Zinc finger E-box-binding homeobox 1 (ZEB1) is required for neural differentiation of human embryonic stem cells

Yuan Jiang1,2,3, Long Yan1,3, Longkuo Xia1,2, Xiaoyin Lu1, Wenliang Zhu1,2, Dewen Ding1,2, Mingxia Du1, Da Zhang1, Hongmei Wang1,2,4, * and Baoyang Hu1,2, *

1State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China; 2University of Chinese Academy of Sciences, Beijing, 100049, China; 3Co-first author; 4Lead Contact.

* Correspondence and reprint requests should be addressed to:

Dr. Hongmei Wang, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, P. R. China; E-mail: wanghm@ioz.ac.cn; Tel: 86-10-64807187; Fax: 86-10-64807189;

Dr. Baoyang Hu, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, P. R. China; E-mail: byhu@ioz.ac.cn; Tel: 86-10-64806251; Fax: 86-10-64806251.

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Abstract

Human pluripotent stem cells hold great promise for improving regenerative medicine. However, a risk for tumor formation and difficulties in generating large amounts of subtype derivatives remain the major obstacles for clinical applications of stem cells. Here, we discovered that zinc
finger E-box–binding homeobox 1 (ZEB1) is highly expressed upon differentiation of human embryonic stem cells (hESCs) into neuronal precursors. CRISPR/Cas9-mediated ZEB1 depletion did not impede neural fate commitment, but prevented hESCs-derived neural precursors from differentiating into neurons, indicating that ZEB1 is required for neuronal differentiation. ZEB1 overexpression not only expedited neural differentiation and neuronal maturation, which ensured safer neural cell transplantation, but also facilitated the generation of excitatory cortical neurons, which were valuable for managing certain neurological disorders, such as Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS). Our study provides useful information on how human neural cells are generated, which may help inform strategies for developing and improving replacement therapies for treating patients with neurological diseases.

Introduction

Human pluripotent stem cells (PSCs), particularly human embryonic stem cells (ESCs) or inducible pluripotent stem cells (iPSCs), owing to their ability to differentiate into all cell types, become the most reasonable sources for transplantation therapy (1-4). In recent years, progress has been made in direct neural differentiation of human PSCs, especially embryonic stem cells, into certain functional cell types, (5-9), which provides an ideal resource for pathogenesis research and clinical therapies of neurodegenerative diseases, such as Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and other disorders in nervous system. However, some problems hinder the application of human ESCs, such as time-consuming differentiation, low yield, heterogeneous nature of derived neuronal populations and a risk of tumor formation upon transplantation in vivo. Therefore, further efforts are required to guarantee both efficiency of differentiation and maturation.

During the differentiation from hPSCs into neurons, hPSCs undergo substantial morphological alterations, first from adherent hESC colonies to cell aggregates, then to neural rosettes, neural stem cells and neurons or glia, a course very similar to that occurs in epithelial-mesenchymal-transition (EMT). It is highly possible that molecules related to EMT may play important roles in neural differentiation. We made a screening and found that ZEB1 was expressed along with neural differentiation.

ZEB1, also named as ZFHX1A or ΔEF1, together with its fellow member ZEB2 (also called ZFHX1B or SIP1), belongs to the zinc finger E-box binding homeobox family (ZFH family). ZEB1 is putatively involved in cell morphological change during EMT. Accordingly, ZEB1 has been extensively studied in various cancers (10-13). Members of ZEB family facilitate neural fate decision through tuning TGFβ/BMP signaling or as targets of miR-200 family (14-16) and ZEB1 has an essential role in neural stem cell survival in vitro (17). In mouse models, Zeb1 has been shown to be strongly expressed in the progenitor cells of the ventricular zone (VZ) and the subventricular zone (SVZ) of the developing cortex and that Zeb1 is necessary for the proliferation of the VZ and SVZ progenitors (19, 20). Recently, Singh et al. reported that Zeb1 is required for tangential migration of unpolarized cerebellar granule neuron progenitors, and downregulation of Zeb1 is necessary for maturation and polarization of the cerebellar granule neurons (21).

However, whether ZEB1 is involved in human neural differentiation still remains
unclear. Here, by using a model system of neural differentiation from hESCs (22, 23), we carried out a series of functional assays to explore the role of ZEB1 in human neural differentiation.

Results

ZEB1 was progressively expressed during neural differentiation of hESCs-To inspect the expression of ZEB1 during neural differentiation, we differentiated hESC line WA09 (H9) as shown in Figure 1A, and harvested cells at the indicated stages for real-time RT-PCR and western-blotting analysis. mRNA level of ZEB1 was hardly detected in hESC (Day 0). Upon neural differentiation, ZEB1 started expression and reached a peak on day 15, remained at a high level thereafter (Figure 1B). The expression pattern of ZEB1 was also verified by western-blotting and immunostaining together with cell type specific markers at different stages of differentiation on samples taken from Day 0, 15, 25 and Day 35 (Figure 1C and D). We also detected the expression of other fellow molecules of EMT in the same set of experiments. Except for N-cadherin, which was up-regulated over the progression of differentiation, no other EMT-related molecules, such as ZEB2, Snail, Slug and Twist2 was detected (Figure S1). These results together indicate ZEB1 may play a role during neural differentiation, which was not previously documented.

ZEB1 was required for the intrinsic neuronal differentiation-To examine whether ZEB1 was required for neural and neuronal differentiation, we generated ZEB1-knockout hESCs based on a H9 hESC line that harbored a cassette of inducible Cas9 (iCas9) and a verified sgRNA targeting ZEB1 locus (sgRNA1) (Figure S2A). We designed three sgRNAs targeting ZEB1 (Table S1), and identified the indel percentages with T7EI assays for each target in HEK293T cells (data not shown), then sgRNA1 which targeted the third exon (CCDS53506.1) was selected as the most efficient one. In H9ESCs, Restriction Fragment Length Polymorphism (RFLP) assay revealed approximately 21% indel rate (Figure S2B). hESCs were then dissociated into single cells. By picking colonies, RFLP analysis and sequencing, we ultimately obtained a hESC line with homozygous mutant of ZEB1 (Clone 9, Figure S2C and S2D). Potential off-target sites were identified at MIT (http://crispr.mit.edu/), and only one possible off-target with four mismatches fall in gene coding sequences (NM_001130675), indicating the specificity of the selected sgRNA1 locus.

Deletion of ZEB1 was confirmed at protein level at different stages during neural differentiation (Figure 2A). During the normal differentiation of hESCs into neurons, cells spreaded out and formed rosettes at the early stage, and then differentiated into neuron phenotype at the late stage (Figure 2B, upper panel). ZEB1 knockout cells did not exhibit distinguishable difference from control cells at hESCs stage and early phase of neural differentiation (Figure 2B, middle panel). However, with neural differentiation proceeded, most of the ZEB1-depleted cells died at the stage when neuronal differentiation occurred (Figure 2B, middle panel). We then overexpressed ZEB1 in the ZEB1 KO hESCs cells, some of them underwent morphological changes at the early stage of differentiation, and eventually became neuron cells (Figure 2B, lower panel). We did not observe any significant difference by evaluating cells proliferation with BrdU antibody (Figure 2C). However, we observed more cells that were...
undergoing apoptosis in ZEB1-depleted group in comparison to the control group using PE Annexin V/7-AAD Kit (Figure 2D). Re-expressing of ZEB1 in ZEB1-KO cells rescued the neural differentiation (Figure 2B, lower panel). In addition to the rescue effects, forced expression of ZEB1 in ZEB1-KO caused discernable dispersing of the differentiated cells at early stage of neural differentiation (Figure 2B, right of the lower panel), which was consistent with the phenomenon detected in ZEB1 overexpression cell lines (mention below). Taken together, these data indicated that ZEB1 expression may not be critical for early neural specification, but was essential for the conversion from neural precursors to neurons.

Forced expression of ZEB1 promoted the neural differentiation of hESCs-We next overexpressed ZEB1 (with 3× Flag fusion to the N-terminus) in hESCs with lentivirus tet-on 3G inducible expression system (Figure S3A). The insertion of mNeonGreen-P2A-Blasticidin (BSD) fragment facilitated picking of single colony hESCs (Figure S3B). Selected colonies of H9ESC lines were verified using GFP or antibodies against ZEB1 or Flag upon doxycycline induction (Figure S3C and S3D and Figure 3A). Forced expression of GFP itself in hESCs neither affected the morphology of the hESC colonies nor changed the expression of pluripotent marker such as OCT4 (Figure S3C). However, when overexpressing ZEB1 in hESCs, although the expression pattern of OCT4, NANOG and SOX2 was not changed, these colonies exhibited unique fragmentation upon long-term culture (Figure 3B, S3D and S4). For some hESC colonies, continual overexpression of ZEB1 elicited neurite-like out-growth which was immunostained for the neuron-specific antibody TUJ1 (Figure S3E).

Extra amount of ZEB1 accelerated the neural differentiation of hESCs-To further investigate the effect of ZEB1 in neural differentiation, we induced the expression of ZEB1 once the hESCs were lifted for differentiation. At day 15, most of the wild type cell aggregates formed neural rosettes within colonies (Figure 3C, left panel). However, in the colonies of hESCs with ZEB1 overexpression, only a small number of rosettes were identified. Quite a few number of cells migrated from the colonies and extended neurite-like structures surrounding the cells (Figure 3C, left panel).

Decreasing of neural rosettes may reflect a result of inhibited neural differentiation or alteration of the progress of differentiation. We then stained the cells with neuron-specific and dentsrites antibodies TUJ1 and MAP2 to address this issue. In the control group, only sporadic TUJ1+ cells were detected (~ 2%) on day 15 (Figure 3C and 3D, P<0.001), and the vast majority of TUJ1+ neurons did not appear until day 25. In the ZEB1-overexpressed group, however, TUJ1+ cells appeared as early as Day 15 (up to 30%) (Figure 3D, P<0.001), and the number of TUJ1+ cells continually increased when neural differentiation proceeded. One week after the cells were attached for neuronal differentiation, only 16% of TUJ1+ cells express more mature neuronal marker MAP2 in the control group, while approximate 32% among the TUJ1+ cells began to express MAP2 in ZEB1-overexpressed group (Figure 3C and 3E, P<0.001). Thus, we deemed that forced expression of ZEB1 promoted the neural differentiation by altering the phase of neural differentiation.

