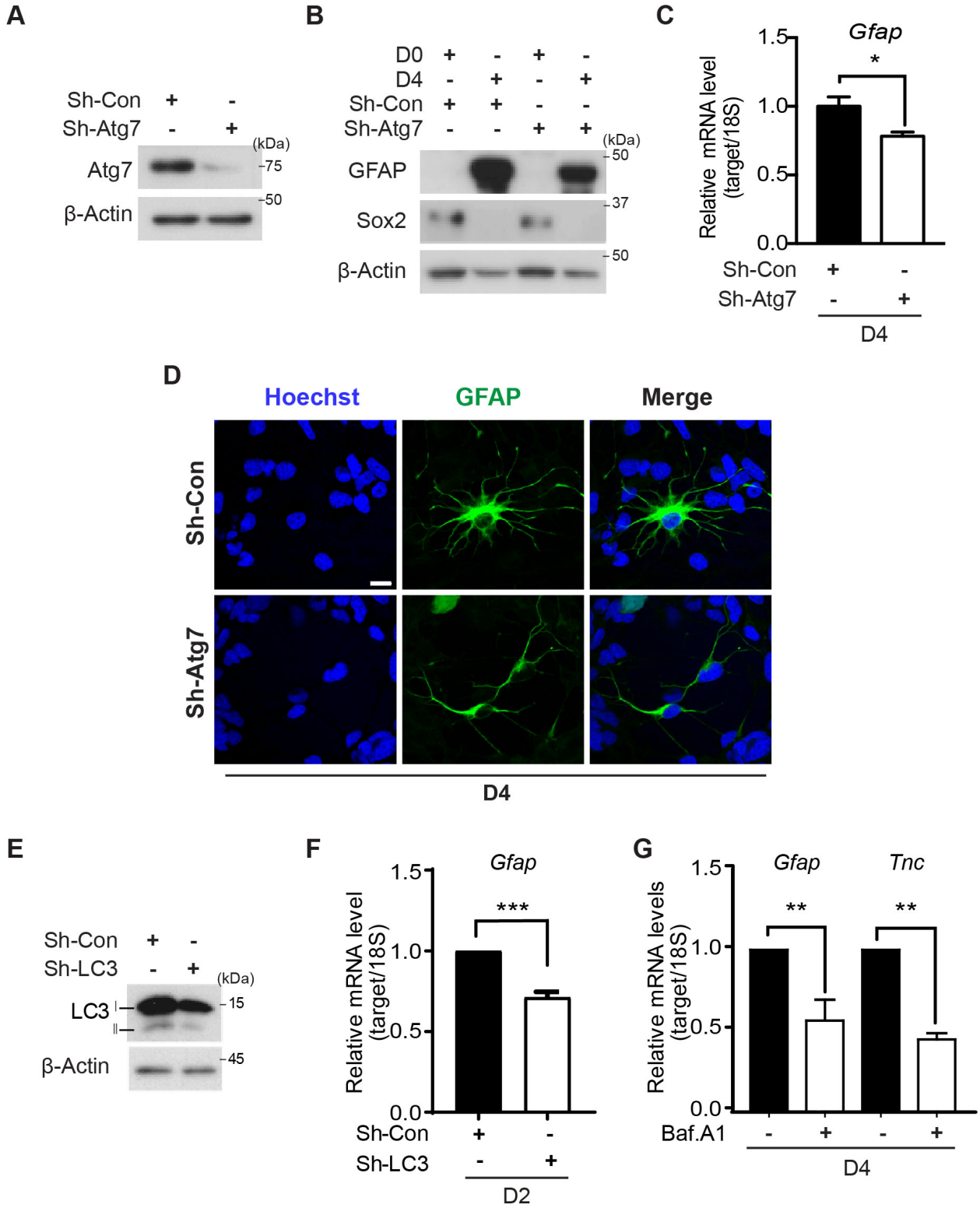

Fig. 4. Impaired astrogenesis by the suppression of autophagy in HCN cells. (A) Verification of Atg7 knockdown by Western blotting analysis. (B, C) Decrease in GFAP protein (B) and mRNA (C) levels in Sh-Atg7 cells compared with Sh-Con cells. (D) Impaired astrocyte morphology in Sh-Atg7 cells compared with Sh-Con cells at D4. (E) Verification of LC3 knockdown by Western blotting analysis. (F) Decrease in *Gfap* mRNA level in Sh-LC3 cells. (G) Reduction of *Gfap* and *Tnc* mRNA levels by Baf.A1 treatment. Baf.A1 (0.5 nM) was added at D0 and D2 and half of media was changed at D1 and D3. Scale bar, 25 μm. *p<0.05, **p<0.01, and ***p<0.001. n≥5.

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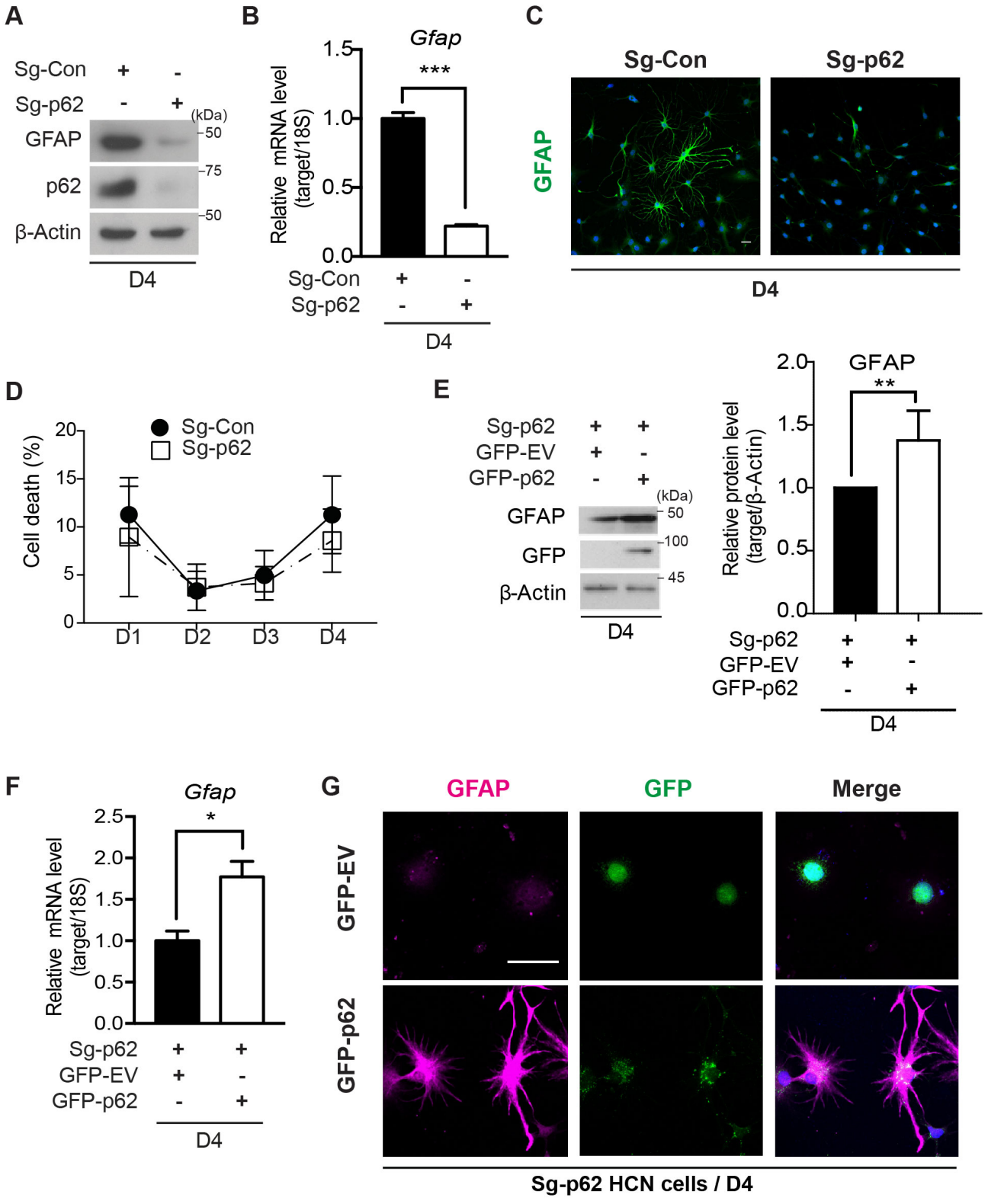

Fig. 5. Impaired astrogenesis in *p62* knockout HCN cells. (A) Genetic deficiency of *p62* abrogated an increase in GFAP expression in differentiated *p62* knockout HCN cells (Sg-p62) in comparison with control cells (Sg-Con) at D4. (B) A dramatic decrease in *Gfap* mRNA expression level in Sg-p62 cells in comparison with Sg-Con cells at D4. (C) Impaired astrocyte morphology in Sg-p62 cells in comparison with Sg-Con cells at D4. Scale bar, 25 μm. (D) The absence of induction of cell death during astrogenesis. (E~G) Recovery of GFAP protein (E), mRNA (F) levels, and astrocyte morphology (G) by expression of GFP-p62, but not GFP-EV (empty vector) in Sg-p62 cells at D4. Scale bar, 25 μm. *p<0.05, **p<0.01, and ***p<0.001. n≥3.

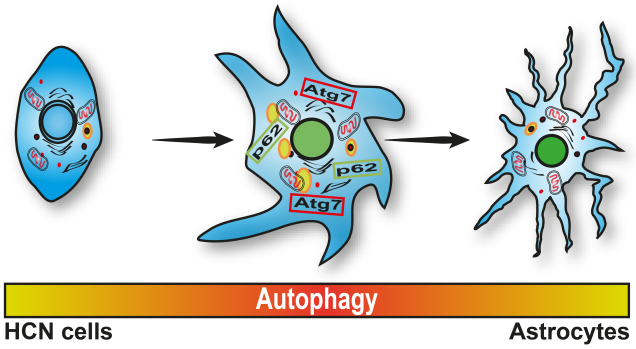

Fig. 6. A schematic diagram illustrating the involvement of autophagy in astrogenesis.

with much fewer processes, whereas Sh-Con cells had normal astrocyte morphology (Fig. 4D). These data show that the key autophagy gene *Atg7* is required for astrogenesis in HCN cells. Furthermore, we tested whether knockdown of LC3 also modulates astrogenesis of HCN cells. Compare to control cells, knockdown of LC3 (Sh-LC3) decreased *Gfap* mRNA level, indicating diminished astrogenesis (Fig. 4E and 4F). Besides genetical suppression of autophagy, we checked whether pharmacological inhibition of autophagy affects astrogenesis. Baf.A1 treatment during 4 days reduced the transcript levels of *Gfap* and *Tnc* (Fig. 4G). Overall, these data provide the evidence supporting our hypothesis that the autophagy is critical for astrogenesis.

Knockout of p62 impairs astrogenesis in HCN cells

To confirm the requirement of autophagy for astrogenesis, we deleted another key autophagy-related gene, *p62*, by using the CRISPR/cas9 system [49]. Successful knockout of *p62* in HCN cells (Sg-p62) was confirmed by Western blotting analysis (Fig. 5A). p62 deficiency blocked astrogenesis, as evidenced by dramatic down-regulation of the GFAP protein and mRNA levels (Fig. 5A and 5B) and impaired astrocyte morphology (Fig. 5C). Since the long-term absence of autophagy may decrease cell viability, we checked cell death rates in Sg-p62 cell lines, but found no significant induction of cell death in comparison with control cells (Fig. 5D). These results also suggest that the reduced number of intact astrocytes did not result from death of autophagy-impaired cells. To examine whether reintroduction of p62 can rescue impaired astrogenesis phenotypes, GFP-fused p62 was expressed in Sh-p62 cells. By Western blotting, qPCR, and immunocytochemical analyses, we confirmed that the expression level of GFAP was restored and astrocyte morphology became normal (Fig. 5E~G). Thus, astrogenesis was recovered by re-expression of p62 in Sg-p62 cells. These results suggest that canonical autophagy mediated by Atg7 and p62 is required for differentiation of HCN cells into astrocytes.

