Biochemical and Biophysical Research Communications 458 (2015) 110–116

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

YAP/TAZ enhance mammalian embryonic neural stem cell characteristics in a Tead-dependent manner

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ARTICLE INFO

Article history:
Received 7 January 2015
Available online 26 January 2015

Keywords:
YAP/TAZ
Tead
Neural stem cells
Brain development

ABSTRACT

Mammalian brain development is regulated by multiple signaling pathways controlling cell proliferation, migration and differentiation. Here we show that YAP/TAZ enhance embryonic neural stem cell characteristics in a cell autonomous fashion using diverse experimental approaches. Introduction of retroviral vectors expressing YAP or TAZ into the mouse embryonic brain induced cell localization in the ventricular zone (VZ), which is the embryonic neural stem cell niche. This change in cell distribution in the cortical layer is due to the increased stemness of infected cells; YAP-expressing cells were colabeled with Sox2, a neural stem cell marker, and YAP/TAZ increased the frequency and size of neurospheres, indicating enhanced self-renewal- and proliferative ability of neural stem cells. These effects appear to be TEA domain family transcription factor (Tead)–dependent; a Tead binding-defective YAP mutant lost the ability to promote neural stem cell characteristics. Consistently, *in utero* gene transfer of a constitutively active form of Tead2 (Tead2-VP16) recapitulated all the features of YAP/TAZ overexpression, and dominant negative Tead2-EnR resulted in marked cell exit from the VZ toward outer cortical layers. Taken together, these results indicate that the Tead-dependent YAP/TAZ signaling pathway plays important roles in neural stem cell maintenance by enhancing stemness of neural stem cells during mammalian brain development.

1. Introduction

During mammalian development, the central nervous system is precisely structured by the adequate generation and organization of diverse neural cells. Neural cell fate specification, which determines neuronal or several glial cell types, is controlled by the cell microenvironment where multiple signaling cues exist. Signaling cues such as secretory factors and cell surface ligands maintain the balance between the number of newly divided stem cells and differentiated daughter cells [1]. At the early stages of brain development, neuroepithelial cells divide symmetrically and expand the neural stem cell population. As development progresses, neural stem cells tend to divide asymmetrically to generate lineage-determined progenitors or postmitotic neurons as well as neural stem cells [2]. The signaling pathways regulating the maintenance or differentiation of neural stem cells are necessary not only for an appropriate neural cell composition, but also for achieving the proper size of a mature brain [3].

Yes-associated protein (YAP) was first identified as a binding partner of the Yes protein tyrosine kinase in chickens, and a year later the human and murine homologues were isolated [4,5]. Transcriptional co-activator with PDZ-binding motif (TAZ) was originally identified as a 14-3-3 binding protein [6] and is also referred to as WW domain containing transcription regulator 1 (WWTR1). Both YAP and TAZ are homologous to Yorkie (Yki) in the *Drosophila* Hippo signaling pathway, and are also key downstream targets of the Hippo pathway in mammals [7,8]. YAP and TAZ have been shown to act as transcriptional co-activators of several PPXY motif-containing transcription factors through the WW domain. For this reason, transcription factors containing the PPXY motif, such as Runx2, AP2, C/EBPα, c-Jun, Oct-4, and p73, were speculated to interact with YAP/TAZ [6]. YAP and TAZ also bind to TEA domain family transcription factors (Teads) lacking the PPXY domain in a WW domain-independent manner [9].

Teads are evolutionarily conserved transcription factors consisting of Tead1 to Tead4 in humans and mice [10], and they share

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http://dx.doi.org/10.1016/j.bbrc.2015.01.077
0006-291X/

the conserved 72-amino acid TEA DNA binding domain at the N-terminal region. Although all Tead proteins are very similar in DNA binding specificity [10] and interacting partners [9], they appear to have distinct roles at different stages of embryonic development. For example, Tead1 knockout mice cannot develop a normal heart and show lethality before embryonic day 12 (E12) [11], and Tead4-deficient mice stop development before E3.5 [12]. In the central nervous system, Tead2 was implicated in mouse neural tube closure [13]. However, the roles of YAP/TAZ and Tead, and their relationship in mammalian brain development that occurs after neural tube formation had remained unclear.

In this study, we show that YAP/TAZ enhance neural stem cell characteristics *in vivo* as well as *in vitro* through interaction with the Tead transcription factor during the mammalian mid-to late neurogenic period.

