Interruption of β-Catenin Signaling

Reduces Neurogenesis in Alzheimer's Disease's Brains

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Although studies show new neurons generated from progenitor cells in a healthy adult neocortex, neurogenic potential derived from stem/progenitor cells in the cortex of Alzheimer's patients still remains unsolved. In this study, we isolated the glial progenitor cells (GPCs) from the cortex of healthy (HC) and Alzheimer's disease (AD). The AD-GPCs displayed reduced capability to renew and decreased production of new neurons. Interestingly, we found significant reduction of β-catenin in AD-GPCs compared to HC while glycogen synthase kinase 3β (GSK-3β) was promoted. Moreover, we found both increased phosphorylation of β-catenin in AD-GPCs and their progeny cells. Amyloid β peptide (Aβ) treatment results in increase of GSK-3β and a reduction of β-catenin, ultimately impairment of neurogenesis of GPCs. Similar results were observed in GPCs from AD transgenic mice. These results suggest that decreased β-catenin signaling by Aβ may be one of causes for the lack of neurogenesis in AD-GPCs.

Studies have illustrated that there are large pools of glial progenitor cells (GPCs) in the human neocortex[1-9]. There are different antigen phenotype classifications for GPCs such as: A2B5-positive cells[4,10] and NG2-expressing cells[11-14]. The predominant cell type in injured brains are NG2-positive cells[1,15]. Although, it is still controversial whether GPCs are able to generate new neurons in their local brain regions[16,17], It is well-known that after environmental limits in healthy brains were depleted, the stem/progenitor cells from non-neurogenic regions could restore self-renewal and generate new neurons[2,4-8,18-20]. Recent studies have shown that $NG2^+$ GPCs isolated from postnatal rodents were able to form neurospheres and generate new neurons and glia[11-14].

Alzheimer's disease (AD) is a neurodegenerative diseases and is characterized by amyloid plaque deposits and neurofibrillary tangles [21,22]. Damage-induced neurogenesis has not been demonstrated in the neocortex after progressive neuronal loss in AD brains[23,24], although significant gliogenesis was observed in the degenerative cortex[25]. The mechanism that underlined the fate of GPCs remains unclear, however several signal transduction pathway cascades have been shown to control progenitor cell fate[26]. One of these pathways, Wnt/β-catenin signal cascade, was involved in progenitor cell lineage decisions[27] in developmental stages[28-30] as well as in adult brains[31]. A reduced Wnt/β-catenin signaling has been reported in AD brains[32,33], and the decreased expression of β-catenin was also found in AD patients carrying presenilin-1-inherited mutations[34-36].

In the current study, we found that new neuron productions of AD GPCs were significantly reduced compared to HC brain samples. Furthermore, reduced β-catenin signaling was observed in AD neurospheres and their differentiated cells. Exogenous Aβ

treating interrupted the neuron fate commitment of HC GPCs. These results suggested that reduced β-catenin signaling causes a decrease in neurogenesis of AD GPCs.

RESULTS

Generation of new neurons reduces from AD glial progenitor cells

Increased Aβ in AD brains is produced by neurons. In order to investigate whether Aβ production is also promoted in AD GPCs, both Aβ40 and Aβ42 levels in GPCs were measured with ELISA. We found an approximate one-fold increase in both Aβ40 and Aβ42 level in AD (n = 3) GPCs to their HC (n = 3) counterpart **(Fig. 1a).** Furthermore, we tested the Aβ level of the daughter cells derived from the reprogrammed GPCs. These results have shown a similar increase in Aβ40 and Aβ42 in cells from AD GPCs as compared to HC cells **(Fig. 1a).** Because Aβ is generated in a vesicular pathway, it has little effect on the extra-vesicular components. Therefore, we tested Aβ level in the medium, and normalized both Aβ40 and Aβ42 to the total proteins of the corresponding cell lysate. As expected, both Aβ40 and Aβ42 levels increased in the medium of AD GPCs, with a prominent increase of Aβ42 level in the medium containing AD daughter cells (**Fig. 1b**).

Whether there are any potential differences in the developing fate of HC and AD GPCs, we observed the cell differentiation toward neurons with doublecortin (DCX) labeling, a neuroblast cell marker, at 2-days in culture (**Fig. 1c**). Respectively, 6% and 2% of scored HC and AD cells displayed DCX positive staining (**Fig. 1d**). To further clarify the immunostaining results, we applied Western blot analysis for the progeny cells. In addition to reduced DCX level in AD progeny cells, polysialic acid-neural cell adhesion molecule

(PSA-NCAM), a neuroblast marker, was reduced at 2 days in culture **(Fig. 1e).** The results revealed a decline in the production of new neurons from AD GPCs.

Thus far, it is not clear whether a decline of new neurons from AD GPCs is due to changes in proneural gene expression. Therefore, we tested the expressions of the proneural genes; neurogenin 2 (Ngn2) and mammalian achaete-scute homolog 1 (Mash1), members of the helix-loop-helix class of transcription factors and neurogenic differentiation factor 1 (NeuroD1) by Western blot. It has been reported that neurogenins inhibit glial differentiation[37], which was reflected in the results that illustrated a significantly reduced expression of Ngn2, Mash1, NeuroD1 in AD differentiated cells at 2 days in culture **(Fig. 1f).** This information suggests that the decline of neurogenic gene expression might be associated with the reduction of new neurons derived from AD GPCs**.**

β-catenin signaling decreases in AD glial progenitor cells and their daughter cells

