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Secreted type of amyloid precursor protein induces glial differentiation by stimulating the BMP/Smad signaling pathway

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ABSTRACT

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases leading to dementia. Although cytotoxicity of amyloid β peptides has been intensively studied within pathophysiology of AD, the physiological function of amyloid precursor protein (APP) still remains unclarified. We have shown previously that secreted APPα (sAPPα) is associated with glial differentiation of neural stem cells. To elucidate specific mechanisms underlying sAPPα-induced gliogenesis, we examined the potential involvement of bone morphogenic proteins (BMPs). BMPs are one of the factors involved in glial differentiation of neural progenitor cells. When expressions of BMP-2, -4, and -7 were examined, upregulation of BMP-4 expression was solely observed as a result of treatment with sAPPα in a time and dose-dependent manner. Furthermore, the treatment of sAPPα promoted phosphorylation of Smad1/5/8, a downstream signaling mediator of BMP receptors. Interestingly, N-terminal domain of APP (1–205) was sufficient to elevate BMP4 expression, resulting in an increase of glial fibrillary acidic protein (GFAP) expression and phosphorylation of Smad1/5/8. However, the application of APP neutralizing antibody and anti-BMP4 antibody significantly suppressed expression of BMP-4 as well as phosphorylation of Smad1/5/8. Thus, our results indicate that sAPPα-induced gliogenesis is in part mediated by the BMP-4 signaling pathway. We also observed upregulation of BMP-4 and phosphorylation of Smad1/5/8 in APP transgenic mice. It is imperative to unravel the mechanisms underlying the role of BMP-4 during APPα-induced glial differentiation in hope of providing novel prevention or treatment for AD.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease increasingly appreciated as one of the most devastating diseases affecting the elderly [26]. The major pathological phenomena are synaptic dysfunction and neuronal loss caused by Aβ deposition, resulting in changes of memory and behavior. Amyloid precursor protein (APP), a hallmark of AD, has various roles in the pathology of an AD patients' brain [10,26–28]. Although some evidence supports that APP and its proteolytic products enhance neurogenesis [5,20], other studies have shown that neurogenesis is impaired in both transgenic AD mice and AD patients [30,31], suggesting that Aβ peptides cause marked dysregulation of adult neurogenesis and gliosis in AD.

In our previous studies, treatment with secreted APP α (sAPPα) or overexpression of APP induced massive glial differentiation of human neural stem cells (HNSCs) and NT2/D1 teratocarcinoma cells *in vitro* [14,15]. Moreover, when HNSCs were transplanted into APP23 transgenic mice, most of them were differentiated into glia rather than neurons. Furthermore, we also have demonstrated that sAPPα induced glial differentiation was mediated via both notch and IL-6/gp130 signaling pathways [16,17]. However, further studies are necessary since it is not clear whether these signaling pathways are enough to instruct cell fate of adult neural stem cells for glial differentiation or if other factors are also required. Therefore, we investigated novel factors that mediate sAPPα induced glial differentiation of neural stem cells in the context of AD. And down's syndrome (DS), where APP is over-expressed by virtue of its presence on chromosome 21.

One of the potential inducers of glial differentiation are bone morphogenetic protein (BMP) family cytokines [24]. The BMPs belong to a subset of the transforming growth factor-β (TGF-β) superfamily, which includes multifunctional peptides that control proliferation, differentiation and other functions in many cell types

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[22]. Like leukemia inhibitory factor (LIF), BMPs are known to promote glial differentiation, although in certain cellular environments they also tend to enhance neurogenesis [7]. In terms of glial differentiation, mouse subventricular zone (SVZ) neural progenitor cells begin to express the astrocytic marker glial fibrillary acidic protein (GFAP) after exposure to BMP2 [9] and BMP4 [19]. Secreted BMPs bind to a receptor complex composed of type I and type II BMP receptors, both being membrane-spanning serine/threonine kinases. Upon binding of the ligands, the type II receptor phosphorylates the type I receptor and, subsequently, one of Smad1, 5, or 8 proteins are phosphorylated by the activated type I receptor, forming homodimers of Smad1, 5, or 8. The phosphorylated Smad homodimers translocate to the nucleus together with Smad4, which is commonly used as a binding partner of Smad homodimers. These Smad heterotrimers themselves form complexes with signal transducer and activator of transcription (STAT) 3 and the transcriptional coactivator p300 to promote gene transcription of GFAP synergistically. In terms of BMP signaling within the pathological environment of AD, $A\beta_{1\text{-}42}$ has been shown to increase BMP6 protein expression in neural progenitors, while BMP6 led to decreased progenitor proliferation, in line with increased BMP6 and impaired neurogenesis in both AD patients and APP transgenic mice [31]. Proteolytic products of APP may therefore exert pathological effects through BMP signaling, leading to the cognitive decline seen in AD.