DISCUSSION

Various intrinsic and extrinsic cues regulate astrocyte differentiation in the developing brain [50]. Being a major process for turnover and recycling of cellular constituents, autophagy is also intimately involved in development; thus, it is not surprising that autophagy is critical for the development of the nervous system. The role of autophagy in astrocyte differentiation in the developing brain was recently studied [51]. However, so far, the role of autophagy in astrocyte differentiation from adult NSCs is very little known. Although the present study was restricted to *in vitro* conditions, it was the first study to investigate the role of autophagy in astrogenesis of adult NSCs. It should be mentioned that deletion of autophagy genes in NSCs *in vivo* will cause degeneration of the nervous system because of long-term deficiency in autophagy, and it is not feasible to perform an in-depth mechanistic study *in vivo*. Therefore, despite the limitations of the *in vitro* cellular model, it is still very useful for detailed study of astrogenesis using HCN cells.

Interestingly, we observed an increase in autophagy flux at relatively early time points during 5-day differentiation of HCN cells into astrocytes. Autophagy flux peaked on days 1 and 2, and then returned to a level similar to that in control HCN cells. However, when fusion of autophagosomes with lysosomes was blocked by Baf.A1, differentiating HCN cells at D4 still displayed similar or higher capacity of autophagy flux, compared with D2. When autophagy was suppressed by a stable knockdown of Atg7 or LC3 using sh-Atg7 or sh-LC3-expressing lentivirus or by genetic knockout of *p62* using CRISPR-Cas9 genome editing, the ability of HCN cells to differentiate into astrocytes was greatly impaired. p62 is an adapter protein that regulates multiple signaling pathways, such as those mediated by nuclear factor erythroid factor 2, mammalian target of rapamycin, nuclear factor-κB, or mitogen activated protein kinase [52]. p62 can also act as a receptor for selective autophagic clearance of protein aggregates and damaged organelles [53]. p62 can serve as a signaling hub through its roles related to recruitment of important signaling molecules to control events such as cell survival, cell death, and autophagy [54]. Hence, our study shows a prominent role of autophagy in HCN cell differentiation into astrocytes and suggests a p62-mediated mechanistic link between autophagy and astrogenesis in these cells (Fig. 6).

In the presence of differentiation cues, autophagy stimulates differentiation of stem cells by regulating a dynamic and highly inducible metabolic process [55]. Autophagy is also a crucial player in intracellular remodeling, and this role of autophagy is particularly important for stem cells. Our results show that autophagy

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is critical for remodeling of adult NSCs. Due to high metabolic demand during differentiation and tissue remodeling, mitochondria are also important for differentiation and tissue remodeling. We previously reported that mitophagy occurs in HCN cells [38]. Autophagy may also regulate astrogenesis of HCN cells by controlling the mitochondrial network during differentiation [56].

Differentiation capability of neural stem/progenitor cells can be used for the development of treatments for various neurodegenerative diseases [57]. Studies using HCN cells can contribute to the molecular dissection of autophagy-mediated neural differentiation and better understanding of the basic and applied biology of adult NSCs.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant (2017R1A2B4004289, 2018M3C7A1056275), the Korea Brain Research Institute basic research program (19-BR-0402), and the DGIST Convergence Science Center Program (19-BD-04) of the Ministry of Science, ICT and Future Planning of Korea.

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https://doi.org/10.5607/en.2019.28.2.229

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