2. Materials and methods

2.1. Plasmid construction

The wild-type and S94A-mutated YAP vectors were purchased from Addgene (plasmid #33091 and #33094) (Cambridge, MA), and subcloned into the MluI site of the retroviral vector MSIG [14]. The TAZ construct was kindly provided by Dr. E. S. Hwang (Ewha Womans University, Seoul, South Korea) [15] and amplified using the primers mTAZ-F (5′-GGACGCGTGCCACCATGCATAATTCAACAGCTCC-3′) and mTAZ-R (5′-CCACGCGTTCAAGCGTAATCTGGAACATCGTATGGGTACAGCCAGGTTAGAAAGGGCT-3′). The Tead2 sequence was amplified using the primers mTead2-F (5′-ACACGCGTGCCACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGG CGGGCAAGGGGGATCCCCGGACTGGG-3′) and mTead2-R (5′-TGACGCGTTCAGTCCCTGACCAGGCG-3′) using embryonic age 13.5 (E13.5) mouse brain cDNA as a template. The VP16 transcription activator domain from herpes simplex virus and the *Drosophila* Engrailed repressor domain were fused to the 3′ end of the TEA domain of Tead2 as previously described [16]. All amplified cDNA sequences were initially cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and then inserted into the multicloning sites of MSIG [14].

2.2. Retroviral vector production and transduction

The method of retroviral vector production has been previously described [17]. Briefly, the retroviral construct was transfected into 293T cells with gag-pol (pCA-gag-pol) and env-expressing vector (VSV-G) using polyethyleneimine (Sigma, St. Louis, MO). Supernatant was collected 48 h after transfection, filtered through a 0.45 μm filter and frozen at 80 °C until used. Concentrated viral stocks were prepared by ultracentrifugation at 25,000 rpm for 90 min at 4 °C in an SW28 rotor (Beckman–Coulter, Fullerton, CA). Pellets were resuspended in 50 μl of PBS at 4 °C for about 12 h, and virus aliquots stored at −80 °C. For viral titration, NIH3T3 cells were seeded at 1×10^5 in 6-well plates on the previous day. Viral supernatants were added in the presence of polybrene (final concentration 8 μg/ml) (Sigma). The viral titer was determined by measuring the percentage of GFP-positive cells transduced with different dilutions of virus stock.

2.3. Animals and in vivo injection into the embryonic brain

All animal protocols were approved by the Institutional Review Board (#2014-36) and conducted in the Laboratory Animal Research Center of Sungkyunkwan University. Timed pregnant CD1 mice (Orient Bio, Osan, Korea) were used for viral injections, and embryos were considered 0.5-days old (embryonic day 0.5, E0.5) when a vaginal plug was detected in the morning. Prior to injection, pregnant mice were anesthetized with pentobarbital sodium (Hanlim Pharm, Gyeonggi, Korea) and ultracentrifuge-concentrated viruses containing polybrene (final concentration 80 μg/ml) were injected into the telencephalic ventricle of E13.5 embryos.

2.4. Neural progenitor cell preparation, and neurosphere assay

Primary neural progenitor cells were prepared from the lateral and medial ganglionic eminences of E14.5 embryos. Dissected brain tissue was minced, washed three times with PBS, and incubated in 0.25% trypsin (Invitrogen) at 37 °C for 5 min. DNase and ovomucoid trypsin inhibitor (both from Worthington, Freehold, NJ) were added, and samples were triturated using a fire-polished Pasteur pipette. Cells were washed twice with DMEM/F12 media (Invitrogen), resuspended in PBS, and run through a 40 μm cell strainer (Falcon, Franklin Lakes, NJ). Prior to neurosphere assays, neural progenitors were transduced with concentrated retroviral vectors. After 48 h, aggregated cells were mechanically dissociated, and 5000 cells were seeded and incubated in DMEM/F12 media supplemented with B27/N2 (both from Invitrogen) and FGF2 (peprotech, RockyHill, NJ). After 7 days of incubation, the numbers of neurospheres larger than 50 μm in diameter were counted under a light microscope (Eclipse TS100, Nikon, Melville, NY).

2.5. Immunofluorescence

Standard immunofluorescence procedures were used for visualization of target gene expression in retroviral vector-injected animals. Briefly, gene-transferred embryonic brains at E17.5 were fixed in 4% paraformaldehyde and cryosectioned. Sections were washed in PBS, then blocked for 1 h with PBS containing 10% fetal bovine serum and 0.2% Triton X-100. Samples were then incubated overnight at 4 °C with anti-GFP (Invitrogen) and with or without anti-Sox2 (Millipore, Temecula, CA) primary antibodies, washed three times in PBS and incubated for 1 h at room temperature with Alexa Fluor-conjugated secondary antibodies (Invitrogen) diluted in blocking solution. Samples were further counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Images were acquired using a Zeiss (Oberkochen, Germany) LSM 700 confocal microscope.

