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Transfection of arginine decarboxylase gene increases the neuronal differentiation of neural progenitor cells

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ABSTRACT

Growing evidence suggests that the clinical use of neural progenitor cells (NPCs) is hampered by heterogeneity, poor neuronal yield and low survival rate. Recently, we reported that retrovirus-delivered *human arginine decarboxylase* (*hADC*) genes improve cell survival against oxidative insult in murine NPCs in vitro. This study investigates whether the induced expression of *hADC* gene in mNPCs induces any significant change in the cell fate commitment. The evaluation of induced *hADC* gene function was assessed by knockdown of *hADC* gene using specific siRNA. The *hADC* gene delivery triggered higher expression of N-CAM, cell adhesion molecule and MAP-2, neuronal marker. However, the *hADC* gene knockdown showed downregulation of N-CAM and MAP-2 expression suggesting that *hADC* gene delivery favors cell fate commitment of mNPCs towards neuronal lineage. Neurite outgrowth was significantly longer in the *hADC* infected cells. The neurotrophic signal, BDNF aided in the neuronal commitment, differentiation, and maturation of *hADC*-mNPCs through PI3K and ERK1/2 activation. The induction of neuron-like differentiation is believed to be regulated by the expression of GSK-3β and Wnt/β-catenin signaling pathways. Our findings suggest that *hADC* gene delivery favors cell fate commitment of mNPCs towards neuronal lineage, bring new advances in the field of neurogenesis and cell therapy.

1. Introduction

Neural stem cells (NSCs) are defined by their ability to proliferate, self-renew and retain the potential to differentiate into neuronal and glial lineages [1–4]. They are responsible for early nervous system development and postnatal nervous tissue regeneration and repair [5–7]. Under normal conditions, neurogenesis in the adult mammalian brain is restricted to two discrete germinal centers: the subgranular layer of the hippocampal dentate gyrus and the subventricular zones of the lateral ventricles [3,8,9]. Studies on NSCs provide a unique model system to understand basic mechanisms of neural differentiation, but also lead to improved strategies for neural tissue repair and cell-based replacement therapies in the nervous system and have the potential to ameliorate parkinson's disease, huntington's disease, stroke, and traumatic brain injury leading to partial functional recovery [10,11]. Although studies using stem cells provide hope, it is necessary to understand how to direct and control differentiation of specific target phenotypes required for replacement and repair in each disease, as well as to improve survival and differentiation levels of stem cells after transplantation [12,13]. Recently, several techniques have been adopted to regulate the differentiation, cell cycling or apoptosis of stem cells by over-expressing antioxidant genes such as ADC which can synthesize agmatine [14–16]. Despite several technical and safety problems, scientists have started to translate their basic scientific findings into therapies for untreatable diseases [17].

A complete understanding of neural stem cells requires the identification of molecules that determine the self-renewal and multi-potent characteristics of these cells. Several signaling pathways such as leukemia inhibitory factor (LIF), Wnt protein and bone morphogenetic proteins (BMPs), CAMs and integrins have been demonstrated to play a role in stem cell fate determination of growth and development [18–20]. However, molecular mechanisms underlying regulation of stem cell fate by these extracellular factors remain unknown.

BMPs are members of the Transforming Growth Factor-Beta (TGF-β) family that play various, sometimes distinct roles throughout the development of the nervous system, often in a context and stage-dependent manner [21]. In early embryogenesis, BMPs inhibit neuro-ectoderm formation [22,23] whereas in late embryogenesis, BMPs promote the

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differentiation of both neuronal cells and astroglial cells [24–26]. BMPs execute their functions by binding to and activating BMP receptors I and II [27]. The BMP-Smad1/5/8 pathway is a major pathways controlling neurogenesis [28,29]. BMP receptor I phosphorylates Smad1/5/8 at the C-terminal SXS motif. Smad1/5/8 then associate with Smad4, move into the nucleus, and turn on BMP-target genes to initiate neurogenesis.

BMP-regulated gene expression is controlled without dependence of cell-type through direct Smad binding and in a cell-type-specific manner via interaction with tissue-specific transcription factors. These Smad-dependent transcriptional targets coupled to cross-talk between the BMP and other signaling pathways likely mediate transcriptional programs associated with cell fate choices [29]. However, little is known about the BMP target genes except inhibitor of DNA binding/differentiation-1 (*Id-1*) that controls neurogenesis. BMP target gene, *Id-1*appears to mediate the inhibitory effects of BMPs on neuronal differentiation at least in mouse embryonic stem cells (ESC) [19]. *Id-1* binds to pro-neuronal transcription factors such as Mammalian achaete-schute Homolog 1 (*Mash1*) to inhibit their function. Wnt signaling has long been implicated in neural crest induction [30], and in differentiation of melanocytes from cultured NSCs isolated from mouse neural tube [31]. Neural crest stem cells (NCSCs) lacking the Wnt signaling component,β-catenin, fail to generate sensory neurons [32]. While embryos expressing a constitutively active form of β-catenin in NCSCs develop sensory neurons at the expense of virtually all other neural crest derivatives [33].

Cell adhesion molecules (CAMs) are transmembrane proteins that are responsible for mediating adhesion of cells to other cells and/or the extracellular matrix via their extracellular domains, while their intracellular domain interacts with the cytoskeleton. Thus, CAMs provide a direct link between the extracellular growth/guiding cues and the intracellular scaffold responsible for morphology and growth. Four subclasses of CAMs are expressed in the brain [34].

Neural cell adhesion molecule (N-CAM), a multifunctional regulator for cell adhesion, intracellular signaling, and cytoskeletal dynamics [35–37], is expressed on the surface of most neural cells and plays a major role in the development of the nervous system. Stimulation of N-CAM results in the phosphorylation of extracellular signal regulated kinase I and II (ERKI/II). Integrins are αβ heterodimeric proteins with roles in adhesion to the extracellular matrix (ECM) and in cell–cell interactions [38]. They play important roles in mesoderm development, epithelial morphogenesis, neural tube closure, anchorage to ECM basal lamina and central nervous system development [39]. An important property of integrins is their capacity to cross-talk with growth factor receptors [40].

