Inhibition of glycogen synthase kinase-3 (GSK3) promotes the neural differentiation of full-term amniotic fluid-derived stem cells towards neural progenitor cells

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ABSTRACT

The amniotic fluid has a heterogeneous population of cells. Some human amniotic fluid-derived stem (hAFS) cells have been shown to harbor the potential to differentiate into neural cells. However, the neural differentiation efficiency of hAFS cells remains low. In this study, we isolated CD117-positive hAFS cells from amniotic fluid and then examined the pluripotency of these cells through the formation of embryoid bodies (EBs). Additionally, we induced the neural differentiation of these cells using neuroectodermal medium. This study revealed that the GSK3-beta inhibitor SB216763 was able to stimulate the proliferation of CD117-positive hAFS cells without influencing their undifferentiated state. Moreover, SB216763 can efficiently promote the neural differentiation of CD117-positive hAFS cells towards neural progenitor cells in the presence of DMEM/F12 and N2 supplement. These findings provide an easy and low-cost method to maintain the proliferation of hAFS cells, as well as induce an efficacious generation of neural progenitor cells from hAFS cells. Such induction of the neural commitment of hAFS cells may provide an option for the treatment of neurodegenerative diseases by hAFS cells-based therapies.

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1. Instruction

Human amniotic fluid-derived stem (hAFS) cells are one of the promising techniques for the treatment of degenerative diseases. The hAFS cells have been found to share some properties with pluripotent stem cells. Currently, unlike human embryonic stem cells (hESCs), there is no evidence showing that hAFS cells form teratomas after injection into nude mice (Cananzi et al., 2012; Perin et al., 2008). In addition, hAFS cells are able capable of faster self-renewal in the absence of feeder layers *in vitro*. These advantages make hAFS cells a suitable resource for stem cell based therapies. For the stem cell-based transplantation, rat AFS cells have

been used to treat stroke in rat models, and it was found that both motor and cognitive deficits were reduced after the transplantation. Furthermore, the cell proliferation in the subventrical zone (SVZ) was increased (Tajiri et al., 2014). However, amniotic fluid-derive cells are a heterogeneous cells population with diverse phenotypes. Accordingly, the isolation of amniotic fluid stem cells is very important for certain therapeutic purposes. Thus far, some methods have been established to isolate hAFS cells, such as a single cell isolation method and a cell surface marker-based sorting (Bai et al., 2012; Cananzi et al., 2012; Phermthai et al., 2010; Roubelakis et al., 2012). The protein CD117 is a receptor tyrosine kinase which is expressed on many stem cells (De Coppi et al., 2007; Edling and Hallberg, 2007; Leong et al., 2008). Therefore, the CD117 cell surface marker was used in our study to isolate hAFS cells from other cell types. In addition, pluripotency-related marker OCT4 was used in this study to examine the "stemness" status of hAFS cells. OCT4 is important for early embryo development, especially it functions in the inner cell mass of blastocyst during gastrulation (Foygel et al., 2008; Li et al., 2010). Additionally, OCT4 has been used to obtain induced pluripotent stem cells (iPSCs) from somatic cells. Apart from immunoreactivity, there are other standards to check the

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pluripotency of stem cells. For instance, formation of embryoid bodies (EBs) is one of the "gold standard" (Gao et al., 2014; Kurosawa, 2007; Vigneau et al., 2007). Furthermore, there are some protocols which have been established to promote the formation of EBs from ESCs or iPSCs, including suspension, hanging drop, and microwell cultures (Cerdan et al., 2007; Konno et al., 2005; Kurosawa, 2007; Zhang and Xia, 2011). In this research, we characterize hAFS cells through immunoreactivity and the formation of EBs. Moreover, we induced the neural commitment of hAFS cells towards neural progenitor cells by activating the Wnt/β-catenin signaling pathway, which is closely related with the development of the central nervous system (CNS) (Rosso and Inestrosa, 2013; Zhao et al., 2014). The small molecule SB216763, which we used to activate the Wnt/β-catenin signaling pathway, is widely used as a GSK3-β inhibitor, and activates the Wnt/β-catenin signaling pathway by causing the accumulation of β-catenin (Chairoungdua et al., 2010; Kim et al., 2011). It has been successfully used in animal models and cell culture to activate the Wnt/β-catenin signaling pathway (Jian et al., 2015), thus it is suitable for the intended purpose of this research.

In this study, we focused on the effects of SB216763 on the neural differentiation of CD117-positive hAFS cells *in vitro*. We found that SB216763 promoted the generation of NESTIN-positive neural progenitors, which also co-expressed GFAP and β-tubulin. In addition, we found that the SB216763 treatment could stimulate the proliferation of CD117-positive hAFS cells. Therefore, this protocol provides a low cost, highly efficient method to induce the differentiation of hAFS cells towards neural progenitor cells.

2. Material and methods

2.1. Samples and origin of the human amniotic fluid cells

We collected human amniotic fluid samples from healthy donors by cesarean delivery performed after 37 weeks or more of gestation. The study protocol was subject to approval by the Ethic Committee of the Affiliated Hospital of Guangdong Medical University (Ref. PJ2015014). Amniotic fluid samples were transported on ice, and the processing time was less than 1 h. