RESEARCH PAPER

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Lin28 overexpression inhibits neurite outgrowth of primary cortical neurons *in vitro*

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Lin28 has been shown to promote proliferation of progenitors and survival of neurons during cortical neurogenesis. However, the role of Lin28 in the terminal maturation of neurons remains obscured. In this study, we investigated the developmental impact of Lin28 overexpression on neurite outgrowth. Lin28 expression was upregulated by *in utero* electroporation at E14.5. Two days later, electroporated cortices were dissociated for culturing primary cortical neurons. We found that Lin28 overexpression, which was confirmed immunocytochemically, led to neurite underdevelopment for all time points during culture. Specifically, Lin28-overexpressing cells displayed significantly fewer primary neurites and a decreased dendritic branching index, compared to GFP-expressing controls. Additionally, Lin28 overexpression in primary cortical neurons induced the expression of high mobility group AT-Hook 2 (HMGA2). Taken together, our study demonstrates that constitutive Lin28 expression disrupts cortical neurogenesis resulting in impaired neurite outgrowth with a concomitant induction of HMGA2.

Key words: Lin28, *in utero* electroporation, primary cortical neuron, neurite outgrowth, HMGA2

INTRODUCTION

Lin28 is an RNA-binding protein containing a cold shock domain and two CCHC-type zinc fingers (Moss et al. 1997). It has two paralogs, Lin28A and Lin28B, which are known to block let-7 miRNA biogenesis (Piskounova et al. 2011). During mammalian development, Lin28 plays various roles in many biological processes including embryogenesis (Yokoyama et al. 2008), skeletal myogenesis (Polesskaya et al. 2007), germ cell development (West et al. 2009), and the regulation of body size and onset of puberty (Zhu et al. 2010). Additionally, the demonstration of Lin28's involvement in glucose metabolism (Zhu et al. 2011), tissue repair (Shyh-Chang et al. 2013), and cancer progression (Ma et al. 2014) suggests that Lin28 is a pleiotropic molecule. Reports have shown that Lin28 is highly expressed in undifferentiated cells, such as stem cells, and downregulated as cellular differentiation proceeds (Xu et al. 2009, Yang and Moss 2003). In line with this, many studies have investigated the role of Lin28 in stem cell reprogramming and neurogenesis. For example, Lin28 is one of several stemness factors that can reprogram somatic cells to pluripotent stem cells (Hanna et al. 2009, Yu et al. 2007). Moreover, Lin28 promotes proliferation of stem cells and progenitors (Bhuiyan et al. 2013, Cimadamore et al. 2013, Xu et al. 2009, Yang et al. 2015). Regarding neurogenesis, Lin28 was shown to contribute to neuronal cell fate choices at the expense of gliogenesis (Balzer et al. 2010) and to enhance survival of cortical neurons (Bhuiyan et al. 2013). Additionally, inhibition of neural stem cell differentiation by Lin28 through regulation of the Sox2/Lin28/let-7 signaling pathway was reported (Morgado et al. 2016). However, the necessity of Lin28 for terminal maturation of neurons has not yet been established.

Therefore, in the present study, we investigated the impact of Lin28 overexpression on neurite arborization

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utilizing primary cortical neuronal culture following *in utero* electroporation. Since physiological neurite extension and the elongation of neurite arbors during development is critical for proper neuronal function, disruption of this process can significantly impact neurodevelopment (Gilbert and Man 2017). By analyzing the number of primary neurites and the branching index we found that Lin28 overexpression significantly impaired neurite outgrowth. In addition, we found that high mobility group AT-Hook 2 (HMGA2) was induced by Lin28 overexpression, which is one of the important Lin28 downstream molecules (Fujii et al. 2013, Morgado et al. 2016, Yang et al. 2015, Zhu et al. 2011). Our findings demonstrate a crucial role for Lin28 in neuronal maturation, which may be achieved through the upregulation of HMGA2 expression.