Agmatine synthesized by ADC is stored in neuronal cells, may have physiological functions as a neuromodulator [41] and exhibits protective effects both in vivo and in vitro [42–45], suggesting that availability of agmatine helps protection. A recent study report suggests that agmatine treatment to subventricular zone neural stem cells increased neurogenesis and suppressed gliogenesis through the regulation of BMPs and Smad1/5/8 expression [46], driving research on exploring the protective effects of the ADC gene [14,47,48].

This study for the first time, investigates the effect of *hADC* gene delivery on the stemness and cell fate commitment in *hADC*-*m*NPCs. The events were investigated by checking the expression levels of microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP) expressions for cell fate commitment. The morphological change induced by *hADC* gene delivery was investigated by checking the expression levels of adhesion molecules, N-CAM and integrin. The *hADC*-*m*NPCs showed modulation of brain-derived neurotrophic factor (BDNF), phosphoinositide 3-kinase (PI3K) and ERK1/2 expressions. Furthermore, the role of Wnt/β-catenin signaling pathway was explored by checking glycogen synthase kinase3β (GSK-3β), BMP-7, BMP-4, Smad1/5/8 and Id-1 protein expressions for the neuronal commitment of *hADC*-*m*NPCs. The effects of the induced *hADC* gene functions were evaluated by knocking down the ADC gene using specific siRNA.

2. Materials and methods

2.1. Animals

Pregnant female ICR mice were used to obtain stage E14 embryos (KOATECH, South Korea). The animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit #: 10–114). All mice were maintained in the pathogen-free facility of the YLARC.

2.2. Mice neural progenitor cells (mNPCs)

mNPCs were cultured as previously described [14]. See Supplemental experimental procedures.

2.3. Infection of retrovirus containing human arginine decarboxylase genes (hADC) into mNPCs

The ***h***ADC-pCMV SPORT6 plasmid (6.4 Kb, purchased from NIH) containing the cDNA of ADC (1383 bp fragment of ***h***ADC cDNA, GenBank accession number AY325129) was amplified using hisADC specific primers (Forward primer: 5′-GAA TTC gtc gcc acc atg cac cat cac cat cac atg gct ggc tac c-3′, Reverse primer: 5′-CTC GAG tga tgc tcg ctg ggg t-3′) for 35 cycles, consisting of an initial denaturation for 5 min at 95 °C followed by 1 min at 95 °C, 1 min at 50 °C and 1 min 72 °C. Following this step, a final extension was carried out at 72 °C for 10 min. The polymerase chain reaction (PCR) products were ligated to a recombinant retroviral expression vector pLXSN (K1060, Clontech). The retrovirus containing the *hADC* genes was infected into mNPCs as described earlier [48]. The clones with the highest titer were selected and stored at −70 °C until use.

2.4. Experimental design

1-week cultured mNPCs were infected with retrovirus containing empty vector (*v*LXSN) or human *arginine decarboxylase* genes (*vhADC*). After 24 h of incubation with *v*LXSN or *vhADC*, the medium was replaced with neural stem cell basal medium containing the differentiation supplement and mNPCs were maintained for another week. mNPCs infected with *vhADC* (*hADC*-mNPCs), mNPCs infected with *v*LXSN (LXSN-mNPCs) and retrovirus-non-infected control mNPCs (control mNPCs) were used for the experiments.

2.5. siRNAassay

For siRNA inhibition studies, *hADC*-siRNAmNPCs was designed as *hADC*-mNPCs transfected with validated *hADC*-siRNA (siRNA No. 1002652, Bioneer) and *hADC*-siRNA control mNPCs as *hADC*-mNPCs transfected with negative control siRNA (SN-1002, Bioneer) using the Lipofectamine protocol (Invitrogen) at 11 DIV. After siRNA transfection for 72 h, the *hADC*-mNPCs were collected at 14 DIV for immunocytochemistry and protein analysis.

2.6. High performance liquid chromatography (HPLC) analysis

The agmatine concentration in control mNPCs, LXSN-mNPCs, *hADC*-mNPCs, *hADC*-siRNA control mNPCs, and *hADC*-siRNA mNPCs were measured by HPLC as previously described [48]. See Supplemental experimental procedures.

2.7. Immunocytochemistry

The mNPCs in all the experimental groups (control mNPCs, LXSN-mNPCs, *hADC*-mNPCs,*hADC*-siRNA control mNPCs, and *hADC*-siRNA

mNPCs) were fixed with 4% paraformaldehyde for 2 h at room temperature followed by washing with PBS. They were blocked using blocking solution for 1 h at room temperature. They were reacted with appropriate primary antibodies overnight at 4 °C. They were incubated with appropriate secondary antibodies conjugated with Fluorescein isothiocyanate (**FITC, 1**:200, Chemicon) or Rhodamine (1: 200, Chemicon) for 2 h at room temperature. Finally, the nuclei of mNPCs were stained with 4′,6-diamidino-2-phenylindole (DAPI, Vector). Images were obtained by LSM 700 Confocal microscopy (Zeiss, Germany).

2.8. Western blot analysis

Equal amounts of proteins (30 μg) were extracted from control mNPCs, LXSN-mNPCs, *hADC*-mNPCs, *hADC*-siRNAcontrol mNPCs, and *hADC*-siRNA mNPCs. Samples were subjected to electrophoresis on 10–12% SDS-polyacrylamide gels. Separated proteins were then electro-transferred onto Immobilion-NC membrane (Millipore). The membranes were blocked for 1 h at room temperature with 5% skimmed milk in tris-buffered saline (TBS) containing 0.1% Tween-20. The membranes were incubated overnight with appropriate primary antibodies. After washing three times with TBS with Tween-20 (TBS-T), blots were incubated with peroxidase-conjugated *anti*-mouse IgG or anti-rabbit IgG secondary antibodies for 1 h at room temperature. Finally, the blots were rinsed and proteins were visualized using an enhanced chemiluminescent protein detection kit (Thermo) according to the manufacturer's instructions. The densitometric measurements of all the detected proteins were performed by Scion Image Analysis Software (version 3.5). All western blots were represented as a mean of at least 3 independent experiments.

2.9. Neurite outgrowth measurement using cell IQ

The mNPCs from all the experimental groups were cultured in Neurobasal medium (Gibco) with 2% B-27 supplement and 5 mM glutamine at 11DIV. To evaluate newly generated neurite projections, three areas were randomly selected in single culture well and were visualized using a digital camera under a 20× objective. Morphological changes of neurospheres were continuously monitored and the images were captured every 30 min for a period of 56 h with Cell-IQ (CM Technologies Ltd., Finland). The data were collected from 4 to 8 different culture wells in each experimental group. The extension of neurite projections from the neurospheres was measured with Cell-IQ Imagen Standalone (ver. 2.9.3) and analyzed using Cell-IQ Analyzer 4 Pro-Write software (ver. AN4.2.1).

2.10. Statistical analysis

All data were reported as mean ± SD except neurite length. Statistical analysis of the data was performed with one-way ANOVA with Tukey HSD test using SPSS Statistics (version 12.0, IBM). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Overexpression of hADC in mNPCs synthesized agmatine endogenously

The *hADC* gene infection into mNPCs was carried out using retroviral vector system. The expression of ADC protein was confirmed in control mNPCs, LXSN-mNPCs (empty vector transfected), *hADC*-mNPCs, *hADC*-siRNAcontrol mNPCs (*hADC*-mNPCs with control siRNA), and *hADC*-siRNA mNPCs (*hADC*-mNPCs with *hADC*-siRNA) by immunocytochemistry and western blotting (Figs. 1A&B). Immunocytochemistry showed higher expression of ADC positive cells in *hADC* infected mNPCs compared with *hADC* non-infected mNPCs (Fig. 1A). In parallel, western blotting also showed higher expression of ADC protein in *hADC*-mNPCs, while less expression of ADC protein was seen in control and LXSN-mNPCs. However, the *hADC*-siRNA treatment to *hADC*-mNPCs blocked the ADC expression (Fig. 1B).

HPLC analysis showed that endogenous agmatine levels were significantly higher in *hADC*-mNPCs compared with control mNPCs and LXSN-mNPCs. However the agmatine concentration was significantly decreased in the *hADC*-siRNA mNPCs compared to *hADC*-mNPCs and *hADC*-siRNA control mNPCs (Fig. 1C). The results suggested that overexpression of *hADC* genes in mNPCs resulted in higher level of agmatine, whereas, agmatine levels were significantly decreased with *hADC*-siRNA treatment.

3.2. The hADC gene delivery to mNPCs induced the expression of cell adhesion molecules, integrin and N-CAM

Integrins and N-CAMs are major groups of cell-surface receptors for both ECM and cell-surface molecules. They are the primary mediators of neural cell behavior on ECM components and control various cell functions including survival [49], migration [50,51], neurite outgrowth [52], and myelination [53]. In the present study, *hADC*-infected mNPCs showed attachment to the culture plate and the cells apparently migrated outward from the periphery of the attached neurospheres. To explore this phenomenon, the expression pattern of cell adhesion molecules, integrin and N-CAM were examined in control mNPCs, LXSN-mNPCs, *hADC*-mNPCs, *hADC*-siRNA control mNPCs, and *hADC*-siRNA mNPCs. Interestingly, immunofluorescence staining and western blotting showed higher expression of N-CAM in *hADC*-mNPCs compared with control mNPCs and LXSN-mNPCs (Fig. 2), whereas the expressions of N-CAM and integrin in *hADC*-mNPCs were decreased through *hADC*-siRNA treatment (Figs. 2B–D).

These results suggest that *hADC* gene delivery to mNPCs, induces morphological differentiation of neurospheres through higher expression of cell adhesion molecules, integrin and N-CAM in *hADC*-mNPCs.

3.3. The hADC genes infection to mNPCs favors commitment of neural progenitor cells to neuron earlier than glia in hADC-mNPCs

The cell fate commitment of neural progenitor cells was determined by checking the expression patterns of neuronal specific marker, MAP-2 and glial specific marker, GFAP by immunofluorescence and western blotting (Fig. 3). To define the effect of ADC gene transfection on the differentiation of neural progenitor cells definitely, we didn't use coated plate. Immunofluorescence staining showed that the number of MAP-2 positive cells was higher in the *hADC*-mNPCs compared with control and mock vector infected mNPCs, while the GFAP expressions were not clear in the all experimental groups. The *hADC*-siRNA treatment to the *hADC*-mNPCs showed almost similar pattern to that of control and LXSN-mNPCs (Fig. 3A). To substantiate these findings, western blotting was performed with MAP-2 and GFAP antibodies in all the experimental groups. Densitometry analysis showed that MAP-2 expression was significantly increased, whereas the GFAP expression was significantly decreased in *hADC*-mNPCs and *hADC*-siRNA control mNPCs compared with control mNPCs, LXSN-mNPCs and *hADC*-siRNA mNPCs (Figs. 3B-D).

3.4. The hADC gene delivery to mNPCs accelerated neurite outgrowth in neuronal culture condition

The length of neurite was measured to find out if *hADC* gene transfection to mNPCs affected neurite outgrowth of mNPCs under neuronal culture condition. Many cells migrated out from neurosphere in *hADC*-mNPCs compared with the other groups (Fig. 4A). The neurite length per cell was increased in *hADC*-mNPCs (32.44 ± 6.34 μm/cell at 56 h under neuronal culture condition) compared to control mNPCs (4.97 ± 0.29 μm/cell), LXSN-mNPCs (15.73 ± 5.32 μm/cell) and *hADC*-siRNA mNPCs (10.80 ± 1.61 μm/cell, Fig. 4B).