The formation of neural rosettes accompanying the precocious TUJ1+ and MAP2+ cells indicated that neural
differentiation was accelerated. Furthermore, we examined the expression of early neural markers such as PAX6, SOX1 and NESTIN as well as the neuronal markers TUJ1 and MAP2 by immunofluorescence staining or western-blotting analysis. In control group, PAX6 was expressed as the earliest transcription factor of neural differentiation from day 6-8 on, which was followed by SOX1 expression from day 8-10 on (Figure 3C and 3F). Early presence of extra ZEB1 repressed PAX6 but increased the expression of SOX1 on day 10-15, endowing cells becoming TUJ1+ neuronal like cells directly within 15 days of differentiation (Figure 3C and 3F). Bypassing the early stage of neural differentiation also accompanied by downregulation of various genes such as ZEB1 itself and PAX6, which likely ensured later smooth transition to an intrinsic neuronal differentiation program (Figure 3F).

**ZEB1 promoted neuronal maturation**-Deletion of ZEB1 impeded neuronal differentiation, while forced ZEB1 expression expedited this process, which prompted us to examine whether ZEB1 could also promote neuronal maturation. We thus continued the neuronal differentiation experiment by placing the hESCs-derived neural progenitor cells on laminin substrate from Day 26 and cultured in neural differentiation medium supplemented with trophic factors such as BDNF, GDNF, IGF1 and cAMP. Four weeks later, the majority of cells in both control and ZEB1-overexpressed groups became elaborately branched and expressed the neuronal marker MAP2 in the cell body and in the neurites (Figure 4A). Considering that the synaptic connection between neurons was an integral component of functional maturity, we then examined the localization of synaptic proteins such as SYNAPSIN-1 in differentiated neurons by immunofluorescent staining. While SYNAPSIN-1 was diffusely distributed in the cytoplasm of neurons in control group, it aggregated as punctuated throughout the more complex dendritic arborizations of neurons when ZEB1 was induced during the neuronal differentiation (Figure 4B).

To further examine whether overexpression of ZEB1 increased the functionality of hESCs-derived neurons, we employed whole-cell voltage-clamp to record electrophysiological activities at week 8 of neural differentiation. Cells from both control and ZEB1-overexpressed groups displayed full-blown Na+ / K+ currents, and these inward currents could be completely blocked by TTX (1 µM) (Figure 4C, i and ii). However, neurons from the control group failed to fire multiple action potentials (APs) in response to a 70 pA current pulse, or displayed single AP or APs with typically faded amplitude in peak value (Figure 4D, i), while trains of sequential APs on the similar spikes were detected in neurons from the ZEB1-overexpressed group (Figure 4D, ii). Neurons in ZEB1-overexpressed group also showed spontaneous inward synaptic currents at 8 weeks of differentiation, which were almost completely eliminated by the non-NMDA antagonist DNQX (20 µM) (Figure 4E). Neurons from the control group did not exhibit spontaneous synaptic currents until ten weeks of differentiation (Figure 4F). These data further validated that ZEB1 promoted the functional maturation and synaptogenesis of the hESCs-derived neurons.

**ZEB1 facilitated the differentiation of excitatory neurons**-To identify which specific types of neurons or glia were differentiated when ZEB1 was overexpressed, we labeled the cells with...
various markers at week 8 of neural differentiation. In control group, hESCs were randomly differentiated into excitatory, GABAergic and dopaminergic neurons at a proportion of 52.2% (± 9.0%), 30.5% (±5.5%) and 8.6% (± 3.7%), according to immunostaining for vGLUT1, GABA or TH, respectively (Figure 5A and 5B). When ZEB1 was overexpressed during neural differentiation, the majority of the cells were distinctly positive for vGLUT1 (84.9 ± 10.2%) (Figure 5A and 5B, *P* < 0.001). Only a small proportion of cells were stained with GABA (4.8 ± 7.9%) and TH (4.8 ± 6.8%) (Figure 5A and 5B). In both groups, we detected a similar proportion of astrocytes (~ 30%) (Figure 5C) but no oligodendrocytes, as revealed by immunostaining for S100β/TUJ1 and MBP (data not shown), indicating that ZEB1 preferably affected the neuronal subtype specification but not glia fate choice. These results collectively demonstrated that forced expression of ZEB1 specifically promoted hESCs to differentiate into excitatory neuron subtypes.

**ZEB1 reduced the overgrowth of hESCs-derived progenitors upon transplantation into mouse brains**-Since ZEB1 accelerated neural differentiation and promoted functional maturation of hESCs-derived neurons, it thus became a plausible lever for safety control of hESCs in regenerative medicine. We then examined whether overexpression of ZEB1 in hESCs-derived neural progenitor cells could reduce the chance of tumor formation upon transplanting into brains of neonatal pups of NOD/SCID mice. Eight weeks after transplantation of hESC-derived neural stem cells (day 15 of differentiation), mice were perfused for brain sections and immunostaining. Monoclonal antibodies specifically recognized human nuclei were used to identify the transplanted human cells. Graft human cells were found in cerebral cortex or nearby hippocampus and there was no difference for their distribution in the mouse brain between two groups (Figure 6A). In the control group, transplanted cells formed substantial rosette-like overgrowth in the resident brain regions (presence in 55.2% of the brain slices) (Figure 6B and 6C). However, in ZEB1-overexpressed group, this percentage was only 18.3% (Figure 6C, *P* < 0.01), and most of the graft cells dispersed across the brain (Figure 6B and 6C). Consistently, much more of grafts differentiated into neurons, with more TUJ1+ cells in ZEB1 group relative to control group (Figure 6D and 6E, *P* < 0.05). These data indicates that ZEB1 over-expression promoted the differentiation of hESC-derived neural stem cells and potentially improved the safety when used for therapy. In accordance with the *in vitro* results (Figure 3C and 3F), lack of rosettes in the ZEB1 group *in vivo* also reflected the loss of a PAX6+ fate in the ZEB1-overexpressed cells.

**ZEB1 knock out prevented neural precursors differentiate into neurons**-To elucidate the mechanism of ZEB1 function during the differentiation from hESCs into neurons, we collected RNA samples of different stages and examined genome-wide changes during the process by performing a RNA sequence analysis. Both clustering and principle component analysis (PCA) indicated the genome expression pattern occurred significantly changes since 25d differentiation, as the 25d samples’ genome expression pattern were more close to 30d and 35d, far from 15d and 10d samples (Figure S5A and S5B). When we turned to the different expression genes (DEGs) at different stages, we found more DEGs since 25d (Figure 7B). Meanwhile, the genome
expression pattern of 25d WT could be easily clustered with 30d and 35d WT, and the ZEB1 KO cell lines clustered together (Figure 7A). To further validate the ZEB1 functioning stage, we picked some of the neuronal related genes and found there was no significant difference in expression level between WT and KO cell lines at the early differentiation stage (Figure 7C), which were then confirmed by real time PCR (Figure 7D). As 25d seemed to be a coteau, we subjected DEGs at 25d into gene ontology (GO) analysis, and found most of the genes accumulated to neuronal related items (Figure 7E), most of these genes were down regulated after ZEB1 KO, such as FGF8, NKX2.1, SIX3 and DLX1 (Figure 7F). Besides, we also observed some DEGs subjected to cell adhesion item in GO analysis, and found most of them were down regulated in the ZEB1 KO cell line (Figure S5C).

**Discussion**

In human neural differentiation, ZEB transcription factors, particularly ZEB2, as direct targets of miR-200 family members play essential roles in fate specification of neuroectoderm (14). In this study, we found that when ZEB1 was depleted, although these cells could commence an early neural differentiation, they failed to differentiate further into neurons and died within a few days. Based on our data and the previous literature, we speculated that during neural differentiation of hESCs, the two members of ZEB family, ZEB1 and ZEB2, synergistically regulated the progression of cell fate conversion, with ZEB2 functioning primarily at the early stage of neural specification while ZEB1 exclusively affecting the neuronal conversion during late neural differentiation.

Though not necessary for early neural specification, forced expression of ZEB1 during early stage did expedite the progress of neuronal conversion, as evidenced by positive staining of a series of markers of neural differentiation and electrophysiological recordings. Overexpression of ZEB1 in hESCs significantly inhibited the expression of PAX6 but increased the expression of TUJ1, a conventional neuron marker, thus prompted a comprehensive revision of cell fate determination differed from the original opinion which believed PAX6 pioneered a cohort of neural determinants (24). The expression of SOX1, another neural fate determinant which usually expressed following PAX6 during neural differentiation of hESCs (24, 25), however, did not shift to earlier expression, suggesting the temporary alteration of cell fate did not completely perplex the intrinsic program of neural differentiation. Interestingly, ZEB1 was not detected at protein level in the following days once it was overexpressed in hESCs at the initiation of neural differentiation. This might due to an instigation of quality control system within cells in response to the forced expression of ZEB1 and the sudden shuffle of cell fate. In both groups, ZEB1 was expressed at high levels from day 10-15 on, indicating its endogenous expression and intrinsic function. These data collectively supported a notion that cells undergoing cell fate rearrangement were able to gear to the intrinsic neural differentiation program when endogenous ZEB1 start to take over.

ZEB1 may play an important role in regulating the differentiation of ESCs into neurons, particularly in the transition from NPCs to neurons, as ZEB1 KO make no significantly difference in the expression of neuronal related genes at the early stage, but after 25 days of differentiation, we detected the remarkable decrease for genes that
associated with neuronal differentiation (FGF8, NKX2.1, SIX3, DLX1). ZEB1 KO also result in cell adhesion related genes down regulation, indicated low ability of adherence maybe another reason destroyed the differentiation from NPCs to neurons in the ZEB1 KO cell line.

We noticed that ZEB1 overexpression promoted the neuronal maturation for over 2 weeks in advance as compared to the regular neural differentiation. As it is known that astrocytes promote neuronal maturation both in vivo and in vitro (26, 27), we doubt whether ZEB1 accelerates neuronal maturation through an indirect regulation of astrocytes. Whether ZEB1 was overexpressed or not, a similar proportion of S100β+ cells were identified at eight weeks of neural differentiation. Thus, the presence of the astrocytic lineage in the culture unlikely caused significant difference of the spontaneous synaptic currents of the hESCs-derived neurons, and the extra ZEB1 expression during neural differentiation autonomously accelerated the neuronal maturation and promoted the occurrence of synaptic currents.

ZEB1 may also play a role in neuronal fate determination, as forced expression of ZEB1 almost exclusively induced hESCs into vGLUT1 + glutamatergic excitatory neurons in which ZEB1 may directly interacting with key transcription factors involving excitatory fate. Nevertheless, it is also highly possible that the acceleration of neuronal differentiation itself accounts for the augmented generation of excitatory neurons. As noted above, forced expression of ZEB1 granted the neural stem cells for directed neuronal conversion and bypassed certain stages of neural differentiation. This may let neural stem cells miss competence to certain morphogens, such as Sonic Hedgehog (SHH) which is critical for ventral patterning and GABAergic neuron differentiation (22, 28-30). Thus, ZEB1 appears to be an essential player that ensures appropriate cellular constitution of brains for proper function.

Transplantation of ESC-derived cells even at late-stage of differentiation still bear risks of overgrowth in brains (31). Expedited neuronal differentiation is valuable for efficient differentiation into functional cells as well as for transplantation safety. In a set of proof of concept neural cell transplantation, we did observe aggregates of cell overgrowth in brains that received transplantation of regular hESCs-derived neural progenitors, which is in accordance with the previous reports (31). Nevertheless, mice that transplanted with hESCs-derived ZEB1 overexpression neural progenies contained much fewer aggregates, suggesting increased safety from overgrowth with ZEB1 overexpression. Thus, ZEB1 and the relevant signaling pathway could be informative to optimize the procedures of differentiating hESCs into neural cells for therapy.

Overall, we identified an important regulator of neural differentiation that coupled the neural differentiation progression and fate choice. However, the molecular mechanism under it still remains elusive and needs further research.
Materials and methods

Ethics statement, Animals
All mouse studies were approved by the Ethics Committee of the Institute of Zoology (research license number AEI-06-07-2014), Chines Academy of Sciences. NOD/SCID mice were purchased from Vital River Laboratory Animal Technology Corp (Beijing, China) and then raised in a sterile room.

Culture and differentiation of human ESCs
H9ESC line (WiCell Institute, Madison, WI, passages 29-50) was cultured with feeder-free media. Neural differentiation from hESCs was performed as described previously (9). See the Supplemental Experimental Procedures for additional details.

Generation of the ZEB1-knockout hESC line using iCRISPR
To generate loss-of-function mutations of ZEB1 in human ESCs, we firstly constructed the CRISPR/Cas9-inducible (iCas9) hESC line by inserting a CRISPR/Cas9-inducible expression cassette in the AAVS1 genomic locus with the TALEN technology (32, 33). For gRNA transfection and transduction in hESCs, we followed the published protocols (34, 35). In brief, iCas9 hESCs were treated with doxycycline (2mg/ml) for 1 or 2 days before and during transfection. Cells were dissociated into single cells using Accutase and transfected the sgRNA using Lipofectamine RNAiMAX (Life Technologies) following the manufacturer’s instructions. The details were described in the Supplemental Experimental Procedures.

Construction of the ZEB1 overexpression hES cell line
To overexpress ZEB1 in H9ESCs and ZEB1-depleted H9ESCs, the tetracycline inducible tet-on lentiviral-expression vectors were used. For the plasmid construction please see the Supplemental Experimental Procedures.

Preparation of lentiviruses and transduction were conducted as previously described (24). hESCs were treated with ROCK inhibitor (1 μM, Calbiochem, 688001) for 1 day before transduction. Cells were dissociated with 0.5 mM EDTA on the following day (UltraPure™, 15575-020, Gibco). Cell pellets were incubated with concentrated virus at 37°C for 1 hr then replated onto matrigel substrate overnight with E8 medium. Two days later, neomycin (50 μg/ml, Sigma, G8168) and blasticidin (2 μg/ml, Sigma, 15205) were added to the culture medium to select drug-resistant clones. To obtain stable transduced monoclonal lines, hESCs were dissociated into single cells with Accutase and the mNeonGreen-positive cells were sorted by FACs. hESCs were treated with the ROCK inhibitor (1 μM) for all the processes. After 1-10 days of cultivation, mNeonGreen-positive colonies were identified and picked.

Real-time quantitative RT-PCR
Total RNA was isolated manually with TRIzol Reagent (Invitrogen, 15596-026). cDNA was synthesized using 1 µg of RNA using ReverAid First Strand cDNA Synthesis Kit according to the manufacture’s instruction (Fermentas, K1662). Quantitative reverse-transcription PCR was conducted with SYBR Premix Ex Taq (Takara, RR430A) and the primers were listed in Table S5.