METHODS

Construction of plasmids

pCAGGS–GFP or pCAGGS–Lin28-GFP were generated as described previously (Bhuiyan et al. 2013). Briefly, Lin28 cDNA was amplified with a 5' primer with an XbaI site (GCTCTAGAGATGGGCTCGGTGTCCAACCAGCA) and a 3' primer containing an overlapping sequence of the GFP gene (CTCACCATATTCTGGGCTTCTGGGAGCAGG). In addition, GFP cDNA was separately amplified with a 5' primer containing an overlapping sequence of Lin28 cDNA (CCCAGAATATGGTGAGCAAGGGCGAGGAGCT) and a 3' primer with an EcoRI site (GTGAATTCTTACTTGTACAGCTCGTCCATGCCGAGAG). Using these two PCR products as templates, PCR was also performed with a 5' primer for Lin28 with an XbaI site and a 3' primer for GFP with an EcoRI site and cloned into a pCR 3.1 vector (Promega, Madison, WI, USA). After verifying the nucleotide sequence, the insert was cloned into the pCAGGS vector (Niwa et al. 1991) at XbaI and EcoRI sites (Lin28-GFP). GFP cDNA was also cloned into the pCAGGS vector at the EcoRI site as a control (GFP).

In utero electroporation

Pregnant C57BL/6N mice were purchased from Koatech (Gyeonggi-do, Korea) and were housed in the animal facility with food and water ad libitum. The day of vaginal plug detection was designated as embryonic day (E) 0.5. All animal experiments were performed according to the Ethics Committee of The Catholic University of Korea and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996). *In utero* electroporation was performed as described previously with minor modifications (Tabata and Nakajima 2001). Briefly, pregnant mice at E14.5 were anesthetized with 1% isoflurane balanced with 30% O_2 and 70% N_2O. After the uterine horns were exposed, 1 µl of DNA solution (1 µg/µl) containing 0.01% fast green solution was injected into the right lateral ventricle using a pulled glass micropipette. Then, electronic pulses (40 V, 50 ms, seven times) at 1 s intervals were applied using an electroporator (CUY21SC, NEPA Gene, Chiba, Japan) with tweezer-type electrodes. Then, embryos were placed back into the abdominal cavity and sutured. During the procedure, rectal temperature of the pregnant mouse was maintained at 36.5–37.5°C. The embryos were allowed to develop normally for 2 days.

Primary culture of cortical neurons

Two days following *in utero* electroporation, when the electroporated embryos were expressing detectable levels of GFP, brains of E16.5 mouse embryos were harvested for cortical neuronal cell culture. High density cultures of dissociated primary cortical neurons were prepared according to our previously established method with minor modifications (Bhuiyan et al. 2012). In brief, under a dissecting microscope, electroporated right cerebral cortices were collected in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS, Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated with 0.025% trypsin for 10 min at 37°C. Enzymatic digestion was terminated by mixing the suspension with DMEM supplemented with 10% FBS (Thermo Fisher Scientific Inc.). After gentle trituration, the cells were passed through cell strainers (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and collected by brief centrifugation. The dissociated cells were suspended in Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 25 µM glutamate, 50 units/ml penicillin and 50 µg/ml streptomycin (Thermo Fisher Scientific Inc.). The cells were then plated on 10-cm and 24-well plates (pre-coated with 10 mg/ml poly-L-lysine) at a density of ≈1200 cells/mm^2 and incubated at 37°C with 5% CO_2. The seeding medium was replaced with maintenance medium (without glutamate) one day after plating and refreshed twice a week. The cells that survived in serum-free culture were mostly neurons (Brewer et al. 1993). These cultures were composed of approximately 99% neuronal cells as estimated by immunocytochemical staining with anti-neuron specific nuclear protein (NeuN) and anti-glial fibrillary acidic protein (GFAP) (Bhuiyan et al. 2011).