2.6. Reverse transcription PCR

Total RNA was prepared from E14.5 embryonic brains using the TRIzol reagent (Invitrogen) and cDNAs were synthesized from 500 ng of RNA sample by using an oligo(dT) primer and M-MLV RTase enzyme (Promega, Madison, WI). PCR was performed according to the Smart Cycler System's (Takara, Shiga, Japan) protocol using the following primers: Tead1 forward, 5′-CTCCGCTTTCCTTGAACAGC-3′, Tead1 reverse, 5′-GTCCACAGATTCGAGCAACG-3′, Tead2 forward, 5′-TGAGCAGCCAGTATGAGAGC-3′, Tead2 reverse, 5′-CAGCAGACGGTACACAAAGC-3′, Tead3 forward, 5′-GATTGCCCGCTACATCAAGC-3′, Tead3 reverse, 5′-ATGCCAACCTGGTATTCCCG-3′, Tead4 forward, 5′-AGCTAAGAACAAGGCCCTGC-3′, Tead4 reverse, 5′-TGCCAAAACCCTGAGATTGC-3′.

3. Results

3.1. YAP/TAZ retain neural cells in the VZ/SVZ of the embryonic brain by enhancing neural stem cell characteristics

To address the role of YAP/TAZ in mammalian brain development *in vivo*, retroviral vectors [14] expressing YAP/TAZ were introduced into the embryonic age 13.5 (E13.5) mouse

telencephalic ventricle *in utero* and the distribution of infected cells was analyzed at E17.5. As shown in Fig. 1A–D, a higher fraction of both YAP- or TAZ-infected cells was detected in the ventricular/subventricular zone (VZ/SVZ). To characterize the YAP-infected cells, immunofluorescence of brain sections was performed using anti-Sox2, a well-known neural stem cell marker [18,19]. As shown in Fig. 1E, most YAP-expressing cells in the VZ/SVZ were colabeled with Sox2 indicating that the YAP^{+} cells in the VZ are neural stem cells. A neurosphere assay [20] was also conducted in parallel to determine whether YAP/TAZ expression affected neural stem cell characteristics. As shown in Fig. 1F, YAP/TAZ-infected cells gave rise to significantly larger neurospheres than control cells. In addition, expression of YAP/TAZ resulted in more than a 2-fold increase in neurosphere frequency (Fig. 1G). These results indicate that YAP/TAZ enhance both self-renewal capacity and proliferative activity, which are neural stem cell characteristics, and changes in cell