In the present study, we report that BMP4 is a novel gliogenic factor for glial differentiation of neural stem cells in pathological conditions such as AD and DS. The analysis revealed that treatment with sAPPα specifically increased BMP4 expression, resulting in GFAP expression via activation of Smad signaling pathway *in vitro* and *in vivo*, while expressions of BMP2 and BMP7 were not affected. Direct antibody-mediated inhibition of BMP4 also impaired both activation of the Smad pathway and glial differentiation as determined by reduced Smad1/5/8 phosphorylation and GFAP expression, respectively. Thus, these results implicate the BMP-induced Smad signaling pathway as a novel mechanism for sAPPα-mediated glial differentiation of neural stem cells and provide further detail of the potential mechanism of sAPPα-induced glial differentiation of neural stem cell in the pathological environments of AD and DS.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant sAPPα protein (Sigma) was solubilized with dH_2O and stored at −80 °C until use. The following antibodies were used: Rabbit anti-GFAP antibody (Promega); mouse anti-APP antibody (22C11) (Chemicon); mouse anti-BMP4 antibody (Chemicon); mouse anti-phospho-Smad1/5/8 antibody (Chemicon); and rabbit anti-β-actin antibody (Cell Signaling). For the secondary Abs, anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratory) were used.

2.2. Cell culture and transfection

Human embryonic teratocarcinoma cells (NT-2/D1) derived from a testicular germ cell tumor have been intensively used as an experimental model for investigating neural differentiation. Human embryonic teratocarcinoma cells (NT-2/D1) derived from a testicular germ cell tumor have been intensively used as an experimental model for investigating neural differentiation and exhibit both undifferentiated markers and multipotent differentiation potential similar to NSCs [32–37]. Thus, unlike post-mitotic CNS neurons or neuroblastoma, NT-2/D1 cells still possess some multipotency and distinctive developmental characteristics, which resemble the nature of neural stem cells. The NT2/D1 cells were seeded (5×10^6 cells per 60 mm petri dish) in Dulbecco's modified Eagle's medium (DMEM/F-12; Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS; Novacell), 1% antibiotic–antimycotic (Invitrogen), 4 mM glutamine (Invitrogen) and maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The cells were passed twice a week by short exposure to trypsin/EDTA (Invitrogen). For the experiments, 1×10^6 NT-2/D1 cells were plated in a 6 well tissue culture plate and subsequently transfected with Lipofectamine™2000 (Invitrogen) based on company's protocol. Transient transfections were performed with vector constructs for pCDNA3.1; pCEP-APP695 (residues 1–695); pCMVIg-APP.1 (residues 1–678), pCMVIg-APP.2 (residues 1–205), [pCMVIg-APP.1 and pCMVIg-APP.2 (generously provided by Dr. Thomas Suhduf, University of Texas Southwestern, USA)].

2.3. Immunocytochemistry and cell imaging

NT2/D1 cells were treated with recombinant sAPPα (100 ng/mL) for 5 days and allowed to differentiate. Then, cells were imaged using a LEICA DMIL differential interference contrast (DIC) microscope. For cell staining, after fixation in 4% paraformaldehyde, the samples were washed in PBS with 0.2% Triton X (PBST), and then incubated in PBST containing appropriate normal sera. Next, samples were incubated overnight (<16 h) with rabbit IgG anti-human GFAP (1:500; Sigma) at 4 °C. Then, samples were incubated with FITC-conjugated secondary antibodies (Jackson Immunoresearch) for 1.5 h in a dark humidified chamber after washing with PBS. Next, the samples were washed in PBS and cover slipped with VECTASHIELD Mounting Media (Vector Laboratories).