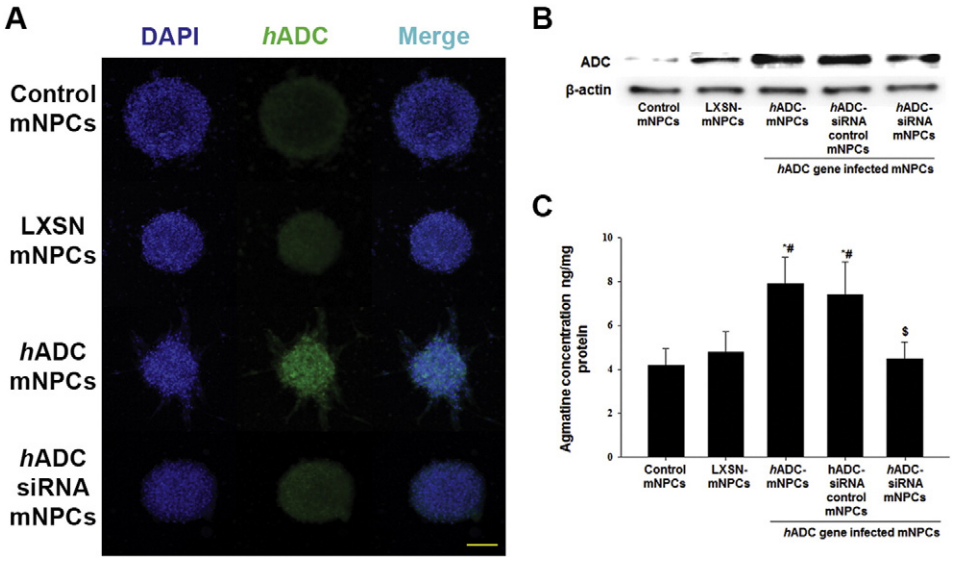

Fig. 1. Confirmation of hADC protein expression and increased endogenous concentration of agmatine in mNPCs. The expression of hADC protein was confirmed in the control mNPCs, LXSN, *hADC*, and *hADC*-siRNA by immunocytochemistry (A) and immunoblotting (B). The endogenous concentration of agmatine measured by HPLC was increased in *hADC*-mNPCs and *hADC*-siRNA control mNPCs (C). Scale bar is 100 μm. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; $, $P < 0.05$ vs *hADC*-mNPCs.

3.5. The retrovirus containing hADC gene delivery to mNPCs induced the brain derived neurotrophic factor (BDNF) expression and initiated differentiation of neurospheres through PI3K and ERK1/2 activation

BDNF is reported to influence hippocampal neurogenesis [54,55], neuronal survival [56,57] and neuroplasticity [58] suggesting that BDNF promotes neurogenesis. We, therefore, investigated the effect of *hADC* gene delivery on the expression of BDNF and the downstream proteins, PI3K and ERK1/2 expressions. Western blotting displayed that in *hADC*-mNPCs and *hADC*-siRNA control mNPCs, the expression levels of BDNF, PI3K and ERK1/2 were significantly increased compared with Control mNPCs and LXSN-mNPCs (Fig. 5). However, ADC knockdown significantly decreased the expression levels of BDNF, PI3K and ERK1/2 compared with *hADC*-mNPCs and *hADC*-siRNA control mNPCs.

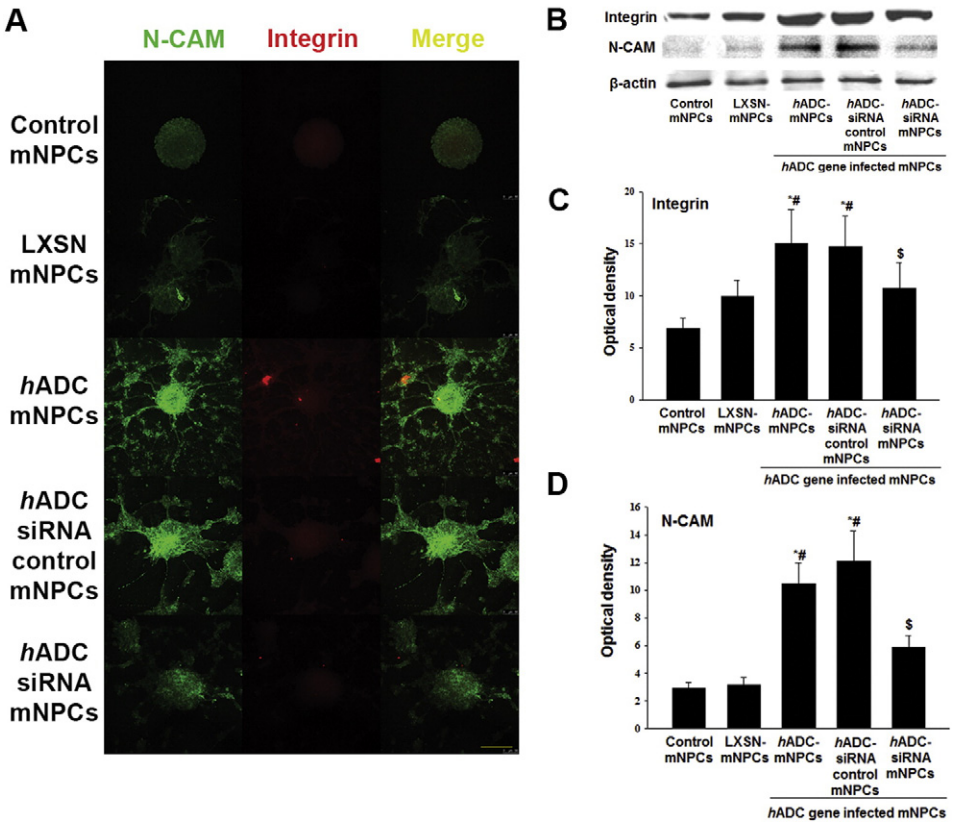

Fig. 2. The retroviral infection of *hADC* genes to mNPCs stimulated attachment of neurospheres by secreting cell adhesion molecules, integrin and N-CAM. Immunocytochemical staining results showed strong expressions of N-CAM in *hADC*-mNPCs and *hADC*-siRNA control mNPCs compared to the other experimental groups (A). The immunoblotting and quantitation of Integrin and N-CAM exhibited significant increase in *hADC*-mNPCs and *hADC*-siRNA control mNPCs compared to the others (B-D). Scale bar is 100 μm. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; $, $P < 0.05$ vs *hADC*-mNPCs.