Western blot analysis
Cell pellets were lysed in RIPA lysis buffer (Thermo, 89900) supplemented with protease inhibitor (Roche Applied Science), then quantified using a BCA protein assay kit (Thermo, 23225). After electrophoresis, proteins were transferred to nitrocellulose membranes. The following primary
antibodies were used: ZEB1 (Millipore, ABN285), ZEB2 (Santa Cruz, sc48789), N-cadherin (Abcam, ab18203), SNAIL (Cell Signaling Technology, #3879), SLUG (Cell Signaling Technology, #9585), TWIST2 (Abcam, ab50887), Flag (Sigma, F3165), OCT4 (Abcam, ab19857), NANOG (R&D, AF1997), SOX2 (Millipore, AB5603), PAX6 (Developmental Studies Hybridoma Bank), SOX1 (Millipore, MAB3369), NESTIN (Millipore, MAB5326), βIII-tubulin (TUJ1, Covance, PRB-435P), GAPDH (Abcam, ab9485) and β-actin (ProteinTech, 60008-1-Ig), β-tubulin (Cell Signaling Technology, # 2148).

**Immunofluorescence staining**

Primary antibodies used for immunostaining were ZEB1 (Millipore, ABN285), OCT4 (Abcam, ab19857), NANOG(R&D, AF1997), SOX2 (R&D, AF2018), PAX6 (Developmental Studies Hybridoma Bank, DSHB), SOX1 (R&D, AF3369), NESTIN (Millipore, MAB5326), βIII-tubulin (TUJ1, Covance, PRB-435P), Sigma, T8660), MAP2 (Abcam, ab11267), S100β (Millipore, 04-1054), SYNAPSIN1 (Synaptic System, 106011), vGLUT1 (Synaptic System, 135302), GAD65&67 (Millipore, AB1511), GABA (Sigma, A0310), TH (Millipore, MAB318), human-Nuclei (Millipore, MAB1281).

Stained cells were mounted and observed on a LSM 780 META microscope (Zeiss, Berlin, Germany).

**BrdU labeling**

BrdU labeling of proliferating cells was performed according to previously published protocols (36). Differentiated cells were treated with BrdU for 24 hours and analyzed using a Cell Proliferation Assay kit (Calbiochem, K306) according to the manufacturer’s instructions. Results were analyzed with a CytoFLEX Flow cytometry (Beckman Coulter, Suzhou, China).

**Apoptosis assay**

Apoptosis analysis was performed using the Annexin V-PE/7-ADD Apoptosis Detection Kit (BD Pharmingen 559763) according to the manufacturer’s instructions.

**Electrophysiology**

Whole-cell patch-clamp recordings of hESCs-derived neurons that were differentiated 8-10 weeks were performed as described previously (26). The transmitter receptor blockers, tetrodotoxin (TTX; 1 µM), 4-aminopyridine (4-AP; 1 mM), bicuculline (20 µM), and 6, 7, - Dinitroquinoxaline-2, 3, - dione (DNQX, 20 µM) were used in the bath solution for the detection of action potentials and spontaneous excitatory postsynaptic currents. Data was analyzed using pClamp version 9.2 software (Molecular Devices).

**Transplantation of hESCs-derived cells in vivo**

Differentiated cells (1×10⁵) from control or ZEB1 groups were transplanted into the lateral ventricles of the brain of P1-2 pups (ice anesthetized), respectively. For ZEB1 group, 2 mg/mL doxycycline was administered orally in the drinking water to induce the expression of ZEB1. The mice were perfused (anesthetized with 40 mg/kg chloral hydrate) with 0.9% saline followed by 4% paraformaldehyde 10 weeks post-transplantation. Consecutive coronal brain sections (30 µm) were sliced using a Leica SM 2000R Sliding Microtome and fixed in 4% paraformaldehyde overnight followed by dehydration in 0.1 M PBS containing 30% sucrose for 2 days at 4 °C. Immunofluorescence staining for the brain sections were performed as protocols used for cultured cells except for using 1% Triton-100 to permeabilize.

**Statistical analysis**
Statistical analyses were executed with two-tailed Student t tests. Data are presented as the mean ± S.D., and values were considered statistically significant at \( p < 0.05 \) (*), \( p < 0.01 \) (**), and \( p < 0.001 \) (***) as described in the figure legends. Diagrams were created by GraphPad Prism 5 software.

**RNA-seq analysis**

For RNA-seq, cells at indicated time points during the differentiation were harvested, total RNA was extracted with TRIzol reagent (1559618, Ambion, USA). After testing the Quality of RNA by an Agilent 2100, 0.2 \( \mu \)g of each sample was sent to Annoroad Genomics for RNA-seq analysis. Raw data and processed data were uploaded to the NCBI Gene Expression Omnibus database under accession number GSE118990. RPKMs at the current sequencing depth were evaluated by using the jackets of the total comparison reads in RSeQC, and a relative error rate was utilized to measure the accuracy of the evaluated RPKM. Unsupervised heat map clustering analysis was performed by using Cluster 3.0. The clustering results were visualized and exported by TreeView.