2.4. RT-PCR analysis

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. One microgram of the total RNA was reverse-transcribed and amplified by the SuperScriptTM ONE-STEPTM RT-PCR system (Invitrogen) with the following primers: BMP2 (+) 5′-CAGAGACCCACCCCCAGCA-3′, (−) 5′-CTGT TTGTGTTTGGCTTGAC-3′; BMP4 (+) 5′-TTCCTGGTAACCGAATGCT-3′, (−) 5′-GGGGCTTCATAACCTCATAA-3′; BMP7 (+) 5′-GTCATGAGCTTC GTCAAC-3′, (−) 5′-AACTTGGGGTTGATGCTC-3′; GFAP (+) 5′-AAGCAG TCTACCCACCTCAG-3′, (−) 5′-ATCCCTCCCAGCACCTCATC-3′; β-actin (+) 5′-GACAGGATGCAGAAGGAGAT-3′, (−) 5′-TTGCTGATCCACATCT GCTG-3′. Ten microliters of the reaction mixtures were then analyzed on a 2% E-gel (Invitrogen). Gel images were captured using a KODAK Image Station 2000MM (KODAK).

2.5. Western blot analysis

Protein samples were prepared by lysing the cells with ice-cold lysis buffer consisting of 1% NP40, 150 mM NaCl, 50 mM Tris pH8.0 and 1× protease inhibitor cocktail (Boehringer). The protein concentration of each sample was measured by Bio-Rad protein assay (Bio-Rad). Then, cell lysates were heated at 70 °C for 10 min in LDS sample loading buffer (1×) and separated on NuPAGETM 4–12 % Bis-Tis Gel (Invitrogen) and transferred to a PVDF membrane (30 V, 60 min). Membranes were blocked with 5% skim milk in PBS for 1 h at RT and probed at 4 °C overnight with primary antibody in 5% skim milk. The membranes were washed 3 times for 5 min each with PBS containing 0.05% Tween 20 (PBS-T, pH7.4) and incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skim milk for 2 h at RT. After 3 times washing with PBS-T, immunoreactive bands were visualized by using ECL plus (Amersham Bioscience).

2.6. Analysis of wild type and APP23 tg mouse brain

8 months old male APP23 transgenic [16,17], and wild-type mice were deeply anesthetized. Then, mice brains were obtained to examine expression of BMP-2, -4, and -7 and phosphorylation status of Smad1/5/8. Three APP23 tg mice were used for the analysis in each experiment. Half of the brains were used for protein and the others for mRNA. Mouse handling procedures were performed according to University of Central Florida Animal Research Committee and National Institute of Health Guidelines.

3. Results

3.1. sAPPα induces astroglial morphology in NT2/D1 cells

To determine whether sAPPα is capable of inducing astrocyte-like morphology in the NT2/D1 cell line, we treated NT2/D1 cells with sAPPα (100 ng/mL) for 5 days. Compared to typical NT2/D1 cell morphology (Fig. 1A), NT2/D1 cells exhibited astrocyte-like morphology (Fig. 1B, C) and also stained positive for the astrocyte marker GFAP (Fig. 1C). These results are consistent with our previous studies showing astrocyte morphology of NT2/D1 cells under sAPPα [38].

3.2. sAPPα induces glial differentiation of NT2/D1 cells by increasing BMP4 expression, and N-terminal domain of APP is sufficient for glial differentiation

To investigate the novel mechanism of glial differentiation of neural stem cells in pathological conditions such as AD and DS, we treated NT2/D1teratocarcinoma cells with recombinant sAPPα (100 ng/ml) for 16 h and examined gene expression of BMP families (Fig 1A). In our experimental condition, mRNA level of BMP4 was specifically up-regulated by exposure to sAPPα while the expression levels of other BMP members were not affected. For further study, we exposed NT2/D1 cells to sAPPα for different time courses (0-3-6-12-24 h). We observed that gene transcription of BMP4 was gradually increased after 3 h in a time-dependent manner (Fig 1B). Additionally, sAPPα promoted protein expression of BMP4 dose dependently (0-3-10-30-100-300 ng/ml) and subsequently induced GFAP expression by increasing phosphorylation of (p-)Smad1/5/8, also in a dose-dependent manner (Fig 1C). Taken together, treatment of recombinant sAPPα significantly induced glial differentiation of Nt2/D1 cells by increasing BMP4 expression in both a time and dose dependent manner. Furthermore, the dose-dependent increase of p-Smad1/5/8 indicates that BMP4-induced glial differentiation is mediated by the Smad signaling pathway. Previously, we reported that the N-terminal domain of sAPPα can adequately induce glial differentiation of neural stem cells by stimulating the notch and IL-6/gp130 signaling pathways [16,17]. Therefore, we also tested which domain of sAPPα (1–205, 1–678, and 1–659 a.a.) can induce BMP4-mediated glial differentiation of NT2/D1 cells (Fig 2D) by transfecting full length and truncated APP expression plasmids (Supplemental Fig. 1). Our data suggest that the N-terminal domain of sAPPα was sufficient to induce GFAP expression by activating the BMP4/Smad signaling pathway. These data indicate that sAPPα-induced glial differentiation of neural stem cells can be initiated by sAPPα via various signaling pathways such as notch, IL-6/gp130 and BMP4/Smad.