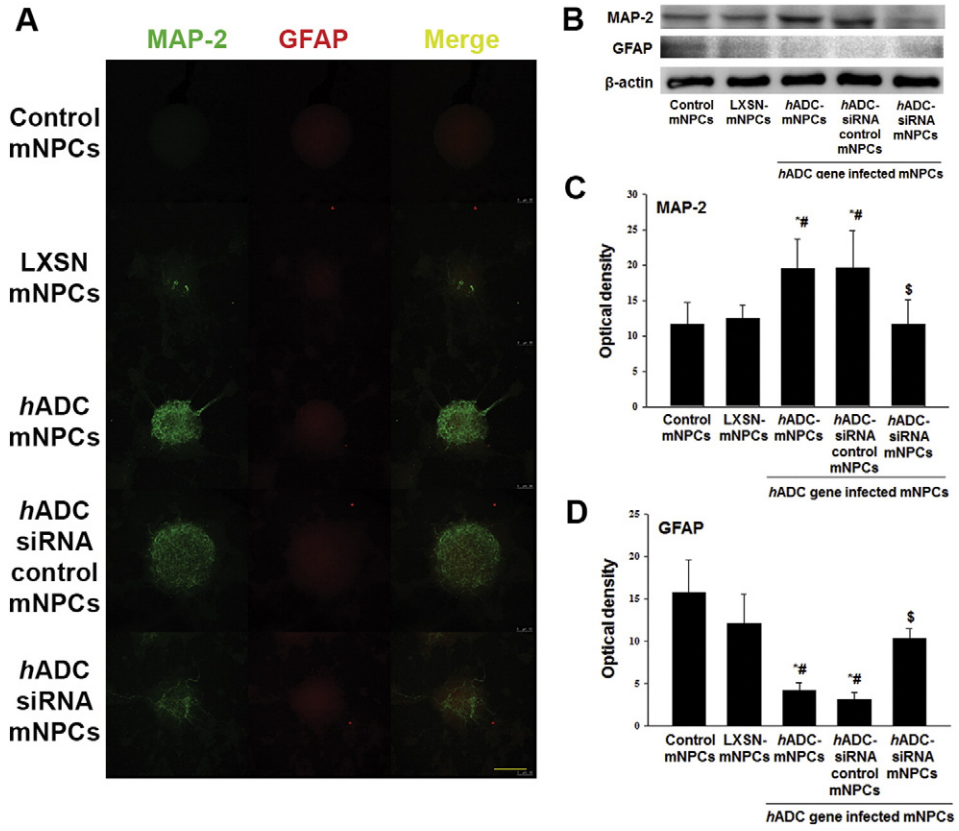

Fig. 3. The cell fate of mNPCs was induced towards neurons earlier than glia by the infection of *hADC* genes. The cell fate commitment of mNPCs due to the delivery of *hADC* genes was determined by immunocytochemistry (A) and immunoblotting (B) of MAP-2 as neuronal specific marker and GFAP as astrocyte specific marker. MAP-2 was expressed intensively in *hADC*-mNPCs and *hADC*-siRNA control mNPCs. The reduced expressions of GFAP were in *hADC*-mNPCs and *hADC*-siRNA control mNPCs, and compared to the others in immunoblotting (C-D). Scale bar is 100 μm. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; $, $P < 0.05$ vs *hADC*-mNPCs.

3.6. The hADC gene delivery to mNPCs triggered Wnt/β-catenin signaling through the recruitment of BMP-4&7, Smad1/5/8, and Id-1 protein expression for the neuronal commitment in hADC-mNPCs.

Several signaling pathways including Wnt/β-catenin signaling have been demonstrated to play key role in stem cell determination and the downstream proteins, BMPs interplay in regulating the cell fate choices in mouse embryonic stem cells [59]. Western blotting showed that the expressions of β-catenin and GSK-3β (Figs. 6A, C and D) as well as BMP-7 and downstream signal, Smad1/5/8 (Figs. 6B, E and G) were increased in *hADC*-mNPCs and *hADC*-siRNA control mNPCs. BMP-4 is known to be related to glial differentiation and Id-1 was reported to mediate the inhibitory effects on neuronal differentiation. Western blotting showed that the expressions of BMP-4 and Id-1 were significantly lower in *hADC*-mNPCs and *hADC*-siRNA control mNPCs compared with other experimental groups (Figs. 6B, F and H). The specificity of the *hADC* gene function was evaluated by knocking down the *hADC* gene with *hADC*-siRNA treatment. The expressions of Id-1 and BMP-4 in *hADC*-siRNA mNPCs were similar to that in control mNPCs and LXSN-mNPCs (Figs. 6F&H).

The overall results demonstrated that *hADC* gene delivery to mNPCs directed cell fate commitment to early neurogenesis at the expense of