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**Author contributions:** Y.J., L.Y., H.W., and B.H. conceived the idea for this project, conducted the experiments and interpreted the data. X.L., M.D., and L. X. assisted with construction of ZEB1 overexpressed cell lines and samples collection. Construction of ZEB1-knockout cell lines was performed with help from W.Z, mice experiments were performed with help from D.D. and D.Z. Y.J, H.W., and B.H wrote the manuscript with inputs from all the other authors.

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Figure legends:

Figure 1. ZEB1 was upregulated in hESCs-derived neural cells. (A) A scheme of neural differentiation of hESCs. Scale bar: 50 μm. (B) Real-time PCR analysis showed the alteration of ZEB1 mRNA levels during neural differentiation of hESCs. The amount of mRNA was normalized to GAPDH levels. Bars represent mean ± SD of three experimental replicates. (C) Western blot showed expression of ZEB1 protein at different stages of neural differentiation from hESCs. β-Actin was a loading control. n=3. (D) Representative images of immunostaining results using indicated antibodies in hESCs-derived neural cells. DAPI was included to show the nuclei (here and after). n=3. Scale bar: 50 μm. hESC: human embryonic stem cell, EB: embryoid bodies, NPC: neural precursors, NSC: neural stem cell, NPC: neural progenitor cell, E8: essential 8 medium, NIM: neural induction medium, NDM: neural differentiation medium.

Figure 2. ZEB1 was necessary for neuronal differentiation. (A) Western blot showed ZEB1 protein levels at day 0, 15 and 35 of neural differentiation from hESCs with or without ZEB1 knockout. β-tubulin was the loading control. (B) Phase contrast images of indicated cells during different days of neural differentiation. n=3. Scale bar: 50 μm. (C) Left, flow cytometry analysis with BrdU antibody using cells after four days attachment of spheres for neuronal differentiation (Day 30). Right, statistical analysis of left results. n=3. (D) Left, cell apoptosis and death analysis with PE Annexin V/7-AAD Kit. Right, Statistical analysis to show the percentage of viable cells (Negative) or cells in early apoptosis (Annexin V+, P < 0.001.), in late apoptosis or already dead (7-AAD+ and Annexin V+ and 7-AAD+, P < 0.001.). n=3. ZEB1-KO: ZEB1-knockout. OE (ZEB1-KO): overexpress ZEB1 in ZEB1-knockout cells.

Figure 3. Sustained overexpression of ZEB1 in hESCs induced neural differentiation. (A) Western blot using indicated antibodies in selected clone #2 of hESCs. GAPDH was the loading control. (B) Representative phase contrast images showing different morphologies of H9ESC colonies with (ZEB1) or without (Control) doxycycline in the medium. n=3. Scale bar: 100 μm. (C) Images showing the phenotypic alterations of cells at various stages of neural differentiation after overexpression of ZEB1. Typical rosettes in control and extended neurite-like structures from colonies with ZEB1 overexpression were observed. Alterations of PAX6 and SOX1 on Day 15, TUJ1 and MAP2 on day 25 were shown as well. Scale bar: 100 μm. (D) and (E) Quantification of TUJ1+ cells and MAP2-positive cells. *** P < 0.001. n=3. (F) Western blot showed the dynamic expression of ZEB1, PAX6, SOX1 and TUJ1 during early neural differentiation in the control and ZEB1 overexpressing cells. GAPDH was a loading control. n=3.

Figure 4. ZEB1 promoted neuronal maturation. (A) Immunostaining for MAP2 along with TUJ1 showed the neuronal differentiation of hESCs with or without overexpression of ZEB1 at eight weeks of neural differentiation. n=3. (B) Representative immunofluorescent images showing the localization of SYNAPSIN-1 (SYN-1) in the cells. The boxed areas at the left were enlarged as shown in the right panel, respectively. n=3. Scale bar: 50 μm (each left panel) and 5 μm (each enlarged right panel). (C-E) Whole cell patch recordings indicated that neurons derived
from hESCs after eight weeks of differentiation were electrophysiological active. n=3. (C) Representative traces of whole-cell currents were elicited by stepwise depolarizations from −80 to 80 mV from a holding potential of −70 mV in neurons of (i) control and (ii) ZEB1 group, respectively. The inset showed sodium currents. (D) Representative traces of action potentials (APs) from a 70 pA current injection were shown for neurons of control (i) and ZEB1 group (ii), respectively. i. APs in control neurons exhibited single or typically diminished amplitude in peak value. ii. Repetitive trains of APs on the similar spikes were detected in ZEB1-overexpressed neurons from neural differentiation. And the fired APs could be blocked completely by TTX (1 µM). (E) Representative voltage-clamp recordings of spontaneous postsynaptic currents (PSCs) from neurons of ZEB1 group after eight weeks of differentiation. And the Glu R currents could be blocked with DNQX (20 µM). Cells were held at −70 mV. (F) After ten weeks of neural differentiation without forced expression of ZEB1, spontaneous PSCs held at −70 mV were detected in the neurons from the control group, and the synaptic activity could be blocked by TTX (1 µM).