3.3. Blocking sAPPα efficiently suppressed glial differentiation of neural progenitor, NT2/D1 cells, via downregulation of BMP4/Smad signaling pathway

For further investigation, we applied a monoclonal 22C11 antibody which recognizes amino acids 66–81 of the N-terminus on APP to neutralize the effect of sAPPα upon BMP4 mediated glial differentiation (Fig 3A) [12]. Treatment of 22C11 antibody (0.25 and 0.5 μg/ml) markedly downregulated sAPPα induced p-Smad1/5/8 as well as GFAP level. On the other hand, blocking BMP-4 by an anti-BMP4 antibody (5 and 10 μg/ml) also potently suppressed sAPPα induced p-Smad1/5/8 and GFAP level (Fig 3B). Thus, these data demonstrate that sAPPα induced BMP-4 is a crucial mediator of glial differentiation of NT2/D1 cells.

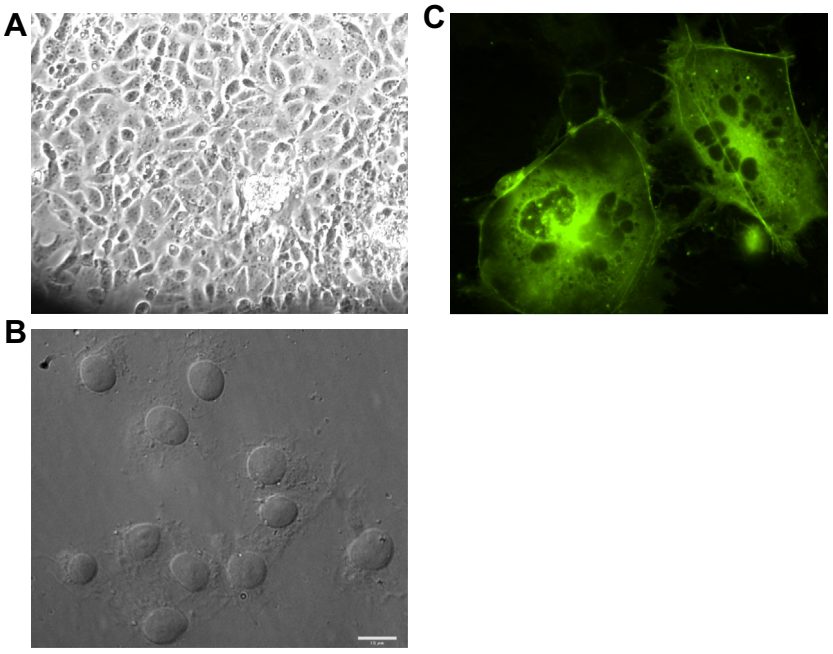

Fig. 1. sAPPα induces astrocyte-like morphology of NT2/D1 cells. (A) Typical NT2/D1 cell morphology (100× total mag). (B) Some gain of astrocyte morphology by NT2/D1 cells after 5 days of sAPPα exposure (400× total mag). (C) Astrocyte morphology of NT2/D1 cells immunostained for astroglial marker GFAP (630× total mag).