Immunocytochemistry

Primary cortical neurons grown on 12-mm glass coverslips were washed with Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 15 min. Then, the cells were permeabilized with 0.15% Triton X-100 for 15 min, followed by DPBS washing three times for 5 min. After blocking with 10% normal goat serum for 2 h at room temperature, the cells were incubated with rabbit anti-GFP (1:1000, Thermo Fisher Scientific Inc.) overnight at 4°C. After several rinses in DPBS, the cells were incubated with anti-rabbit Alexa Fluor 488 conjugated IgG (1:500, Thermo Fisher Scientific Inc.) for 2 h at room temperature. For double staining, goat anti-Lin28 (1:300; Santa Cruz Biotechnology Inc.), and rabbit anti-high mobility group AT-Hook 2 (1:300, HMGA2; Cell Signaling Technology, Danvers, MA, USA), anti-goat Alexa Fluor 647 conjugated IgG (1:500, Thermo Fisher Scientific Inc.), and anti-rabbit Cy3 conjugated IgG (1:500, Jackson ImmunoResearch Laboratories) were used. The primary and secondary antibodies were diluted in 3% normal goat serum. After a final DPBS wash three times for 5 min, glass coverslips were mounted onto slides with ProLong™ Gold fluorescent mounting media and visualized under a confocal microscope (LSM 510 Meta, Zeiss, Oberkochen, Germany).

Quantitative analysis of neurite outgrowth

Quantitative analysis of dendritic arborization was performed by assessing the number of primary neurites and their branching index (Perycz et al. 2011, Song et al. 2002). Briefly, each image of cultured cortical neurons acquired from a series of z-stack confocal images was flattened into a single image using maximum projection. Using ImageJ software (NIH), we defined a neurite or a branch as a protrusion >10 μm to exclude dendritic filopodia and spines. All neurites that extended directly from the cell body were counted as primary neurites. For dendritic branching index analysis, we counted total primary branch points along a 50 μm length of the most complex dendrite near the cell soma. The dendritic branching index was calculated as the total number of primary branch points per 10 μm length of a dendrite. A minimum of 15 cells were evaluated for each analysis.

Intensity measurement analysis

For assessing the expression of Lin28 and HMGA2 in cortical neurons, images of GFP-expressing cells were captured at 3 days *in vitro* (DIV). Using ImageJ software, Lin28 or HMGA2 expression in the cell body was analyzed by measuring the fluorescence intensity at each pixel in the image and averaged to show mean fluorescence intensity. A minimum of 25 cells per each group were analyzed.

Statistical analysis

Data were expressed as the mean ± standard errors of the mean (SEM). All statistical analyses were carried out using Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was assessed with the Mann-Whitney U test to compare GFP and Lin28-GFP groups as normal distribution was not assumed. $p<0.05$ was considered to be statistically significant.

RESULTS

Impaired neurite outgrowth by Lin28-GFP overexpression in primary cortical neurons

In order to investigate cortical neuronal development, we dissociated the electroporated cerebral hemisphere and cultured the neurons. When we examined temporal maturation of GFP-expressing cells, we found that GFP-electroporated cells started to sprout and extend neurites at DIV2 (Fig. 1A). Additional primary neurites sprouted, extended and branched out to form complex dendritic arbors in GFP-electroporated neurons through DIV13. In contrast, Lin28-GFP-overexpressing cells sprouted very few neurites at DIV2 and did not branch out primary neurites after DIV5 (Fig. 1B). These data indicate that Lin28 overexpression during cortical neurogenesis can inhibit neurite development.

Quantitative analysis of neurite arborization in primary cortical neurons overexpressing Lin28-GFP

We performed further quantitative analysis of neurite morphogenesis by assessing the number of primary neurites (Fig. 2A) and their branching indexes by measuring a 50 μm length of the most complex neurite (Fig. 2C). The mean number of primary neurites in GFP-expressing neurons was 3.60±0.39 at DIV2 and 10.59±1.61 at DIV13 (Fig. 2B). On the other hand, protrusions of primary neurites in Lin28-GFP-overexpressing neurons were only observed at the early time point of DIV2 to DIV5 (2.75±0.24 to 4.18±0.37, respectively). After DIV5, there was no significant increase in the number of primary neurites with Lin28 overexpression (Fig. 2B). Compared to GFP-expressing neurons, the mean number of primary neurites in Lin28-GFP-overexpressing