As studies have shown, Wnt/β-catenin signaling can trigger the differentiation of GPCs into neuron phenotype cells[27]. The over-expression of Wnt3 was significant enough to increase neurogenesis from adult human progenitor cells, however, a blockade of Wnt3 signaling reduced neurogenesis [31]. Reduced Wnt/β-catenin signaling has been reported in those with AD[32,33]. Therefore, it was postulated that deficiency in the Wnt/β-catenin cascade could be the reason why there was a reduction in neuron production from AD GPCs. In order to examine whether Wnt/β-catenin signaling pathway is involved in a different cell fate decision between HC and AD GPCs, the signaling molecules in the GPCs and their daughter cells were tested by Western blot. We found no significant differences in Wnt3 and Wnt3 receptor frizzled expression between AD and HC GPCs as well as their progeny cells **(Fig. 2a).** However, β-catenin, which is the downstream effector

of Wnt3 signaling, was significantly decreased in AD GPCs and their progeny cells compared to those from HC samples (**Fig. 2a**), although the cause of the decline is unclear. As previously mentioned, GSK-3β is an enzyme which phosphorylates β-catenin[38-40]. Adding Aβ promoted GSk-3β expression and activity in neurons[39], which, in turn, induced β-catenin phosphorylation to degradation. In order to examine whether β-catenin reduction is also associated with GSk-3β levels in neuronal induction, we tested the expression of GSk-3β in AD and HC GPCs and their daughter cells by Western blot. We found a significant increase in GSk-3β expression in AD GPCs and their differentiated cells **(Fig. 2a).** It is still unclear whether the increased GSk-3β level might promote the phosphorylation of β-catenin in AD GPCs. The phosphorylated β-catenin was detected with an antibody against phosphorylated β-catenin by Western blot. We have also found a significant increase in phosphorylated β-catenin in AD GPCs and the progeny cells (**Fig. 2a**). Due to nuclei translocation of β-catenin, so we need to demonstrate whether decreased β-catenin is also found in the nucleus. Immunohistochemistry demonstrated that the expression of β-catenin was decreased in the nuclei of AD daughter cells (**Fig. 2b**). Meanwhile, we isolated the nucleus and cytoplasm of differentiating cells and also found a similar decrease in β-catenin level in both the nucleus and cytoplasm of AD progeny cells. We observed trace levels of GSK-3β and phosphorylated β-catenin in the nucleus extraction, however, a significant increase in the level of GSK-3β and phosphorylated β-catenin were found in the cytoplasm of AD progeny cells (**Fig. 2c**).

It has also been reported that Ngn1 and Ngn2 are the proneural genes that are controlled by β-catenin[41]. Over-expression of β-catenin could significantly increase the luciferase activity in the reporter assay[41]. We found that β-catenin levels in AD GPCs and

their daughter cells were significantly lower than HC cells. Therefore, it would be interesting to find out whether these pro-neuronal genes could be lowered at mRNA level. We performed RT-PCR to examine the mRNA level of Ngn1 and Ngn2. The results showed that both Ngn1 and Ngn2 mRNA levels were decreased in AD daughter cells, indicating a decrease in β-catenin level in the cells derived from AD GPCs causes a corresponding reduction in pro-neural gene expression, which lowers its ability to differentiate towards neurons (**Fig. 2d, e**).

Amyloid-β peptide impairs neuronal induction by reducing β-catenin level

Increased Aβ production and deposits are the hallmarks of AD progression[21,22]. To find out whether Aβ toxicity could have an inhibitory effect on the neuron-generated capability of $NG2^+$ GPCs, HC spheres were treated with different concentrations of Aβ1-42 peptide, the primary component in the medium of AD progeny cells **(Fig. 1 b),** for 2 days, then Aβ was removed and the cultures were tested at either 7 or 14 day periods. At 0.1 μM of Aβ42, no decrease in βIII $tubulin^+$ neurons was observed as compared to the sample without Aβ42 treatment **(Fig. 3a, b)**. A similar result was also found by MAP-2 staining (data not shown). When treated the cells with 1 μM of Aβ42, we found βIII $tubulin^+$ new neurons were significantly decreased (* $P < 0.05$), however, when treated with 5 μM of Aβ42, βIII $tubulin^+$ and $MAP\text{-}2^+$ neurons were greatly reduced **(Fig. 3a, b, **** $P < 0.01$**)**. Our results indicated that exogenous Aβ could inhibit GPCs capability to generate neurons in a dose-dependent manner.

To understand whether the decreased production of new neurons by Aβ42 treatment is due to the change of proneural genes, HC progeny cells were exposed to

Aβ 42 for 2 days and the culture was kept for 14 days after the removal of Aβ 42. After 14 days, the cells were tested by Western blot. The results demonstrated reduced expressions of the neurogenic genes Ngn2, Mash1, and NeuroD1 in the cells with Aβ42 treatment (**Fig. 3c**), suggesting that transient treatment of Aβ42 could have a long lasting effect on the neuron-fate decision.

Since treatment with Aβ42 could interrupt neuron induction, we further examined whether Aβ treatment could affect the same signal pathway we found in the cells from AD GPCs. The Western blot detected Wnt3/β-catenin signaling molecules. Similar to the results we found in the cells derived from AD GPCs, we did not observe any significant change in Wnt3 and frizzled expression between cells with and without Aβ42 treatment **(Fig. 3d)**. However, in Aβ42 treated HC progeny cells, we found that the expression of β-catenin was significantly decreased in a dose-dependent manner (**Fig. 3d**). We also investigated the phosphorylation of β-catenin and GSK-3β expression along with Aβ treatment. As expected, the expression of phosphorylated β-catenin and GSK-3β was enhanced along with 5 μM of Aβ42 treatment in HC progeny cells **(Fig. 3d).** Meanwhile, Aβ treatment also further promoted GSK-3β expression of AD cells. However, significant changes were not observed in the expression of β-catenin and phosphorylated β-catenin between AD cells with and without Aβ42 treatment (**Fig. 3d**). These data indicate that Aβ up-regulates GSK-3β expression, which, in turn, reduces β-catenin levels in newly generated cells.

β-catenin transfection restores the neuronal induction of AD GPCs

To establish a causal relationship of reduced neurogenesis derived from AD GPCs, we observed whether the restoration of neuronal differentiation in AD neurospheres by promoting β-catenin in the Wnt signaling pathway. Suspended $NG2^+$ progenitor cells from AD brains (n = 2) were transfected by the virus with β-catenin for 5 h followed by a 14-day extension of the culture. The spheres infected the virus with β-catenin were dissociated into single cells and cultured in the differentiating medium for 4 days. The controls were non-transfected β-catenin cells HC (n = 2) and AD (n = 2) and Western blot analysis has demonstrated that the expression level of β-catenin was partially restored **(Fig. 4a).** Immmunostaining with βIII tubulin has shown a significant increase in the neuron number with β-catenin introduction **(Fig. 4b, c**).

DISCUSSION

Recently the statement, "neurons do not regenerate during adulthood," has been challenged, and studies have found evidence that mature brains are capable of regenerating neurons[42]. There are more studies now showing GPCs, such as isolated $NG2^+$ progenitor cells from postnatal, as well as adult rodents, were able to form neurospheres and generate mature neurons and glial cells[12,43]. However, newly generated neurons have not been observed in the neocortex after progressive neuron loss[23,24] due to damage by toxic Aβ deposits[44,45]. Although neural progenitor cells both *in vivo* and isolated cells were reported[46-49], how the fate of the progenitor cells was affected, especially from AD patients has yet to be investigated. In the current study, even at older ages (over 80 years old), the progenitor cells isolated from the neocortex still have the competence to renew and generate new neurons. However, in the brains of AD patients, the self-renewal ability reduced gradually with time in comparison to the rate of renewal like those from the

healthy brain samples. The neurogenesis was dramatically reduced in AD and Aβ treating HC cells; *in vitro* experiments showed such impairment might be due to Aβ down-regulated β-catenin level therefore inhibited the neuron induction of GPCs.

It has been reported that Aβ promotes neuronal apoptosis in AD by activating GSK-3β, leading to the degradation of β-catenin and inactivation of Wnt signaling[50]. Activation of Wnt signaling rescues neurodegeneration and behavioral impairments induced by β-amyloid fibrils[51,52]. Similarly, studies have shown that the activation of PKC by phorbol 12-myristate 13-acetate protected rat hippocampal neurons from Aβ toxicity. Such effect was accomplished by the inhibition of GSK-3β activity, which led to the accumulation of cytoplasmic β-catenin and transcriptional activation via β-catenin/T-cell factor/lymphoid enhancer factor-1 (TCF/LEF-1) of Wnt target genes[53]. In contrast, inhibition of Ca_2^+-dependent PKC isoforms activated GSK-3β and offered no protection from Aβ neurotoxicity[53].

One pathway, Wnt/β-catenin signaling pathway, has been known to promote self-renewal in a variety of tissue stem cells, including neural stem cells, and hematopoietic stem cells. However, activation of the Wnt/β-catenin pathway promoted the inhibition of the pathway and prevented the differentiation of neural precursor cells[31,54]. We have found that Aβ can also cause permanent damage to GPCs by decreasing β-catenin level, so they can decrease the production of new neurons even though a transient toxic dose of Aβ was applied, suggesting Aβ may play a toxic role in neurons and progenitor cells in a similar manner[55-58]. Amyloid beta (Aβ) toxicity on neurons could be reversed by reagents like PKC agonist or Lovastatin, a reagent that could affect cholesterol synthesis and reduce Aβ production[50,53], whether these reagents could be used on GPCs to restore

their capability of neuron induction remains to be further studied.

In conclusion, the potential capability of GPCs and other neural precursor cells could be disrupted due to the effect of Aβ toxicity by lowering cellular signaling molecule β-catenin level. Therefore, there are still GPCs that exist inside the brain, and fail to generate new neurons to compensate for the neuron loss caused by Aβ aggregation. Also, due to the lack of necessary growth factors in AD brains, it could be possible that GPCs primarily differentiate into glial cells, which is consistent with previous studies[25]. Finally, how to lower the toxic effect of Aβ and/or elevate β-catenin level of AD GPCs by the inhibition of GSK-3β^{40} or other neuron progenitor cells will be the key to restore its neuron development fate **(Fig. 5).**

METHODS

Collection of human cortex tissue. Brain tissues were obtained from rapidly autopsied brains of geriatric patients (n = 14 HC, n = 14 AD) enrolled in the Brain Donation Program at Sun Health Research Institute. The average age was 84.3 and 83.7 years old in HC and AD patients, respectively. The tissues were taken and experimental manipulations were performed with sex-matched in HC and AD **(**supporting formation **Table 1).** The average postmortem interval (PMI) was less than 3 h. In order to avoid deep formations, the superficial grey matter of the superior temporal cortex was harvested.

Cell culture and Aβ treatment. To generate primary spheres, single NG2-expressing cells were cultured at 1x10^3 per ml density on uncoated 96-well plates. Suspended cultures were grown in Neurobasal A medium containing 5 ng/ml of bFGF, 10 ng/ml of EGF and 20 ng/ml of PDGF-AA. The spheres were harvested at 14-days in culture. To

generate secondary neurospheres, primary spheres were dissociated with papain, and re-suspended in identical growth medium for another 14 days. For differentiation, neurospheres were seeded on 8-well chamber slides (VWR, Lab Tek II CC^2) and grown in Neurobasal A medium supplemented with 2% of B27, 1% of N-2 supplement, and 10 ng/ml of BDNF, without mitogenic growth factors. For observation of the effect of amyloid peptides on cell differentiation, cultures were exposed to Aβ1-42 (pre-aggregated overnight at 37°C, PP69, Calbiochem)[55] at the concentrations of 0**,** 0.1**,** 1.0**,** 5.0 μM, respectively, 3 h after plating [55] for 2 days. The medium, neurotrophic factors and culture supplements were obtained from Invitrogen.

RT-PCR. For RT-PCR, The sequences of primers for Ngn1 are: 5'-AGTGACCTATCCGGCTTCCT, 5'-AGCGTCTGATTTTGGTGAG; for Ngn2 the primers are 5'-GCTGGGTCTGGRACACGTT and 5'-AACACTGCCTCGGAGAAGAG. RT-PCR was performed by one-step RT-PCR kit from Invitrogen, according to the manufactory's instruction.

β-catenin recombinant retrovirus preparation. The β-catenin full cDNA clone was purchased from OpenBiosystems. The open reading frame was subcloned to pLXRN retrovirus vector from Clontech. Retrovirus was produced by cotransfecting pLXRN vector with β-catenin open reading frame, VSV-G expression vector to the GP2-293 packaging cell line by calcium phosphate transfection (Clontech). The recombinant virus was collected and stored at -80°C until ready to use.

β-catenin transfection. Suspended $NG2^+$ cells from 4 AD brains were cultured in the

medium supplemented with growth factors for 14 days. The spheres were dissociated into single cells. Cells were put into the growth medium containing about $1x10^6$ virus particles for 5 hours. The medium was changed and the culture was extended for 14 days. The neurospheres infected virus with β-catenin plasmid were harvested and dissociated into single cells, and 80% cells were plated in one well of 6-well plate for Western blot and 20% cells in one well of 8-well plate for immunochemistry after 4 days culture.

Immunofluorescence. To observe GPC proliferation, secondary spheres of 14 days in suspension culture were exposed to 10 μM of BrdU for 12 h and plated for 3 hours. Cells were denatured with 2 N HCl and permeabilized with 0.3% Triton X-100. Primary antibodies were applied as follows: nestin (Santa Cruz Technology, 1:40) and BrdU (BioSource, 1:4000). NG2 proteoglycan (1:400, Chemicon) [12], DCX (Abcam, 1:400), PSA-NCAM (Chemicon, 1:500), βIII tubulin (Covance, 1:500), MAP-2 (Chemicon, 1:400), GFAP (DAKO, 1:3,000), O1 antibody (R&D, 1:2,000). Fluorescent-labeling 488 or 568 secondary antibodies against rabbit IgG, mouse IgG or IgM were used for detection (Molecular Probes, 1:1,000).

Nuclear extraction. Cell nuclei were extracted based on the protocol of Nuclear Extraction Kit by IMAGENEX Corporation (Cat: 10081K). In brief, cells were collected and re-suspended in 300 μl of 1x Hypotonic Buffer. The suspension was added to 15 μl of the 10% Detergent Solution. The whole cell lysate was centrifuged at 14,000 rpm for 20 min and the supernatant was cytoplasmic fraction. The remaining pellet was a nuclear fraction and was re-suspended in 30 μl of Nuclear Lysis Buffer. The suspension was centrifuged and the supernatant (nuclear fraction) was collected.

Western blot. Secondary neurospheres from HC (n = 11) and AD (n = 10) of 14 days in culture were collected for testing. To detect cell fate decision, similar spheres from HC (n = 6) and AD (n = 8) brains were grown on a 35-cm dish coated with laminin and deprived of growth factors. Neurosphere cells or their daughter cells were lysed by cell lysis buffer (10 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1 M Na_3VO_4, 0.5%Triton X-100). 25 μg of protein were separated on an 8% SDS-PAGE gel and transferred to PDVF membrane. Proteins were probed with polyclonal antibodies against nestin (Santa Cruz, 1:100), Dlx2 (Chemicon, 1:5,000), Wnt3 (Chemicon, 1:2,000), frizzled (Santa Cruz, 1:5,000, H-300), β-catenin (Chemicon, 1:10,000), GSK-3β (Zymed, 1:5,000), β-catenin [phosphoserines 33 and 37] (Zymed, 1:2,000). PARP (Santa Cruz, sc-7150), β-actin (Sigma, 1:20,000). Similar procedures were applied in transgenic Alzheimer mice and repeated 3 times independently.

Quantification of immunoreactive structures. Digitized images were obtained with a DEI-470 digital camera (Optronics, Goleta, CA) on a Leica fluorescence microscope (Leica, Germany). To determine the number of cells/neurosphere, secondary spheres of 4 and 7 days in free-floating culture, were plated for 3 h and stained with DAPI. The cellular nuclei of spheres were counted (n = 3-4 spheres/culture, 3 separate cultures per condition). Secondary sphere cells were scattered with papain and seeded with $1x10^5$ cells/ml. Differentiating cells were immunostained with specific antibodies at 2, 7 as well as 14 day *in vitro.* Immuno-positive cells were counted in four 40x microscope fields (n = 3 AD and HC separate cultures per condition). Blue nuclei (DAPI) were counted as total cell number. Results were analyzed with Image-pro Plus Analysis by an investigator who was blinded to

the experimental subjects and treatment.

FIGURE LEGENDS

Figure 1 Newly generated neurons reduce from AD progenitor cells. (**a**). Promoted Aβ40 and Aβ42 levels were observed in AD GPCs and their daughter cells. (**b**). An increase in both Aβ40 and Aβ42 levels were observed in the medium of GPCs and their daughter cells. **(c**). Immature neuronal cells were verified with antibody against DCX from both HC and AD GPCs at 2 days in cultures. **(d).** A significant decline of DCX-expressing cell number were observed in the cells from AD GPCs (* $P < 0.05$). **(e).** Western blot showed significantly decreased expressions of neuroblast markers PAS-NCAM and DCX in AD progeny cells. **(f).** Proneural gene expressions of Ngn2, Mash1, NeuroD1 decrease in AD progeny cells of 2-day culture. Scale bar represents 50 μm in **c** (40x magnification).

Figure 2 β-catenin signaling reduces in AD progenitor cells and their daughter cells. (**a**). In AD GPCs, Western blot showed a reduction of β-catenin level and a promotion of both phosphorylated β-catenin and GSK-3β level, without significant differences of both Wnt3 and frizzled expression. In the progeny cells from AD GPCs, Western blot showed similar results. (**b**). Immunostaining showed a decrease in β-catenin expression in AD differentiating cells. (**c**). In nucleus extraction of AD newly generated cells, β-catenin expression decreased without detectable GSK-3β and phosphorylated β-catenin. In the cytoplasm of AD progeny cells, β-catenin also decreased, however, with a promotion of GSK-3β and phosphorylated β-catenin level. **(d).** RT-PCRs showed that proneuronal genes Ngn1 and Ngn2 level reduced. **(e).** Spot densometrics were analyzed by Fluchem8900 software. Ngn1 and Ngn2 expression levels were normalized to the respective β-actin level and relative expression levels were then shown in bar graph (* $P <$

0.05, n = 3).

Figure 3 Aβ treatment decreases the production of new neurons and changes the expression of β-catenin signaling. **(a).** Immunostaining at 7-day culture showed a number reduction of βIII tubulin$^+$ (red) immature neurons from HC GPCs with the treatment of Aβ1-42 for 2 days. **(b).** Statistics showed that the number of new neurons decreased significantly with Aβ1-42 application at the concentration of 1.0 μM or greater (*p < 0.05, **p < 0.01) in contrast to control (0 μM) at 7- and 14-day cultures, student's t-test. **(c).** HC GPCs exposed to 5 μM of Aβ1-42 for 2 days showed reduced expressions of Ngn2, Mash1 and NeuroD1. **(d).** HC progeny cells were treated with 5 μM of Aβ1-42 for 2 days and the culture was extended to 7 days without Aβ. Western blot showed a reduction of β-catenin expression, an increased level in both GSK-3β and phosphorylated β-catenin, correspondingly. In AD cells, the blot did not show any significant level change of β-catenin and phosphorylated β-catenin but a significant increase in GSK-3β expression level. However, in AD and HC cells, no significant differences of both Wnt3 and frizzled expression were observed with or without Aβ treatment (n = 3). Scale bar represents 200 μm in **a** (10x magnification).

Figure 4 β-catenin induction restores neuronal production of AD GPCs. Suspended NG2$^+$ cells from AD brains were transfected by the virus with β-catenin for 5 h and the culture was extended to 14 days. The neurospheres infected virus with β-catenin were harvested and dissociated into single cells and cultured in the differentiating medium for 4 days. **(a).** The expression level of β-catenin was restored in part by Western blot test. **(b).** Neurons

were demonstrated by βIII tubulin staining (green) and the nuclei were counterstained with DAPI (blue) in 4-day progeny cells (n = 2 per group). Scale bar represents 50 μm. **(c).** Neuron number was significantly promoted with β-catenin introduction.

Figure 5: The schematic presentation shows that Aβ elevates GSK-3β, which in turn promotes the phosphorylation and degradation of β-catenin. Reduced β-catenin signaling down-regulates the expression of proneural genes.

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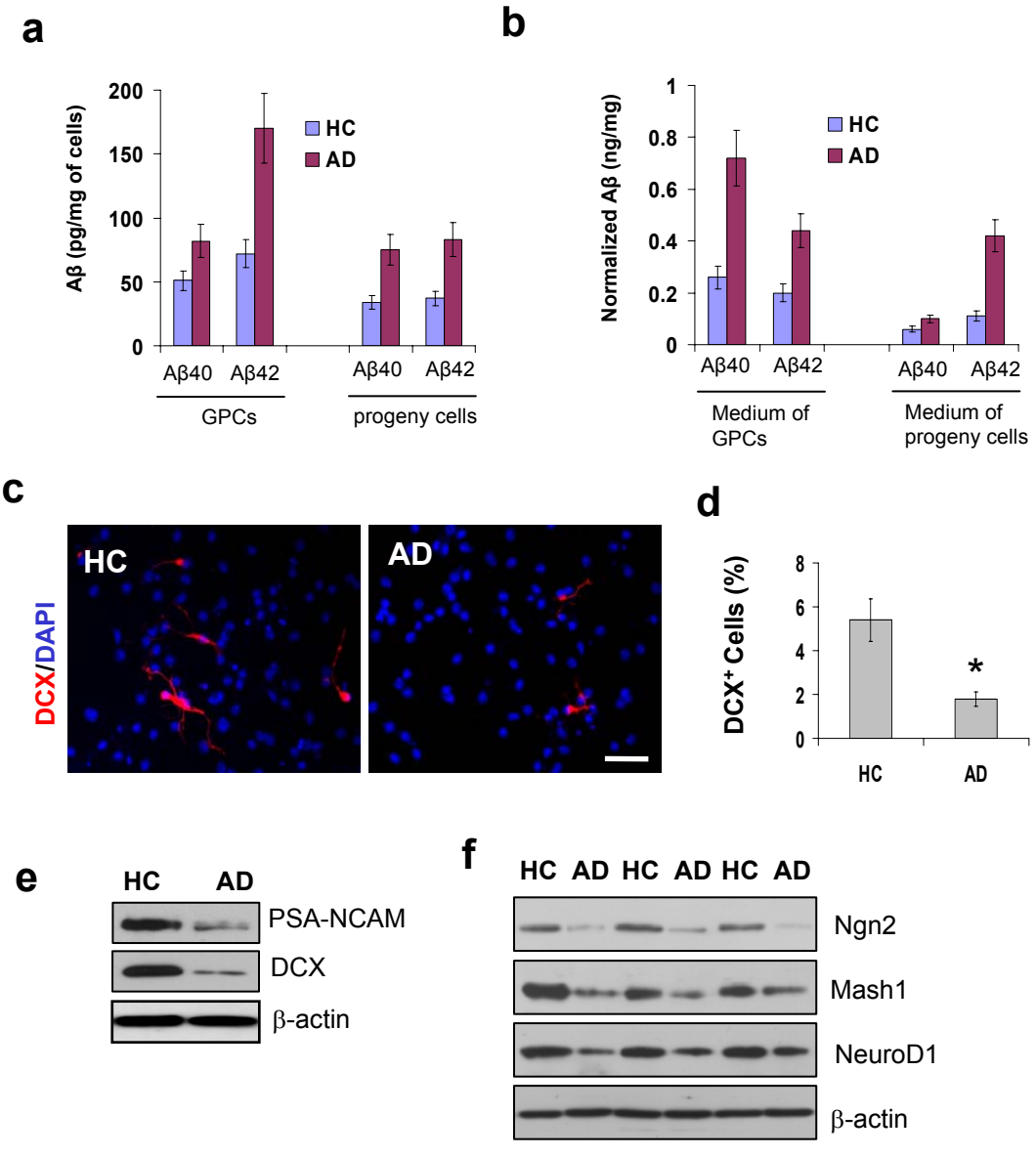
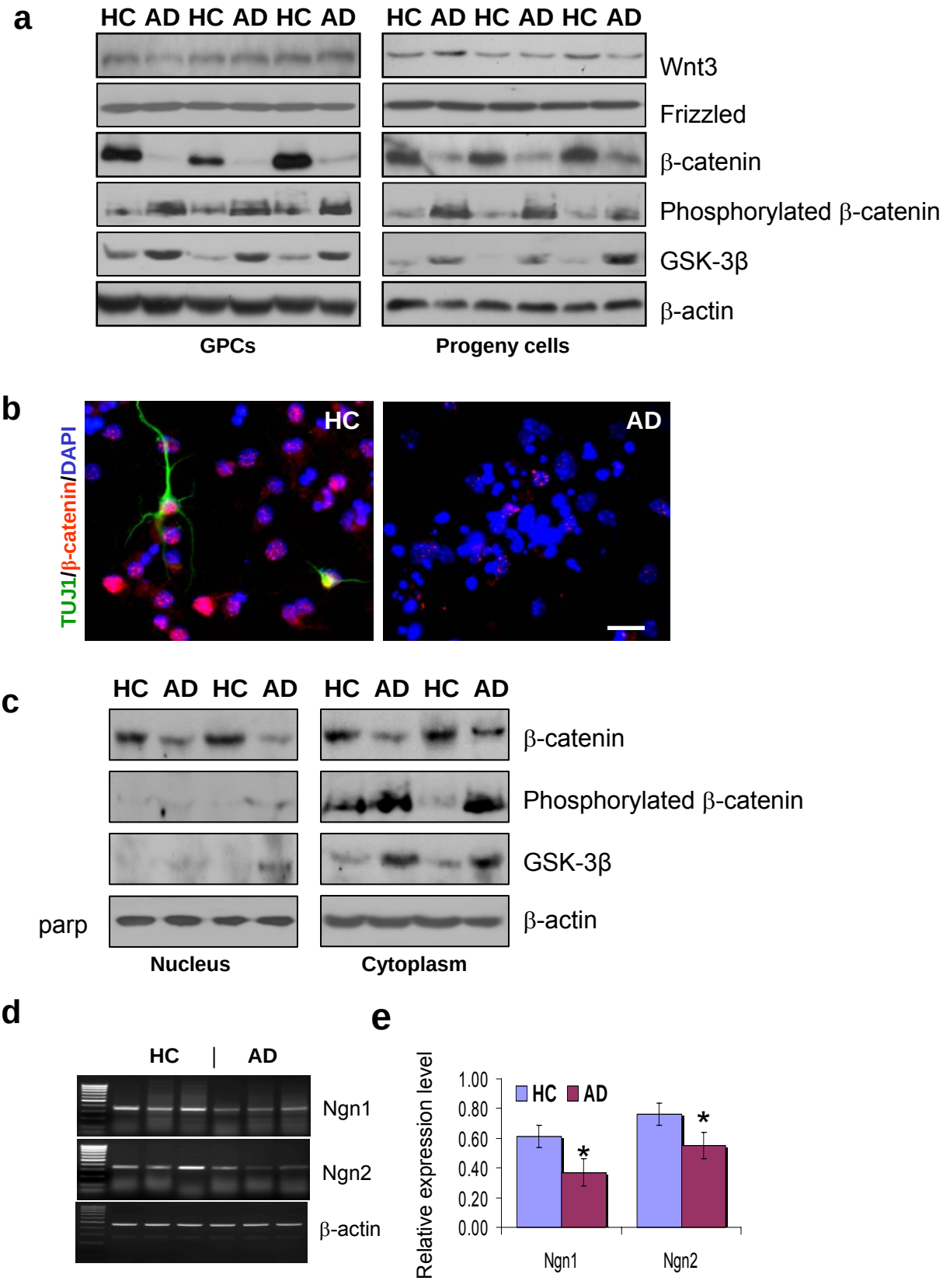
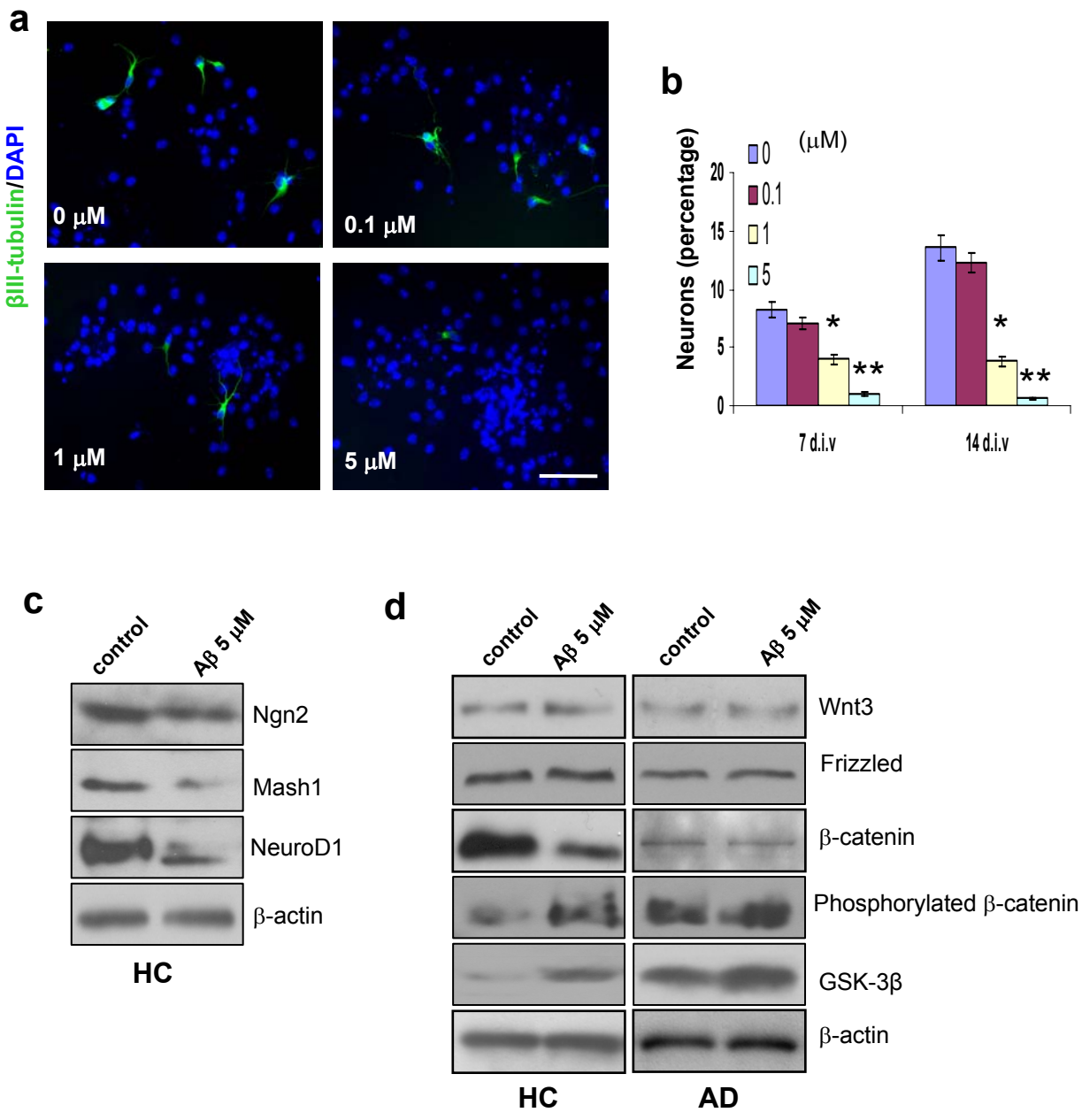
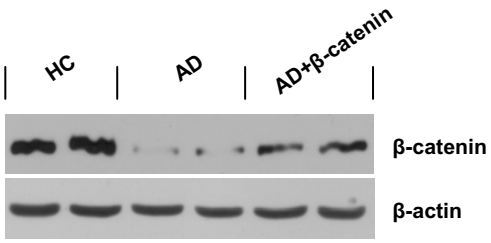