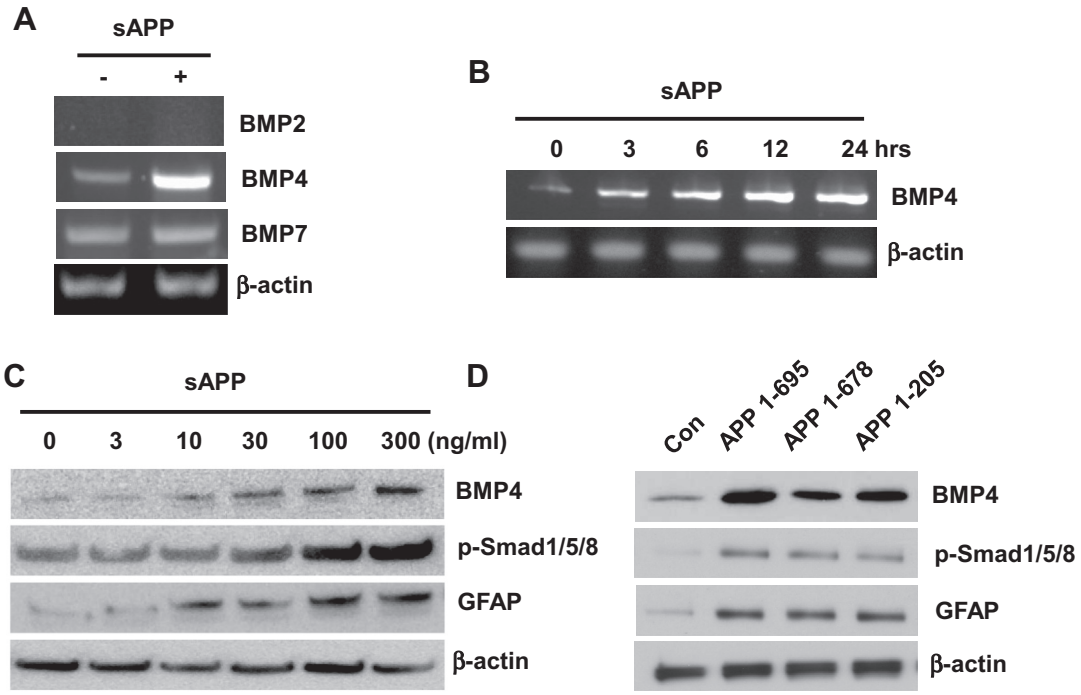

Fig. 2. sAPPα induces glial differentiation by increasing BMP4 expression in NT2/D1 teratocarcinoma cells. (A) mRNA expression profiles of BMP families (BMP-2, -4, and -7) under the exposure to sAPPα (100 ng/ml) for 16 h in NT2/D1 teratocarcinoma cells. Once mRNA was collected with Trizol™ solution, 1 μg of mRNA was used for cDNA synthesis for RT-PCR. (B) mRNA level of BMP-4 was analyzed with RT-PCR. To examine time-dependent effect of sAPPα upon BMP-4 expression, sAPPα was supplemented to NT2/D1 cells for 0–3–6–12–24 h. Then, mRNA of BMP-4 expression level was determined by RT-PCR. (C) Dose-dependent effect of sAPPα (0–3–10–30–100–300 ng/ml) upon BMP-4 mediated glial differentiation was also examined by measuring GFAP expression. Moreover, expression of BMP4 and phosphorylation of its downstream signaling molecule (Smad1/5/8) were examined. Expression of β-actin was examined as a loading control. (D) The cells were transfected with truncated N-terminal domains of APP (1–695, 1–678, and 1–205 a.a.) (diagram available in Fig. S1) and the protein extracts analyzed by Western blot with anti-BMP4 Abs, anti-p-Smad1/5/8 Abs and anti-GFAP Abs. Expression of β-actin was examined as a loading control.

3.4. BMP4/Smad signaling pathway is activated in the APP23 AD transgenic mouse model

In our previous studies, we have reported that transplanted human neural stem cells were preferentially differentiated into glia in a transgenic (tg) AD mouse model (APP23). To investigate whether the BMP-4/Smad signaling pathway is also important for glial differentiation of neural stem cells *in vivo*, we assessed gene transcription levels of BMP families and p-Smad1/5/8 levels in wild type and APP23 tg mouse brains (Fig 4A and B). Consistent with our *in vitro* data, significant increases of BMP-4 and subsequent increases in p-Smad1/5/8 were observed in APP tg mouse brains, suggesting that the BMP-4/Smad signaling pathway plays an important role for sAPPα induced glial differentiation of human neural stem cell differentiation *in vivo*.

4. Discussion

Neural stem and progenitor cells reside in microenvironmental niches such as the adult SVZ that are modified by environmental stimuli to regulate multiple aspects of precursor development [11,21]. Of particular interest are various pathological cues under such diseases as AD and DS that can modulate neural stem cell differentiation. In our previous studies, we demonstrated that neural stem cells can be preferentially differentiated into glia instead of neurons in a tg AD mouse disease model by stimulating multiple signaling pathways such as notch and IL-6/gp130 signaling pathways [16,17]. Physical interaction between sAPPα and notch increased intracellular cleavage of notch (thereby increasing notch intracellular domain, or NICD) and subsequently upregulated gene expression of Hairy and Enhancer of Split (Hes) 1, a synergistic target of NICD and nuclear protein CBF1/Su(H)/Lag-1 (CSL), resulting in glial differentiation. Moreover, sAPPα can induce glial differentiation of neural stem cells via protein–protein interaction with gp130, an important component of the IL-6 receptor complex, thereby stimulating the JAK/STAT signaling pathway. Although it is not clear how these multiple signaling pathways are orchestrated by sAPPα, all were significantly activated by high levels of sAPPα, proteolytic cleavage products of APP, and resulted in induction of GFAP expression in NT2/D1 cells as well as in human neural stem cells (hNSCs) injected into APP23 tg AD mouse model brains. In addition, our previous studies further demonstrated that chemokines such as monocyte chemoattractant protein 1 (MCP1) and MCP-1 induced protein (MCPIP) can promote glial differentiation of neural progenitor cells via APP signaling [29], suggesting a role for inflammation in APP expression and function. In the present study, to extend our understanding regarding the role of sAPPα upon glial differentiation of neural stem cells, we aimed to investigate whether the BMP/Smad signaling pathway is also involved in sAPPα-induced glial differentiation of neural stem cells under pathological conditions such as AD and DS.

BMP signaling is an important regulator of cell proliferation and fate commitment throughout development and within the adult SVZ and SGZ neurogenic niches [1–3,6,18,23]. Binding of BMPs to their receptors initiates phosphorylation and nuclear translocation of Smad1/5/8, prompting transcription of target genes for cell cycle exit and astrocytic fate commitment [25]. Among BMP families, an increasing number of reports has shown that BMP-4 plays a critical role for glial differentiation of neural stem cells. Recently, Kim et al. demonstrated that neurospheres can be differentiated into glia by activation of phosphotidylinositide (PI)-3-kinase signaling-mediated up-regulation of N-Cadherin [13]. Under pathological