cells was significantly decreased at all time points examined (Mann-Whitney U test; DIV2, U=1012.50, p=0.011; DIV5, U=813.50, p<0.001; DIV9, U=300.00, p<0.001; DIV13, U=212.50, p<0.001). Moreover, the dendritic branching index (Fig. 2C, D), an indicator of complex dendritic arborization, was markedly reduced in Lin28-GFP-overexpressing neurons compared to GFP-expressing controls from DIV5 to DIV13 (Mann-Whitney U test; DIV2, U=129.00, p=0.087; DIV5, U=75.00, p<0.001; DIV9, U=38.00, p<0.001; DIV13, U=16.00, p<0.001). Together, these data demonstrate that the overexpression of Lin28-GFP in primary cortical neurons resulted in fewer primary neurites and simpler dendritic trees.

Increased expression of HMGA2 by Lin28-GFP overexpression

To gain insight into the molecular mechanisms of neurite underdevelopment found with Lin28-GFP overexpression, we performed immunocytochemistry. At DIV3, GFP-electroporated cells showed a signal for GFP but no signals for Lin28 or HMGA2, while Lin28-GFP-electroporated cells showed immunoreactivity for both Lin28 and HMGA2 (Fig. 3A). Fluorescence intensity was then assayed for HMGA2 and Lin28 expression for quantitative analysis (Fig. 3B, C). Both HMGA2 and Lin28 expression were significantly increased by Lin28-GFP overexpression (Mann-Whitney U test; U=0.00, p<0.001 for HMGA2, U=0.00, p<0.001 for Lin28). Taken together, these data demonstrated successful Lin28 overexpression and indicate a possible molecular mechanism induced by constitutive Lin28 expression during cortical neurogenesis.

DISCUSSION

In the present study, we demonstrated that constitutive Lin28 expression led to neurite underdevelopment in primary cortical neurons *in vitro*. Specifically, Lin28-overexpressing cells showed a reduction in the number of primary neurites and the dendritic branching index. Lin28 overexpression was further confirmed by immunocytochemistry. The immunocytochemical analysis also demonstrated that Lin28 overexpression induced HMGA2 expression.

Neurite outgrowth in developing neurons is a fundamental feature for neural network formation. Using an established primary cortical neuronal culture method (Bhuiyan et al. 2013), we were able to faithfully track individual neuronal development including neurite morphogenesis. We found that constitutive Lin28 expression during cortical neurogenesis resulted in the inhibition of neurite maturation, suggesting that Lin28 may be a novel regulator of neurite outgrowth. Specifically, Lin28 overexpression hindered both neu-