a
Aβ (pg/mg of cells)
200
150
100
50
0
HC
AD
Aβ40
Aβ42
Aβ40
Aβ42
GPCs
progeny cells
b
Normalized Aβ (ng/mg)
1
0.8
0.6
0.4
0.2
0
HC
AD
Aβ40
Aβ42
Aβ40
Aβ42
Medium of GPCs
Medium of progeny cells
c
DCX/DAPI
HC
AD
d
DCX+ Cells (%)
8
6
4
2
0
*
HC
AD
e
HC
AD
PSA-NCAM
DCX
β-actin
f
HC AD HC AD HC AD
Ngn2
Mash1
NeuroD1
β-actin

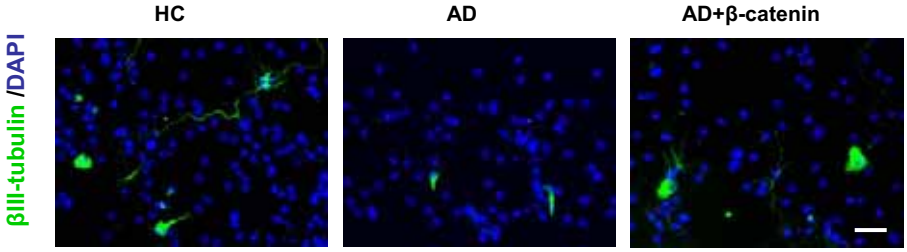

a
HC AD HC AD HC AD
HC AD HC AD HC AD
Wnt3
Frizzled
β-catenin
Phosphorylated β-catenin
GSK-3β
β-actin
GPCs
Progeny cells
b
TUJ1/β-catenin/DAPI
HC
AD
c
HC AD HC AD
HC AD HC AD
β-catenin
Phosphorylated β-catenin
GSK-3β
parp
β-actin
Nucleus
Cytoplasm
d
HC | AD
Ngn1
Ngn2
β-actin
e
Relative expression level
1.00
0.80
0.60
0.40
0.20
0.00
HC
AD
*
*
Ngn1
Ngn2


a
βIII-tubulin/DAPI
0 μM
0.1 μM
1 μM
5 μM
b
Neurons (percentage)
(μM)
0
0.1
1
5
20
15
10
5
0
*
**
*
**
7 d.i.v
14 d.i.v
c
control
Aβ 5 μM
Ngn2
Mash1
NeuroD1
β-actin
HC
d
control
Aβ 5 μM
control
Aβ 5 μM
Wnt3
Frizzled
β-catenin
Phosphorylated β-catenin
GSK-3β
β-actin
HC
AD

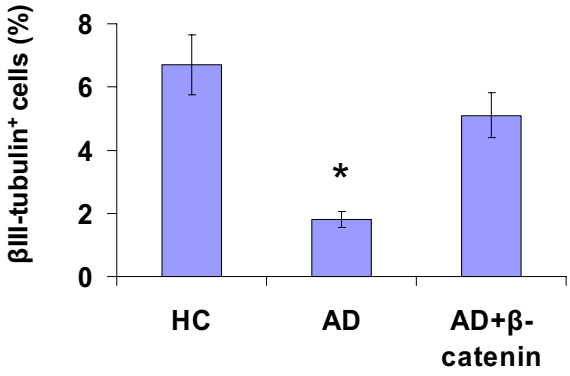
a

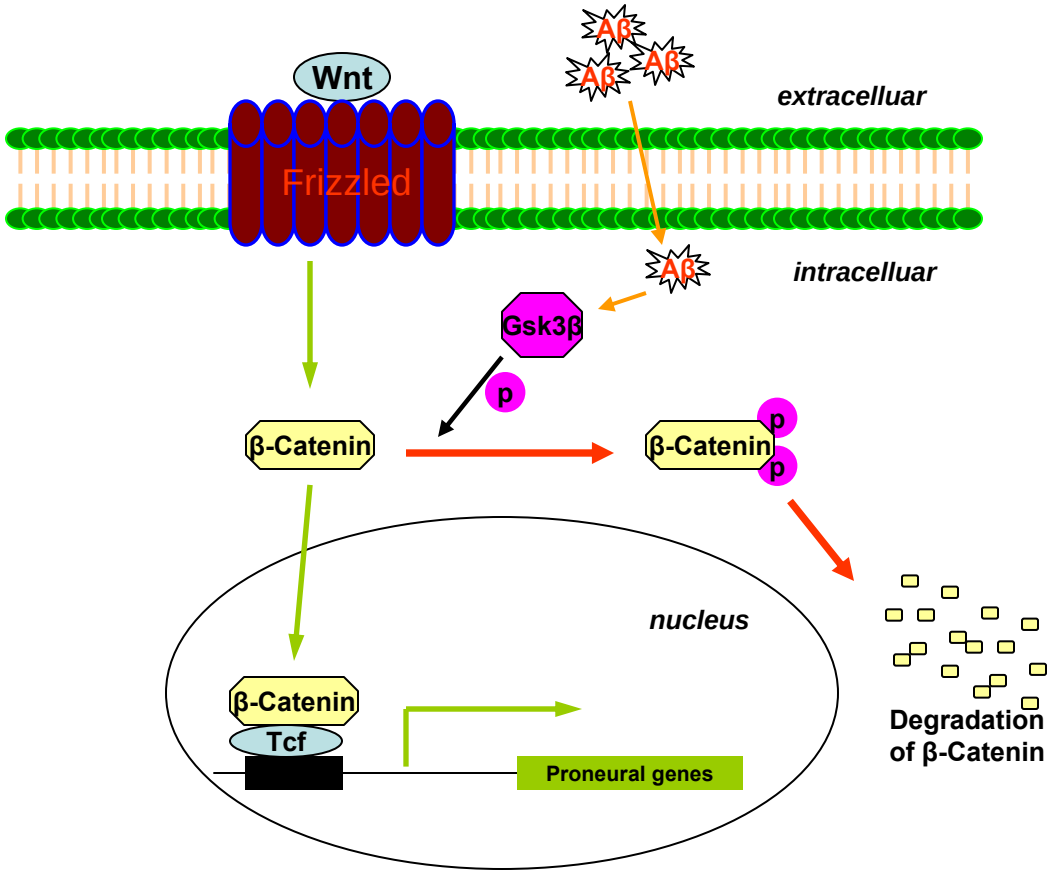

HC
AD
AD+β-catenin
β-catenin
β-actin

b


HC
AD
AD+β-catenin
βIII-tubulin /DAPI

c


βIII-tubulin+ cells (%)
8
6
4
2
0
*
HC
AD
AD+β-catenin


Aβ
Aβ
Aβ
Wnt
extracelluar
Frizzled
intracelluar
Aβ
Gsk3β
p
β-Catenin
β-Catenin
p
p
nucleus
β-Catenin
Tcf
Proneural genes
Degradation
of β-Catenin