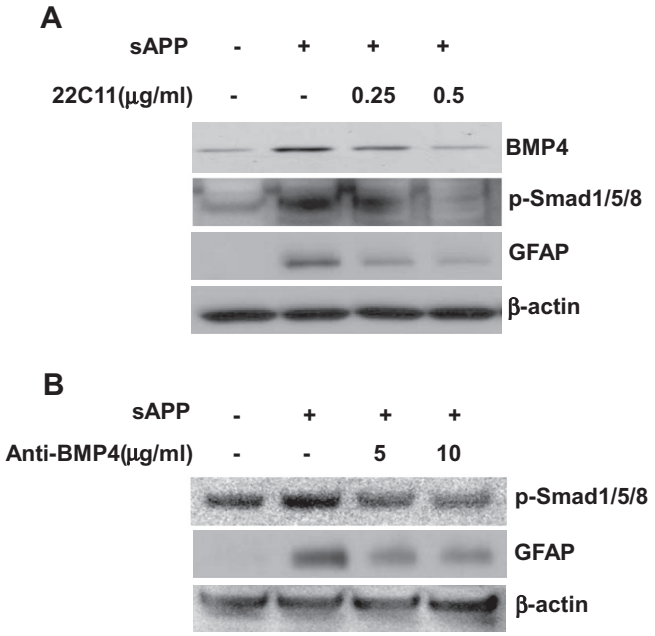

Fig. 3. Blocking sAPPα efficiently suppressed glial differentiation of NT2/D1 cells via downregulation of BMP-4/Smad signaling pathway. (A) To inhibit the function of sAPP upon BMP-4 mediated glial differentiation, NT-2/D1 cells were preincubated with 22C11 Abs (0.25 and 0.5 μg/ml) for 1 h. Subsequently, 100 ng/ml of sAPPα was supplemented to NT-2/D1 cells for 16 h. Then, western blot analysis was performed to detect expression of BMP-4 and GFAP as well as phosphorylation of Smad1/5/8. Treatment of 22C11 Abs markedly suppressed Smad1/5/8 phosphorylation as well as expression of GFAP and BMP-4 in a dose-dependent manner. (B) To elucidate whether BMP4 is crucial gliogenic mediator, anti-BMP-4 Abs (5 and 10 μg/ml) were applied to neutralize secreted BMP4. Likely, co-treatment of anti-BMP-4 Abs significantly downregulated expression of GFAP as well as p-Smad1/5/8 level, increased by sAPP, in NT2/D1 cells.

conditions such as demyelination and oxygen-glucose deprivation, expression of BMP-4 was significantly increased and subsequently induced glial differentiation of SVZ neural stem cells [4]. Exercise has been shown in an earlier study to mitigate the deleterious effects of various mouse models of neurodegenerative diseases, primarily through insulin-like growth factor (IGF)-1 [3]. Exercise has also been shown to reduce BMP-4 expression and Smad signaling as well as increase hippocampal neurogenesis and cognitive function of wild-type mice [8]; in BMP-4 tg mice, elevated BMP-4 markedly diminished hippocampal neurogenesis and impaired cognitive function [8], whereas noggin (a BMP-4 antagonist) tg mice demonstrated significantly elevated hippocampal neurogenesis [2,8] and improved cognitive function, mimicking the effects of exercise [8]. Although further studies are required, these reports suggest that BMP-4 is a negative regulator of neuron production in the brain by not only inducing glial differentiation but also suppressing neurogenesis of neural stem cells. Moreover, exercise may favor inhibition of BMP-4 and Smad signaling, resulting in increased neurogenesis, and underlie the idea that exercise may protect against AD.

In the present study, we observed that treatment of sAPPα specifically upregulated BMP-4 expression *in vitro* and *in vivo,* while BMP-2 and -7 expressions were not changed, and was followed by glial differentiation of neural stem cells via phosphorylation of Smad1/5/8. Conversely, application of anti-APP antibody (22C11) and anti-BMP-4 antibody potently inhibited GFAP expression and phosphorylation of Smad1/5/8, suggesting that sAPPα-induced GFAP expression is mediated by the BMP-4/Smad signaling pathway. Therefore, regulation of BMP-4 is important to control neural stem cell differentiation, especially, in pathological conditions such as AD and DS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.139.

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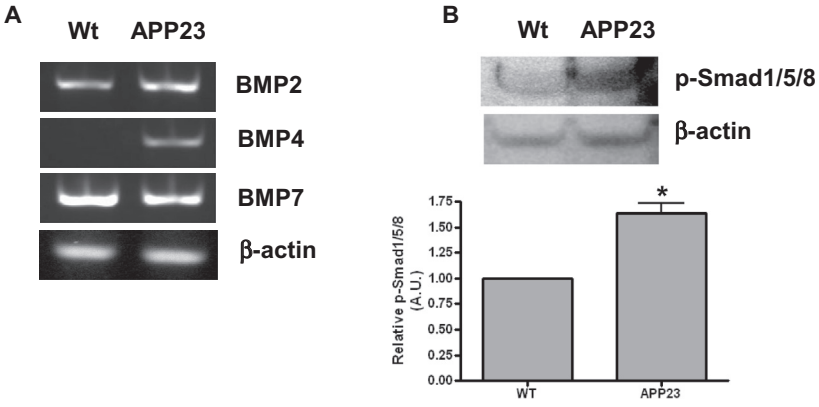

Fig. 4. BMP-4/Smad signaling pathway is activated in APP23 AD tg mouse model. (A) mRNA expression profiles of BMPs were examined in wild type and APP23 tg mouse brain samples with RT-PCR analysis. In APP23 tg mouse brain, transcription level of BMP-4 showed significant increase. (B) Furthermore, phosphorylation status of Smad1/5/8 was examined in wild type and APP23 tg mouse, showing significant increases in APP23 tg mouse ($^{*}=p < 0.05$).

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