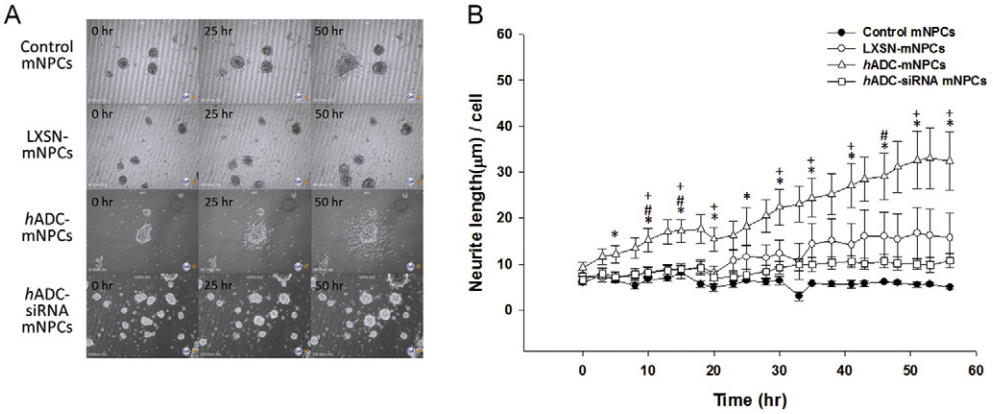

Fig. 4. The transfection of *hADC* genes accelerated neurite elongation in mNPCs. Many neurites of *hADC*-mNPCs were shown to be elongated in serial images taken for a period of 56 h (only three time points represented, A). The quantitative analysis exhibited that neurite length was significantly longer in *hADC*-mNPCs than the others (B, $n = 6–8$ culture wells (4 culture wells for Control mNPCs)). Data were expressed as mean ± SE. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; +, $P < 0.05$ vs *hADC*-siRNA mNPCs.

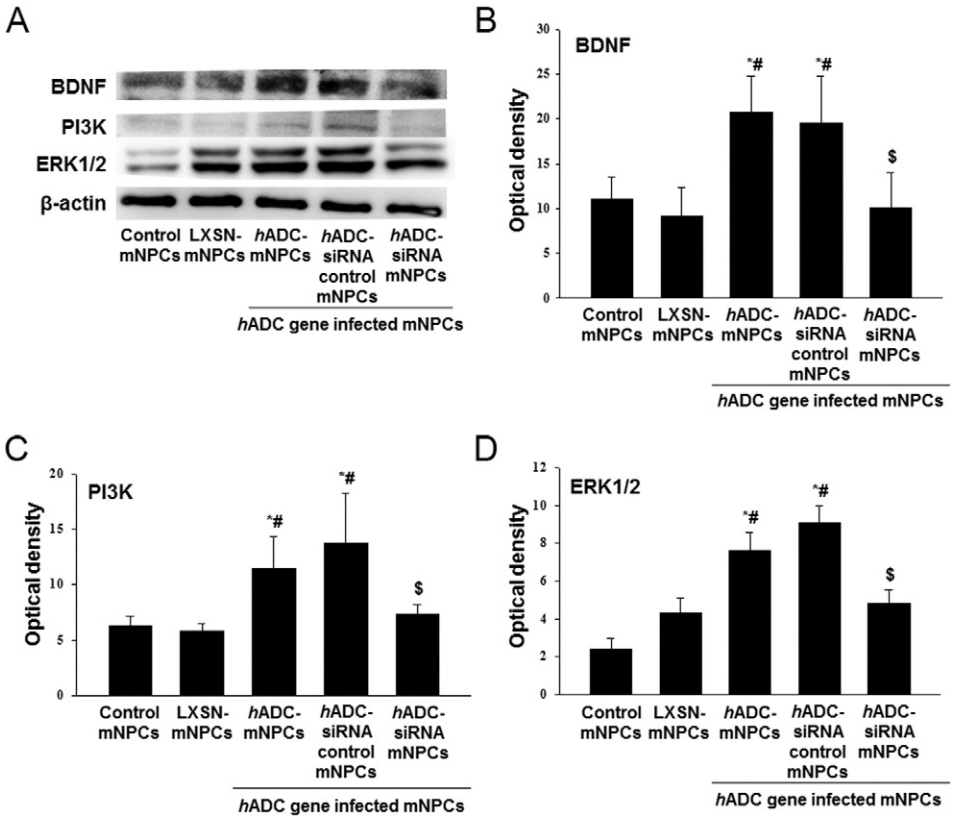

Fig. 5. The transfection of *hADC* genes to mNPCs triggered the secretion of BDNF and promoted PI3K and ERK1/2 activation. Immunoblotting of BDNF, PI3K and ERK1/2, and the quantitation of protein expressions displayed that the delivery of *hADC* genes into mNPCs induced the expression of neurotrophic factor, BDNF (A&B) and activated the downstream proteins, PI3K (A&C) and ERK1/2 (A&D) in *hADC*-mNPCs and *hADC*-siRNA control mNPCs. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; $, $P < 0.05$ vs *hADC*-mNPCs.

astrogenesis through the involvement of Wnt/β-catenin signaling pathway.

4. Discussion

The neurosphere culture system is widely used to propagate multipotent CNS precursors, but their differentiation into neurons, astrocytes and oligodendrocytes is rather poor [60,61]. Further, neural stem/precursor cells (NSPCs) have limited proliferative and neurogenic potential, which may restrict their therapeutic application [62]. Investigations to identify ways for directing stem cells towards a specific phenotype have focused on providing signals to the cells to recapitulate normal development. Mouse NSCs were directed towards dopaminergic neuronal differentiation by over-expressing *Ngn2* and *Nurr1* genes [63,64]. Following grafting, these cells areparticularly vulnerable and have poor survival. Hence, studies are required to enhance neurogenic potential and cell survival of the stem cells to combat the pathological conditions that prevail in the microenvironment of CNS diseases. Our earlier investigations suggested that *hADC*infection to mNPCs and NIH3T3 cell line conferred protection against oxidative insult [14,48].

This present study intended to investigate whether *hADC* gene delivery to mNPCs could induce modulation in stem cell fate and characteristics.