**Figure 5. Forced expression of ZEB1 facilitated differentiation of excitatory neurons.** (A) Representative immunofluorescent images of specific subtypes of neurons in control and ZEB1-overexpression group after eight weeks of neural differentiation from hESCs using indicated antibodies. n=3. Scale bar: 50 µm. (B) Quantification and statistical analysis of different types of neurons as representatively indicated in (A) respect to the number of TUJ1+ cells. n=3. Scale bar: 50 µm. (C) Statistical analysis of the S100β-positive cells respected to total cells. For statistical analysis, cells were counted under 20× objective by selecting 10 fields randomly for each group from each experiment. Bars represent mean ± SD of three experimental replicates. *** P < 0.001, ** P < 0.01.

**Figure 6. Overexpression of ZEB1 decreased the risks of tumor formation of transplanted cells derived from hESCs.** (A) Representative images showing the distribution of transplanted cells in vivo based on a human nuclei antibody immunostaining along with Hoechst staining (nuclei) ten weeks after transplantation of hESCs-derived cells. Scale bar: 2 mm (left panel), 50 µm (middle and right panels). Arrows indicated the region of graft cells distribution with human-nuclei staining. (B) Immunostaining images showing the difference of grafted cells from control and ZEB1-overexpressed groups in vivo. Scale bar: 50 µm. (C) Statistical analysis of the percentage of human nuclei-positive rosettes-like structure in the control and ZEB1 overexpression groups as representatively shown in (B). (D) Representative images of grafted cells (human nuclei-positive) expressing the neuronal marker βIII-tubulin (TUJ1) after transplantation. Arrows indicated the typical co-labeled neurons. n=3. Scale bar: 50 µm. (E) Statistical analysis for the percentage of brain slices with TUJ1+ cells in the two groups as representatively shown in (D). For the statistical analysis, 100-200 brain slices from 9 mice were analyzed, and the brain slices without human cells were discarded directly. ** P < 0.01, * P < 0.05. Bars represent mean ± SD of three experimental replicates.
Figure 7. Whole-genome RNA sequence analysis of gene expression profiles. (A) Hierarchical clustering analysis of the whole-genome profiles during the differentiation. RNA were harvested at the indicated time points. (B) Histogram showing the DEGs between WT and ZEB1 KO cell lines at the indicated time points. (C) Hierarchical clustering analysis of specific neuronal related genes at early stage between the 2 cell lines. (D) Quantitative real time PCR analysis confirm the genes expression referred in (C). (E) GO analysis of the significantly differentially expressed genes at 25d of the differentiation. (F) Quantitative real time PCR analysis confirm the genes expression picked from DEGs related to neuronal development.
Fig. 1

A

hESC  EB  NSC  NPC  Neuron

Day 0 4 7 10 15 15-28 35-55

B

ZEB1

mRNA level relative to Day 0

Day of differentiation

0 4 7 10 15 25 35

C

Day 0 15 25 35

ZEB1  200 KD

β-Actin  42 KD

D

Day 0  Day 15  Day 25  Day 35

ZEB1

Merge

ZEB1, post-Ts, anti-Ts, ZEB1, Tuj1, Tuj1, Tuj1, Tuj1, Tuj1

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Fig. 2

A

|          | Day 0 | Day 15 | Day 35 |
|----------|-------|--------|--------|
| Control  | ZEB1-KO | Control | ZEB1-KO | Control | ZEB1-KO |
| ZEB1     | ![Image](image1.png) | 200 KD | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| β-Tubulin | ![Image](image5.png) | 55 KD | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |

B

|          | Day 0 | Day 15 | Day 35 |
|----------|-------|--------|--------|
| Control  | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) |
| ZEB1-KO  | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) |
| OE (ZEB1-KO) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) |

C

MUD sample: All Events

- Control
- ZEB1-KO

Percentage of BrdU positive (%)

|          | Control | ZEB1-KO |
|----------|---------|---------|
| P1       | ![Graph](graph1.png) | ![Graph](graph2.png) |
| P2       | ![Graph](graph3.png) | ![Graph](graph4.png) |

D

Control: All Events

- ![Graph](graph5.png)
- ![Graph](graph6.png)

ZEB1-KO: All Events

- ![Graph](graph7.png)
- ![Graph](graph8.png)
Fig. 5

A

|                  | vGLUT1/Tuj1 | GABA/Tuj1 | TH/Tuj1 | Hoechst/S100β/Tuj1 |
|------------------|-------------|-----------|---------|---------------------|
| Control          | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| ZEB1             | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |

B

Percentage of neuronal subtype %

- vGLUT1: Control 40 ± 5, ZEB1 80 ± 5
- GABA: Control 20 ± 2, ZEB1 60 ± 5
- TH: Control 10 ± 2, ZEB1 10 ± 2

C

Ratio of S100β + %

- Control: 40 ± 2, ZEB1: 20 ± 2
Zinc finger E-box-binding homeobox 1 (ZEB1) is required for neural differentiation of human embryonic stem cells
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