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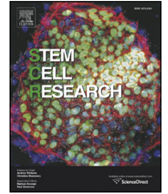

Transfection of arginine decarboxylase gene increases the neuronal differentiation of neural progenitor cells

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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.

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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

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