The induced expression of *hADC* was confirmed by immunostaining and western blotting (Fig. 1A&B). The over-expression of *hADC* genes significantly increased endogenous agmatine (Fig. 1C). Adherence of cells is important for differentiation as well as for proliferation, migration, neurite outgrowth, and synaptogenesis [65]. The cell adhesion molecules, integrin and N-CAM, play important roles in proliferation, migration, and invasion in various cell types and also control cell-cell contact and cell-matrix adhesion during angiogenesis and neurite outgrowth [66]. Integrins play a role in NSC maintenance [67] and ensure the neuron–glial recognition that is essential for neuronal migration during cortical development [50]. N-CAM induced intracellular signaling pathways to induce the extension of neurites in neuronal cell lines or primary neurons [68]. From this approach, a large amount of data has emerged concerning N-CAM signaling in vitro. Another end point of N-CAM-mediated signaling in neurons is neuroprotection [69]. During our culture conditions, the *hADC* gene delivery stimulated earlier differentiation of neurospheres compared to control mNPCs and LXSN-mNPCs. Since cell adhesion molecules, N-CAM and integrin mediate the attachment of neurospheres and differentiation, the expressions of N-CAM and integrin were checked in all experimental groupsusing immunocytochemistry and western blotting (Fig. 2). Immunocytochemistry (Fig. 2A) as well asquantitative analysis of western blotting showed significant increase of N-CAM and integrin expression in *hADC*-mNPCs, suggesting that *hADC* gene transfection to mNPCs stimulates differentiation of NPCs in neurospheres through secretion of integrin and N-CAM. However, *hADC* knockdown group showed decreased expression of the cell adhesion molecules compared with*hADC*-mNPCs (Figs. 2B-D).

Generally to differentiate NPCs, coated plate should be used, but we used uncoated plate to clealy observe the effect of ADC gene infection on the differentiation of neural progenitor cells in this study. Interestingly, *hADC* gene delivery not only mediated early differentiation of neurospheres but also induced cell fate commitment of the neurospheres toeards neuronal lineage in vitro.

The cell fate of neurospheres in all the experimental groups was determined by choosing the neuron specific marker, MAP-2 and the astrocyte specific marker, GFAP in all the experimental groups using immunofluorescence and western blotting (Fig. 3). Immunofluorescence and western blotting studies showed significant increase in the MAP-2 expression in *hADC*-mNPCs compared with control mNPCs and LXSN-mNPCs (Figs. 3B–D). The *hADC* gene functions were attenuated

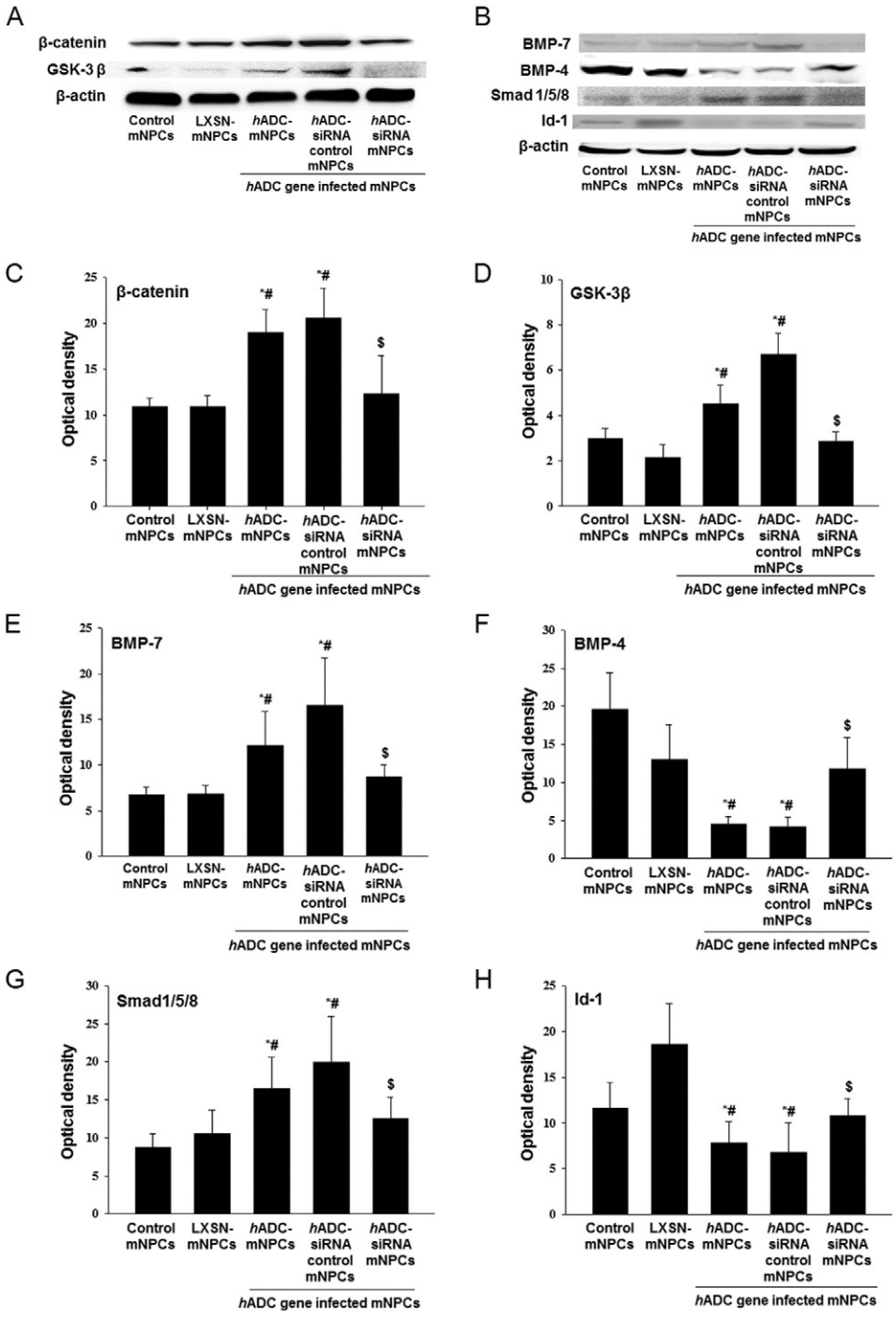

Fig. 6. The viral infection of *hADC* genes into mNPCs changed the protein expressions of β-catenin, GSK-3ß, BMPs, Smad1/5/8 and Id-1 related to neural differentiation. The protein levels of β-catenin (A&C) and GSK-3ß (A&D) were significantly increased in *hADC*-mNPCs and *hADC*-siRNA control. The BMP-7 (B&E) and Smad1/5/8 (B&G) were shown to be increased in *hADC*-mNPCs and *hADC*-siRNA control mNPCs, but the BMP-4 (B&F) and Id-1 (B&H) were presented to be decreased in *hADC*-mNPCs and *hADC*-siRNA control mNPCs. *, $P < 0.05$ vs control mNPCs; #, $P < 0.05$ vs LXSN-mNPCs; $, $P < 0.05$ vs *hADC*-mNPCs.

in the *hADC* knockdown group, *hADC*-siRNA mNPCs. These results suggest that *hADC* gene infection to mNPCs, directes the neurospheres cell fate commitment towards neurons earlier than astrocytes in *hADC*-mNPCs.