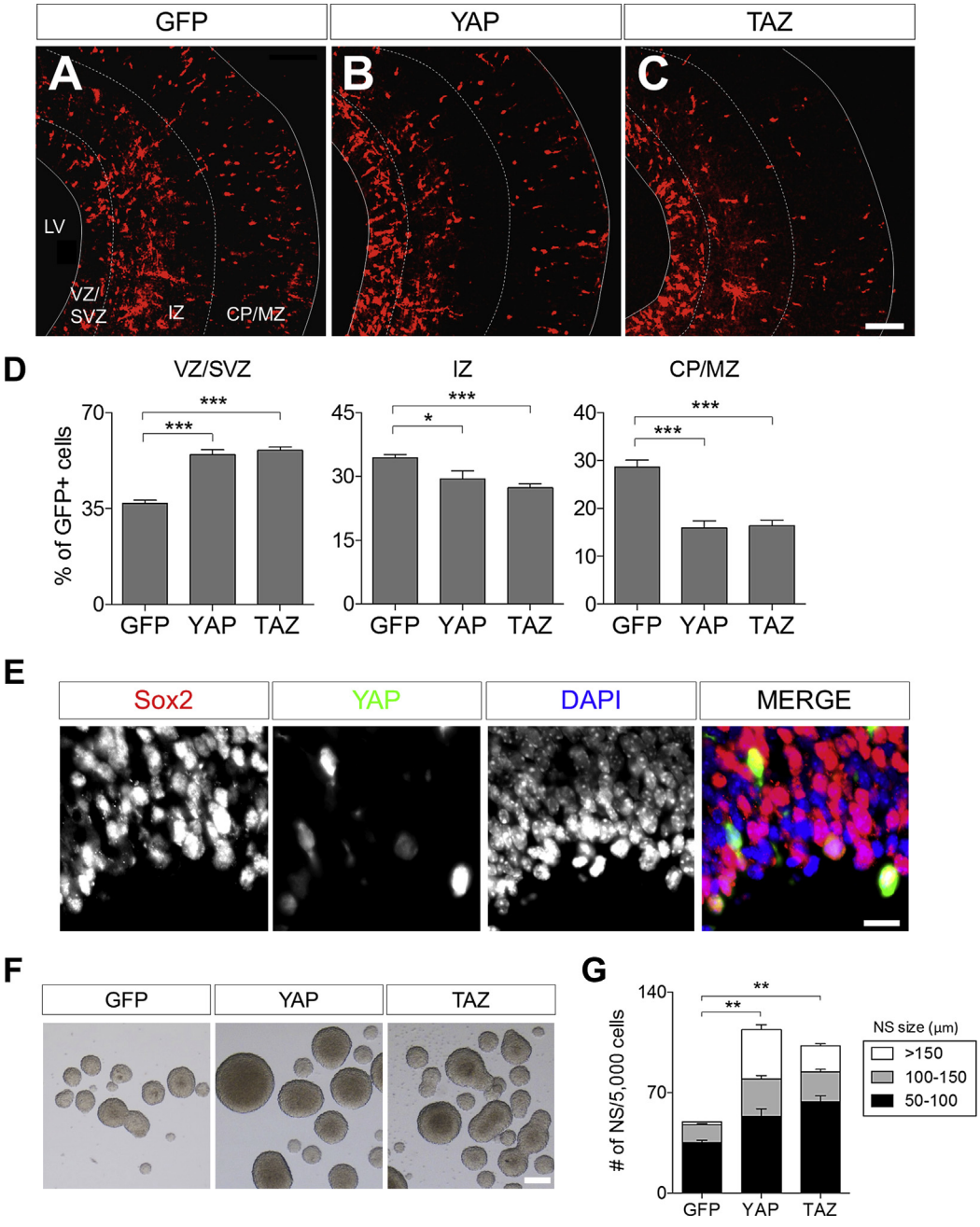

Fig. 1. YAP/TAZ retain neural cells in the VZ/SVZ of the embryonic brain by enhancing neural stem cell characteristics. (A–C) Fluorescent microscopy of coronal sections of E17.5 embryonic brains that were intraventricularly injected at E13.5 with a retroviral vector expressing YAP/TAZ. Gene-transferred cells (red) were labeled with anti-GFP and Alexa 555-conjugated secondary antibodies. (D) Quantification of GFP^{+} cell positions in (A–C). LV, lateral ventricle; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, marginal zone. Scale bar, 100 μm. (E) Double labeling reveals that YAP-transduced cells in the VZ are neural stem cells. YAP-transduced cells in E17.5 embryonic brains that were intraventricularly-injected at E13.5 were double labeled using anti-GFP (green) and anti-Sox2 (red) primary antibodies. Scale bar, 20 μm. (F) Effect of YAP/TAZ on neurosphere formation. E14.5 primary neural progenitors transduced with the retroviral vectors were cultured for 7 days in serum-free media supplemented with B27/N2 and FGF2. (G) Quantification of neurosphere (NS) frequency and size. Scale bar, 100 μm. All error bars represent SEM. Student's *t*-test was used to determine statistical significance. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n \geq 3$.

distribution in the embryonic brain caused by YAP/TAZ are due to the increased stemness of infected cells.

3.2. YAP/TAZ-induced enhancement of neural stem cell characteristics is dependent on association with Tead

Next, we tested whether Tead-binding mediates YAP/TAZ-induced enhancement of neural stem cell characteristics. An alanine residue in place of serine at position 94 (S94A) of YAP reduces binding affinity to Tead [21] and this mutation has been used to determine whether molecular and cellular changes induced by YAP/TAZ expression are Tead-dependent or not [22]. When this Tead-binding-defective YAP mutant was introduced into the mouse embryonic brain, infected cells lost the ability to reside in the VZ/SVZ (Fig. 2A–D). Consistent with this *in vivo* result, YAP S94A displayed a greatly reduced ability to increase the frequency and size of neurospheres (Fig. 2E, F).

3.3. A transcriptionally active form of Tead2 mimics the effects of YAP/TAZ

To further elucidate the role of Tead in YAP/TAZ-mediated regulation of neural stem cells, we first examined the expression pattern of Tead family members during the mid-neurogenic stage. Total RNA was extracted from the E14.5 mouse brain and the expression of Tead1 to Tead4 was analyzed by reverse-transcription polymerase chain reaction (RT PCR). Strong expression of Tead2 was found to be sustained until the mid-neurogenic period (Fig. 3A), consistent with a previous study showing that Tead2 is the most abundantly expressed Tead gene in the mouse neural tube [23].

Without coexpression of YAP/TAZ that contain the transactivational domain, wild-type Tead alone cannot induce target gene expression, and this can cause difficulties in precisely examining the *in vivo* role of Tead. To overcome this, the activation domain of

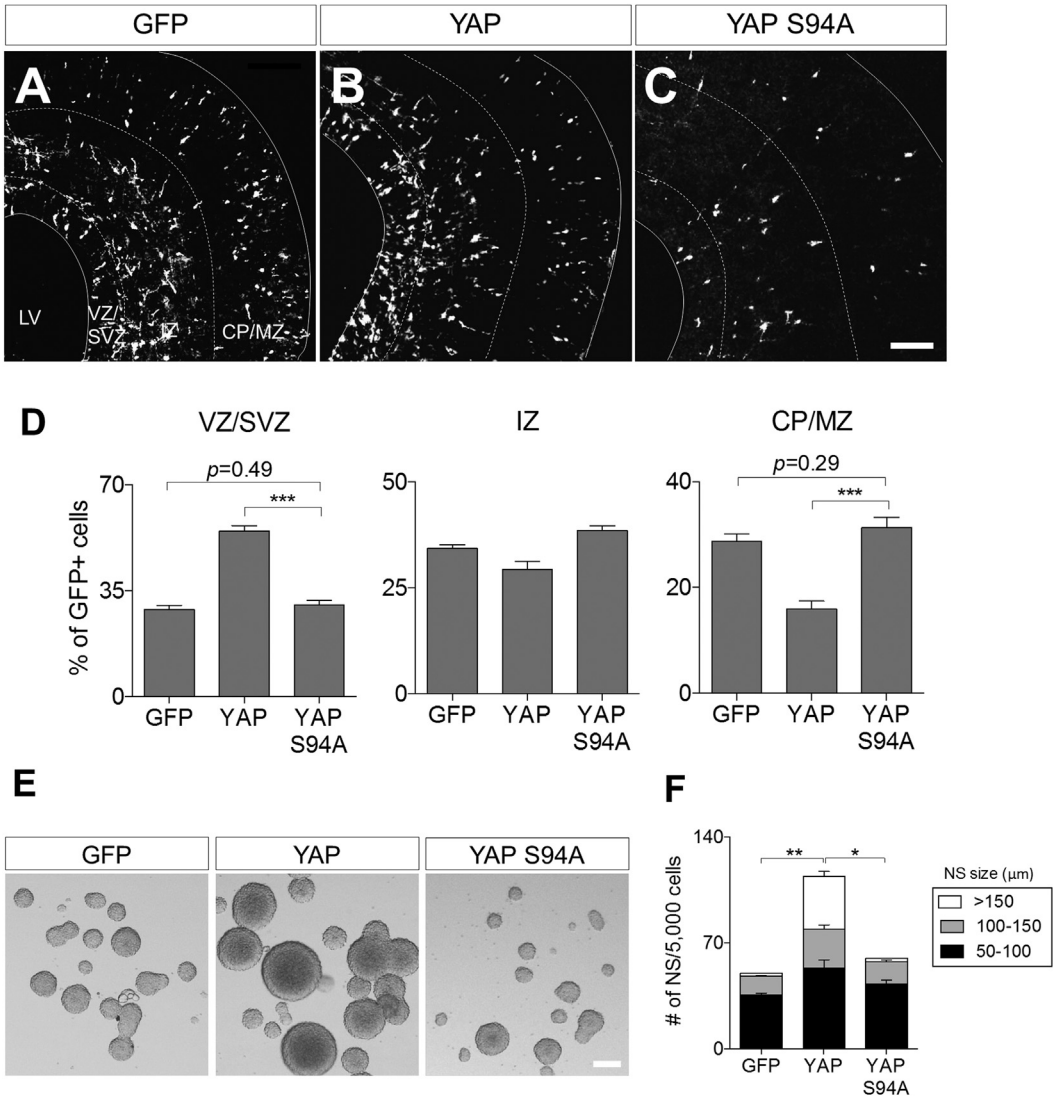

Fig. 2. YAP/TAZ-induced enhancement of neural stem cell characteristics is dependent on association with Tead. (A–C) Fluorescent micrographs of coronal sections of E17.5 cortices that were injected at E13.5 with retroviruses expressing the wild-type or S94A-mutated YAP gene. Gene-transferred cells were visualized by immunostaining for GFP (white). (D) Quantification of sections for the proportion of GFP-positive cells located in the VZ/SVZ, IZ, and CP/MZ. (E) Representative images of neurospheres derived from wild-type or YAP S94A-transduced cells. (F) Quantification of neurosphere frequency and size. Scale bars, 100 μm. Error bars represent SEM. Student's *t*-test was used to determine statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n \geq 3$.

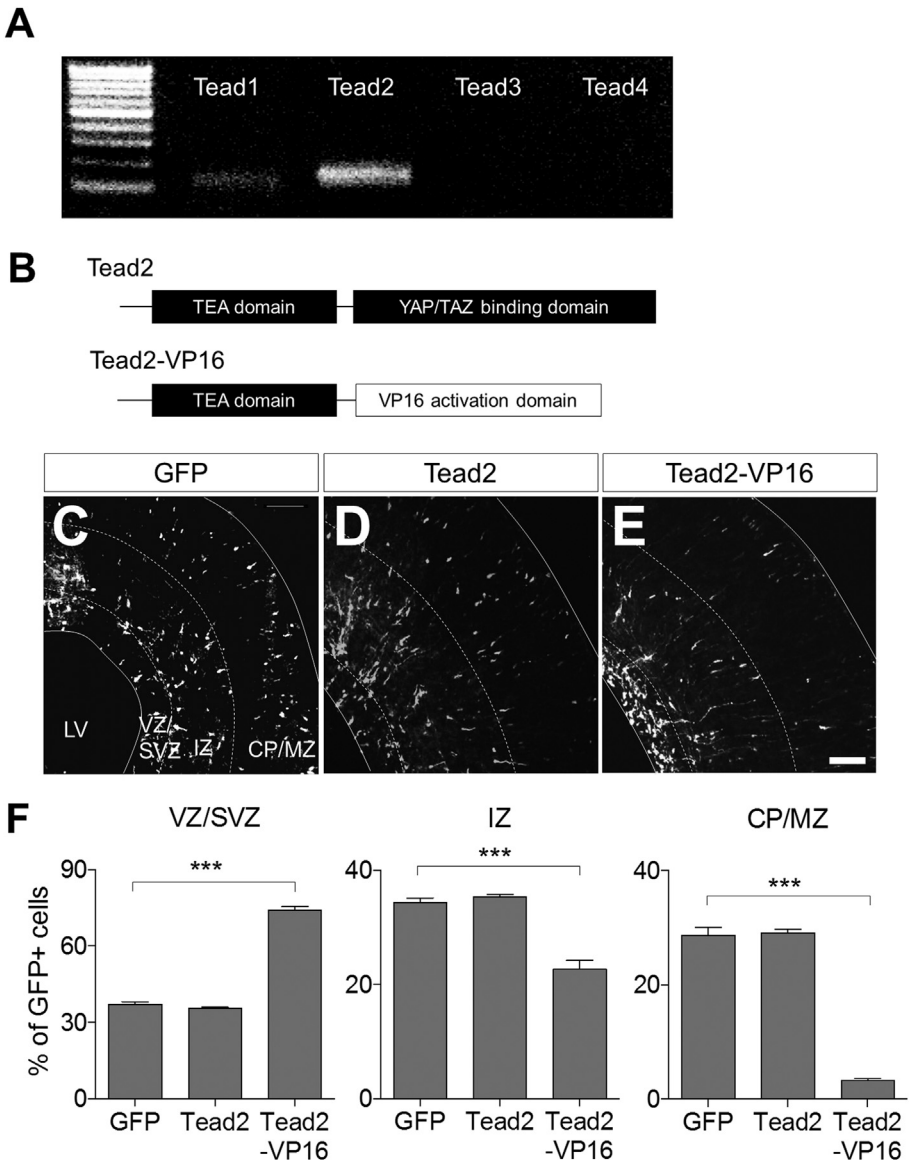

Fig. 3. A transcriptionally active form of Tead2 mimics the effects of YAP/TAZ. (A) Expression pattern of TEA domain family genes in E14.5 primary neural stem cells. Total RNA was extracted from E14.5 neocortices and expression of TEA domain family members was assessed by reverse-transcription polymerase chain reaction (RT PCR). (B) Schematic diagram of full length Tead2 and transcriptionally active form of Tead2 (Tead2-VP16) genes. (C–E) Fluorescent immunofluorescence microscopy of coronal sections of E17.5 brains that were injected with a retrovirus expressing Tead2 or Tead2-VP16 at E13.5. Infected cells were visualized by immunostaining for GFP (white). Scale bar, 100 μm. (F) Quantification of the proportion of GFP-positive cells in the VZ/SVZ, IZ and CP/MZ. Error bars represent SEM. Student's *t*-test was used to determine statistical significance. ***$p < 0.001$; $n \geq 3$.

the herpes simplex virus VP16 was fused to the C-terminus of Tead2 lacking the YAP/TAZ binding domain (Tead2-VP16) as designed in a previous study [24] (Fig. 3B). Indeed, the effects of *in vivo* YAP/TAZ expression were exactly reproduced by Tead2-VP16: a significantly higher number of Tead2-VP16-expressing cells were observed in the VZ/SVZ (Fig. 3C–F).

3.4. Suppression of Tead-dependent transcription results in a decrease of neural stem cell characteristics

Finally, a loss-of-function approach was used to examine the importance of Tead. A fusion protein composed of the TEA DNA binding domain of Tead2 and the *Drosophila* Engrailed transcriptional repressor domain (Tead2-EnR) was generated as designed in Cao et al., 2008 [24] (Fig. 4A). When Tead2-EnR was introduced into embryonic brains, expression of Tead2-EnR resulted in marked cell exit from the VZ/SVZ toward outer cortical layers indicating increased differentiation of Tead2-EnR-expressing cells (Fig. 4B–D). Tead2-EnR expression also had pronounced effects on neurosphere formation. Tead2-EnR-expressing cells produced significantly less and smaller neurospheres indicating reduced self-renewal capacity and proliferative ability of neural stem cells (Fig. 4E, F). Taken all together, our results indicate that Tead is the key transcription factor of the YAP/TAZ signaling pathway during embryonic neurogenesis.

4. Discussion

Our experiments demonstrate an important regulatory role of YAP/TAZ signaling in maintenance of the neural stem cell pool during mammalian brain development. Despite some previous studies suggesting that YAP signaling may be involved in brain

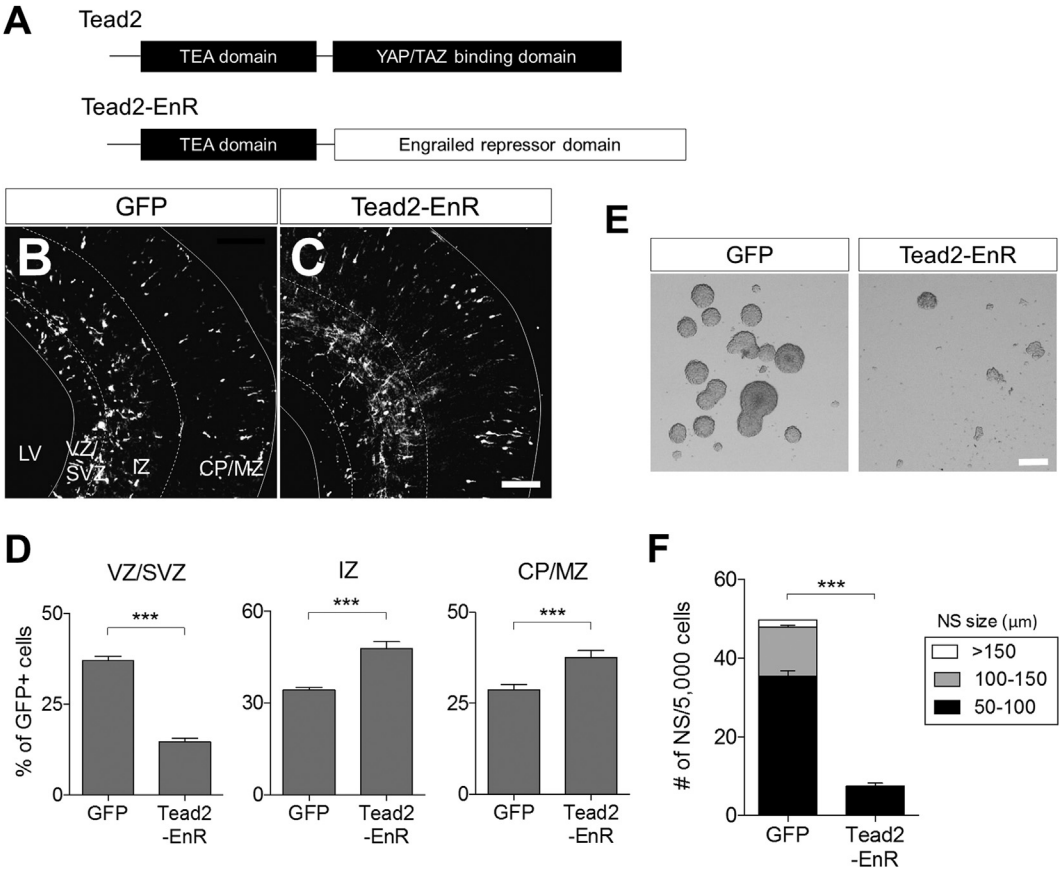

Fig. 4. Suppression of Tead-dependent transcription results in a decrease of neural stem cell characteristics. (A) Schematic diagram of Tead2 and Engrailed repressor domain fusion protein (Tead2-EnR). (B–C) Representative fluorescent microscopy images of coronal sections of E17.5 brains that were injected at E13.5 with retroviruses expressing Tead2-EnR. Transduced cells were immunostained for GFP (white). (D) Quantification of the proportion of GFP-positive cells in the VZ/SVZ, IZ and CP/MZ. (E) Representative images of neurospheres generated from Tead2-EnR-transduced cells, and quantification of neurosphere frequency and size (F). Scale bars, 100 μm. Error bars represent SEM. Student's *t*-test was used to determine statistical significance. $^{***}p < 0.001$; $n \geq 3$.

development, there has been no direct evidence regarding the functional role of YAP/TAZ in the regulation of mammalian neurogenesis. Work in the developing chick system found that YAP regulates progenitor cells in the neural tube [24]. However, much remains to be elucidated regarding the role of YAP at later stages of brain development, between the periods of early- and late-neurogenesis, and regarding the possible differences in YAP/TAZ functions between mammalian and avian species. The Li et al. study strongly supports the *in vivo* relevance of our study; they showed that YAP is expressed in the VZ of the E13.5 mouse brain [25]. However, this study did not investigate the role YAP in the VZ. Recently, Lavado et al. showed that Nf2, which inhibits YAP, limits the neural progenitor pool [26]. However, this study did not provide conclusive evidence of the autonomous effects of YAP and its key downstream factor. Overall, the lack of an appropriate *in vivo* assay system has made it difficult to precisely determine the function of YAP/TAZ in the developing mammalian brain. Our study answers the questions mentioned above by using an *in utero* gene delivery technique to deliver retroviruses into the mouse telencephalic ventricle, and provides direct evidence that YAP and TAZ in the VZ regulate neural stem cells by increasing their stemness in a cell autonomous fashion during the mammalian neurogenic period.

In addition, one of the most important contributions of our study is the identification of TAZ function. Unlike YAP, TAZ has not been extensively studied in the embryonic nervous system, perhaps because TAZ knock-out mice do not develop any observable abnormalities in the brain region [27]. We noticed the *in situ* hybridization data on the Jackson Laboratory website (www.informatics.jax.org/gxd) that demonstrates strong TAZ mRNA expression in the mouse VZ. This TAZ mRNA expression pattern prompted us to explore its role in brain development and led us to conclude that TAZ acts in a similar way to YAP. Hence, the previous observation of less severe changes in the mouse brain lacking TAZ gene is likely due to the redundant functions of TAZ and YAP.

The next question to be addressed was identification of the YAP/TAZ-binding partner that provides target gene specificity. YAP/TAZ are co-activators of various transcription factors such as p73, ErbB-4, Pax3, Runx2, and Tead1-4 [9,28–31]. Among these partner transcription factors, we focused on the Tead proteins based on a report that implicated Tead2 in neural tube formation [23]. However, the upstream element of Tead2 that offers transactivational activity and the role of Tead2 after neural tube formation were unknown. Furthermore, because this study used a genetic model which has Tead2 knocked-out in all brain cells, they could not provide a clear answer as to whether Tead2 acted in a cell autonomous or non-autonomous manner. Our experiments employing *in utero* injection of Tead-binding-defective YAP, and injection of a constitutively active- or dominant negative form of Tead2 into a wild-type genetic background clearly establish a direct link between YAP/TAZ and Tead, and help explain how YAP/TAZ regulate mammalian embryonic neurogenesis. Further studies are needed to determine which downstream target gene of the YAP/TAZ-Tead

transcriptional activation complex plays an essential role, as well as determining potential differences between YAP/TAZ functions during brain development. It would also be worthwhile to study the role of other TEA domain family members in various developmental processes.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (#2012R1A1A2003185).

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