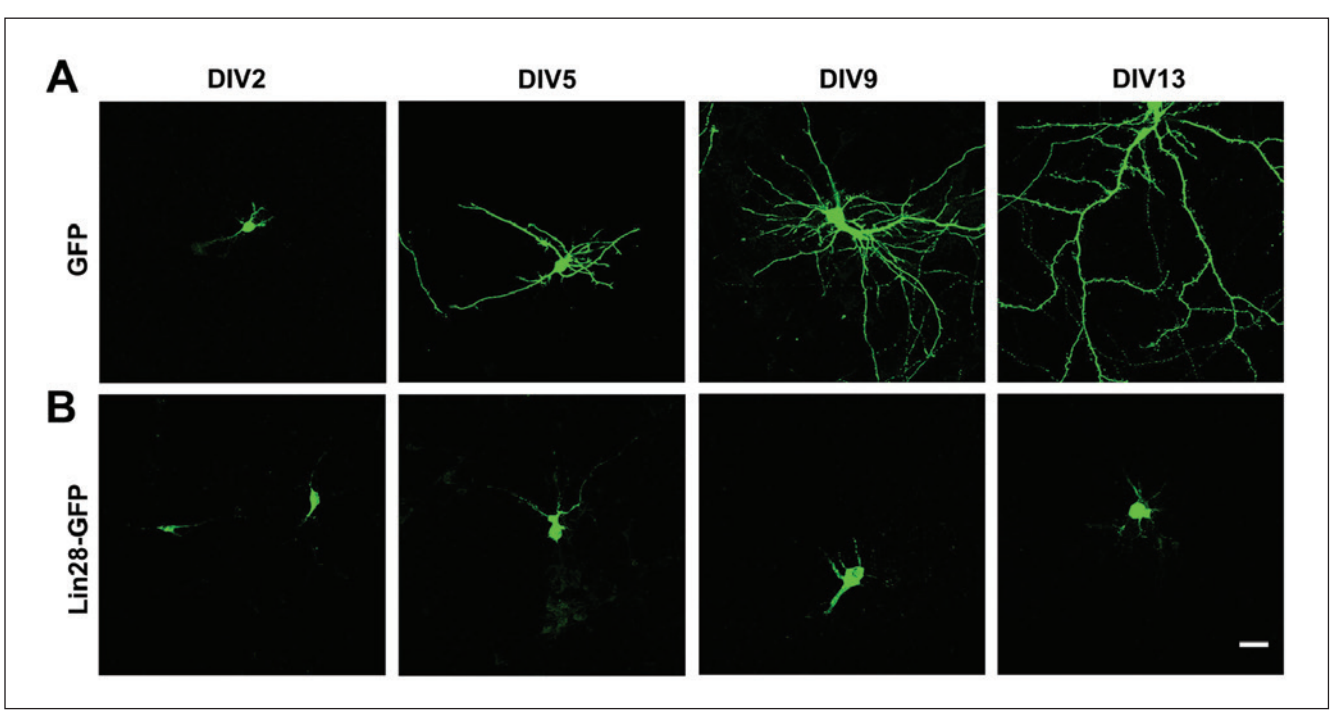

Fig. 1. Lin28-GFP overexpression impaired neurite outgrowth in primary cortical neurons. (A) Representative images of temporal neurite development in GFP-expressing cells. (B) Representative images of temporal neurite development in Lin28-GFP-expressing cells. At E16.5, right cortical tissues that were electroporated *in utero* with GFP or Lin28-GFP vectors at E14.5 were dissociated and cultured in neuron-favorable media. Scale bar=20 μm.

rite extension and dynamic elongation of dendritic arbors, as assessed by the number of primary neurites and dendritic branching index, respectively. Despite a variety of molecules, such as cytoskeleton constituents, growth factors and adhesion molecules, having been identified as modulating neurite outgrowth (Chiu and Cline 2010, Gordon-Weeks 2004, Kiryushko et al. 2004, Mattila and Lappalainen 2008), to our knowledge, our report is the first to demonstrate that mammalian Lin28 can play a direct role in neurite development. Interestingly in support of our findings, overexpression of Lin28 in *C. elegans* hermaphrodite-specific motor neurons inhibited axon extension during development, while loss-of-function mutations of Lin28 promoted precocious axon outgrowth (Olsson-Carter and Slack 2010). Moreover, though indirect evidence, downregulation of miR-145 resulted in a reduction in the number of neurites by increasing Lin28 expression (Morgado et al. 2016). Given that neurite development is a complex process controlled by diverse molecular players, it will