The neurotrophic factor BDNF is essential for proliferation, differentiation and survival of neurons. Following its binding to the TRK-B receptor, several canonical pathways such as the ERK/MAP kinase PI3K/AKT, CREB and JAK/STAT3 are activated [70]. BDNF also promotes synaptic maturation and modulates synaptic plasticity, including long-term potentiation (LTP) [71]. In cultured rat hippocampal neurons, it was demonstrated that depending on the delivery of BDNF, distinct molecular pathways were induced and influenced cell survival, morphology, and functions [72]. The PI3K/AKT signaling pathway, downstream of neurotrophins, is essential for neuronal survival as well as for the development of dendrites. Ras signals via both PI3K/AKT and MAPK pathways co-operate to promote elongation and dendritic filopodia like protrusion in dissociated postnatal hippocampal CA1/CA3 neuronal cultures [73]. Our microarray data (results not shown) revealed higher expression of *BDNF* gene in the *hADC*- infected NPCs. The data was validated by western blotting. Quantitative analysis of western blotting suggests

that *hADC*-mNPCs showed significant increase in the expression of BDNF and the downstream signaling proteins, PI3K and ERK1/2 compared to control mNPCs and LXSN-mNPCs (Fig. 5). However, *hADC*gene knockdown group showed decreased expression of BDNF and its downstream proteins compared with *hADC*-mNPCs. The data suggest that induced ADC gene delivery promotes the secretion of BDNF which might contribute for the early neurogenesis in *hADC*-mNPCs.

The β-catenindependent Wnt signaling pathway is also important in the modulation of cell proliferation, cell survival, and embryo development [74–76] and is also identified as a regulator of stem cell fate determination [77,78]. It was found that phosphorylation levels of c-Myc and β-catenin (targets of GSK-3) were increased at later stages in development (when progenitor proliferation subsides and neuronal differentiation predominates) [79]. As c-Myc and β-catenin are pro-proliferation factors, and their phosphorylation by GSK-3β leads to their degradation, it is suggested that activation of GSK-3β promotes neuronal differentiation [79]. This idea is supported by a study in which *Disc1* knockdown in neural progenitors caused premature neuronal differentiation at the expense of the size of the progenitor pool [80]. When GSK-3β signaling was examined, the levels of phosphorylated β-catenin and tyrosine-phosphorylated GSK-3βwere increased [80]. The activation of the Wnt/β-catenin signaling could modulate the bone morphogenetic proteins which could regulate stem cell differentiation [81].

BMPsare secreted proteins that play key roles inneural tube patterning, mesoderm differentiation [82], regulation of proliferation and differentiation of many different cell types [83] in addition to formation and development of the nervous system [84] and regulation of stem cell fate commitment [29]. BMP-4, in particular, is known to participate in the astrocytic differentiation of NSPCs [85,86]. The expression of BMP-7 is known to increase after CNS injury in motor neurons and might regulate differentiation of glial cells from neural progenitors [87,88]. The expression of BMP-7 was also reported to induce neuronal differentiation from progenitor cells [89].

There is evidence that BMPs also modulate their sub specificationof peripherin-expressing neurons. In primary neural crest stem cells, BMPs induce cholinergic neurons [90]. BMPs also play key roles in regulating fate choices during stem cell differentiation. For example, BMPs direct mesenchymal stem cells to chondrogenic and osteogenic cell lineages [91]. BMPs have also been shown to regulate fate choices in neural crest stem cells [31]. Moreover, the discovery of a key role for BMP in maintaining embryonic stem cell self-renewal [19] is consistent with the notion that this family of secreted factors has broad roles in regulating stem cell biology. Of interest, an important interplay also seems to exist between Wnt signaling and other signal pathways, such as those activated by BMPs during neural development [92–94]. BMP functions through receptor-mediated intracellular signaling and subsequently influences target gene transcription by Smads [95]. Previous report suggested that the BMP signaling pathway networks with Wnt signaling [96]. In corroboration of the earlier investigations suggesting the wnt role in inducing neurogenesis, our western blottingdisplayed that *hADC*-mNPCs showed increased pattern of neuronal differentiation than astrogenesis through Wnt/β-catenin signaling by the recruitment of BMP-4, BMP-7, ERK 1/2, Smad1/5/8 and Id-1 protein expression (Figs. 5&6). However, neurogenesis ceased in the*hADC*-siRNA mNPCs (Fig. 3). In addition, measurement of neurite length in the *hADC* gene infected and non-infected groups for 56 h using Cell-IQ machine suggested that the relative length of the neurites in the *hADC*-mNPCs was longer than the *hADC*-non infected mNPCs (Fig. 4). However, *hADC* knockdown group showed significant decrease in the neurite length (Fig. 4B). These results suggest that *hADC* gene delivery to mNPCs favors cell commitment towards neurons earlier than astrocytes and the neuronal differentaition in *hADC*-mNPCs could be mediated through BDNF and β-catenin signaling.

Overall, our present study demonstrated that *hADC* gene delivery stimulated the attachment of neurospheres via the cell adhesion molecules. *hADC* gene infection induced neuronal differentiation earlier than astrogenesis via GSK-3β andWnt/β-catenin signaling pathways. In NSCs, the function of Smad1 elevation seems to promote neuronal differentiation [97]. The link to BMP-Smad signaling and Id-1 expression may also underlie other aspects of development modulated by *hADC* gene transfer.

Author disclosure statement

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.08.009.

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