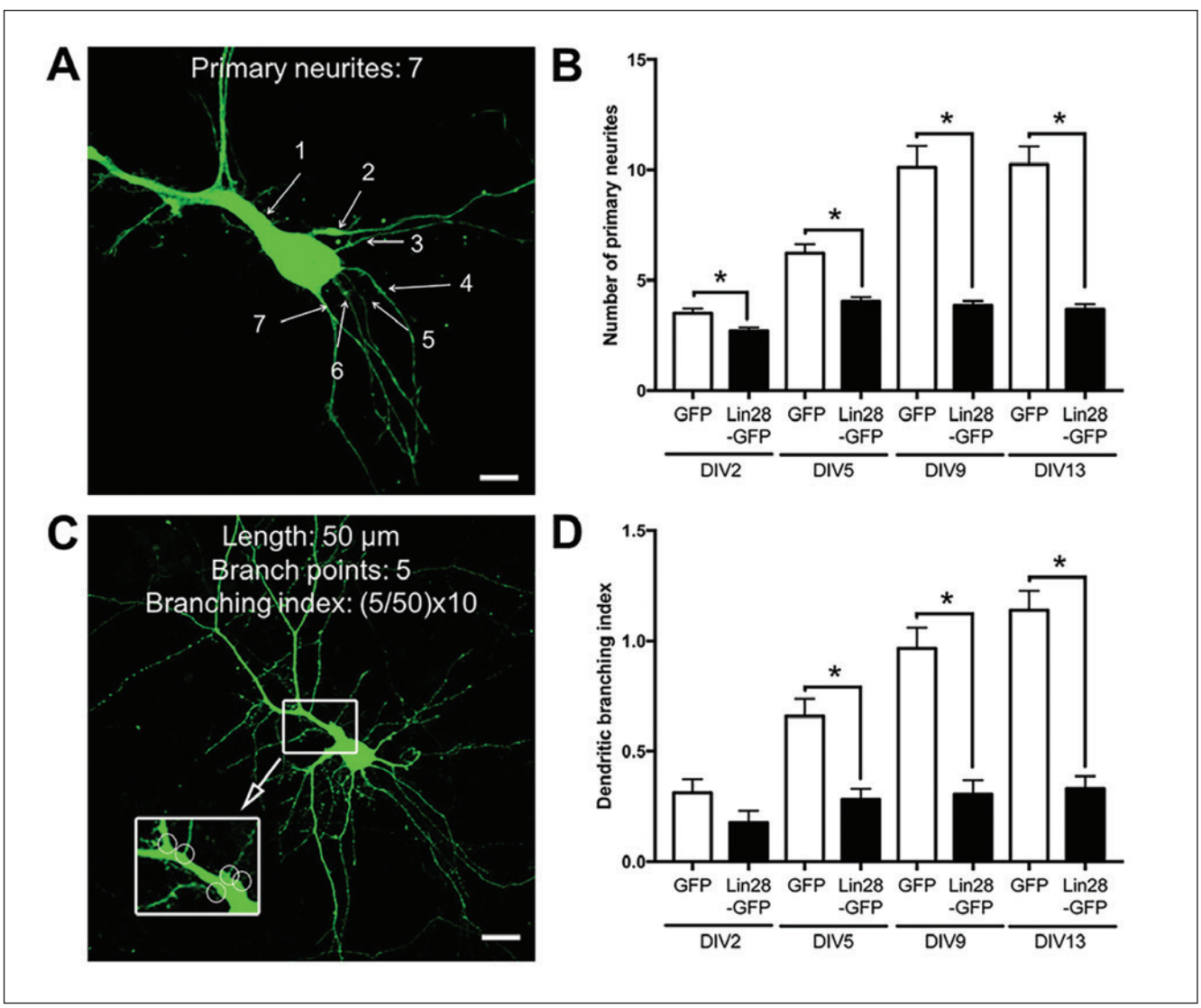

Fig. 2. Lin28-GFP overexpression significantly decreased neurite sprouting and dendritic branching. (A) A representative image of how primary neurites were analyzed. The number of primary neurites that were at least 10 µm long was counted. Scale bar=10 µm. (B) A graph showing the number of primary neurites. Note that Lin28-GFP-expressing cells had fewer primary neurites than GFP-expressing controls at each time point examined. * p<0.05 by Mann-Whitney U test between GFP- and Lin28-GFP-electroporated cells. Sample size for GFP group was 46 (DIV2), 32 (DIV5), 33 (DIV9), 32 (DIV13), and for Lin28-GFP groups was 61 (DIV2), 106 (DIV5), 80 (DIV9), and 97 (DIV13). Data represent mean ± S.E.M. (C) A representative image of how to calculate dendritic branching index. The number of branching points of an apical dendrite for a 50 µm length was counted and the value was presented as dendritic branching number per 10 µm. Scale bar=20 µm. (D) A graph showing dendritic branching index. Lin28-GFP-expressing cells showed decreased values for branching index, compared to controls. * p<0.05 by Mann-Whitney U test between GFP- and Lin28-GFP-electroporated cells. Sample size for GFP group was 21 (DIV2), 17 (DIV5), 18 (DIV9), 19 (DIV13), and for Lin28-GFP groups was 18 (DIV2), 25 (DIV5), 24 (DIV9), and 21 (DIV13). Data represent mean ± S.E.M.

be interesting to investigate how Lin28 interacts with established regulators of neurite outgrowth.

In order to elucidate molecular mechanisms of Lin28-derived neurite underdevelopment, we performed immunocytochemistry targeting a molecule, HMGA2, which is downstream of Lin28 (Fujii et al. 2013, Morgado et al. 2016, Yang et al. 2015, Zhu et al. 2011). We found that HMGA2 expression was in-

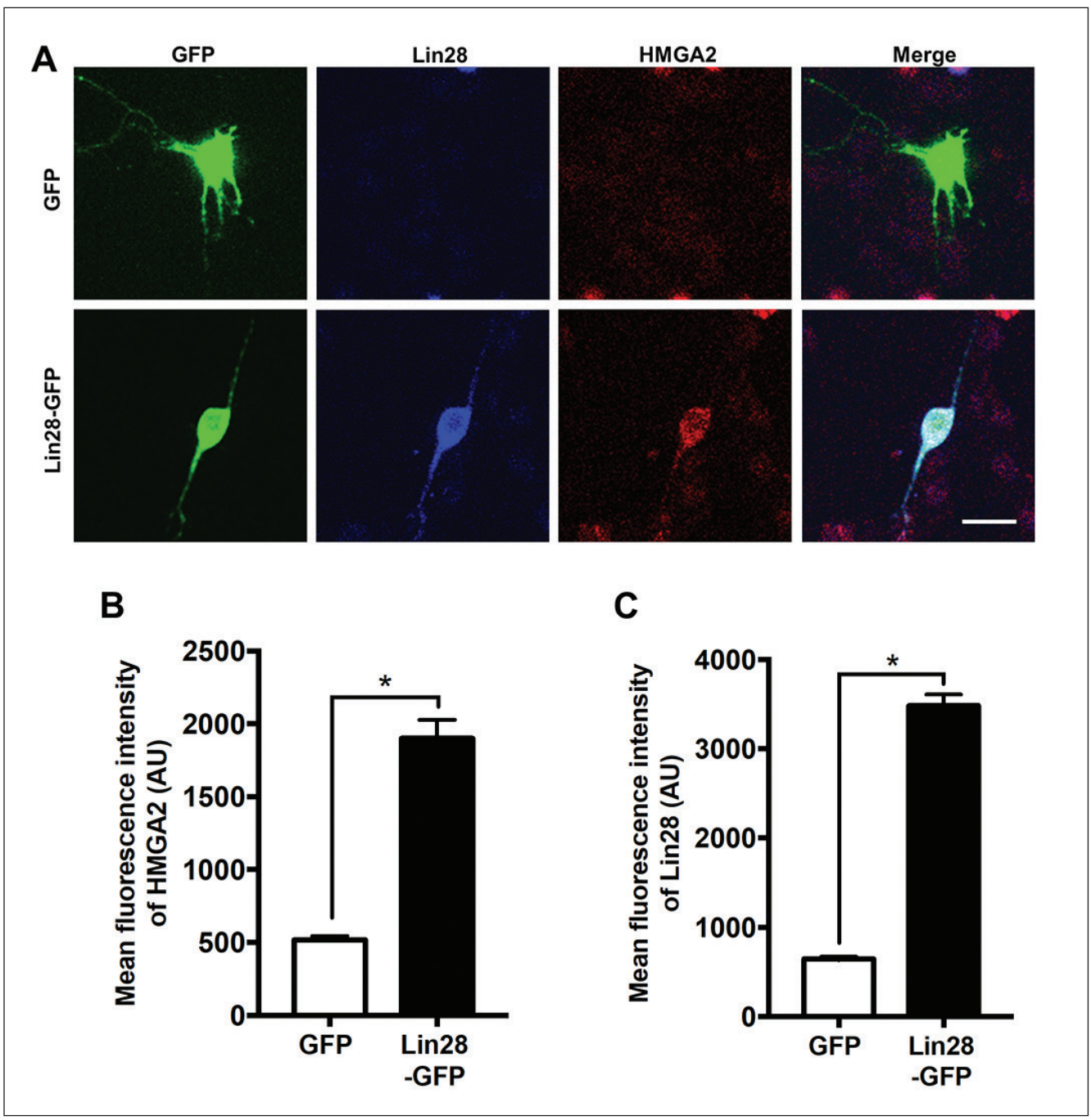

Fig. 3. Lin28-GFP overexpression induced high mobility group AT-Hook 2 (HMGA2) expression. (A) Representative fluorescent images showing GFP (green), Lin28 (blue), and HMGA2 (red) expression in electroporated cells. Scale bar=20 μm. (B) A graph showing mean fluorescence intensity of HMGA2 immunoreactivity. Note that HMGA2 expression was significantly increased in Lin28-GFP-overexpressing cells. * p<0.05 by Mann-Whitney U test between GFP- and Lin28-GFP-electroporated cells. Sample size for each group is 25. Data represent mean ± S.E.M. (C) A graph showing mean fluorescence intensity of Lin28 immunoreactivity. Note that Lin28 expression was significantly increased in Lin28-GFP-overexpressing cells, whereas no signal was detected in GFP-expressing controls. * p<0.05 by Mann-Whitney U test between GFP- and Lin28-GFP-electroporated cells. Sample size for each group is 25. Data represent mean ± S.E.M.

duced by Lin28 overexpression. HMGA2 is a nonhistone chromatin-associated protein containing three AT hooks (Zhou et al. 1996). HMGA2 is predominantly expressed in the ventricular zone of the telencephalon and is downregulated as development proceeds (Hirning-Folz et al. 1998, Nishino et al. 2008). Although the major function of HMGA2 in the central nervous system is reported to be promoting proliferation of neural stem cells and progenitors (Nishino et al. 2008, Vukicevic et al. 2010, Yang et al. 2015), several reports have demonstrated the neurogenic potential of HMGA2, suggesting HMGA2 may be involved in cellular differentiation (Kishi et al. 2012, Sanosaka et al. 2008). Moreover, HMGA2 overexpression increased insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) in neural progenitors, increasing neurogenic potential (Fujii et al. 2013). It is additionally noteworthy that HMGA2 can be negatively modulated by let-7 miRNAs, one of the well-known targets of Lin28 (Lee and Dutta 2007, Nishino et al. 2008). Considering that let-7 miRNAs are required for neurite maturation due to their blocking of Lin28 (Morgado et al. 2016, Petri et al. 2017), it is plausible that a Lin28/let-7/HMGA2 axis may critically function in neurite outgrowth, as our results have suggested. Further studies are required to understand the detailed molecular mechanisms involved in Lin28-associated terminal maturation of cortical neurons.

CONCLUSION

Lin28 has garnered increasing scientific interest since it was reported to be one of the pluripotency factors (Yu et al. 2007). However, besides reprogramming potential, extensive research has demonstrated its central role in diverse biological processes such as regulation of development, proliferation, differentiation, glucose metabolism, tissue repair and tumorigenesis (Bhuiyan et al. 2013, Olsson-Carter and Slack 2010, Polesskaya et al. 2007, Thornton and Gregory 2012, Shinoda et al. 2013, Shyh-Chang et al. 2013, Yang et al. 2015, Zhu et al. 2011). In this study, we provided evidence for a novel function of Lin28, showing that constitutive Lin28 expression during cortical neurogenesis can inhibit neurite extension and complexity of dendritic arbors, possibly through upregulation of HMGA2. Impaired neurite development can result in maldevelopment of brain connectivity, as is frequently observed in neurodevelopmental disorders (Geschwind and Levitt 2007), thus understanding all of the molecules that may play a role in regulating the balance of neurite development is critical. Our findings demonstrate effects of disrupted neurite development during cortical neurogenesis and highlight the potential involvement of Lin28 in this process.

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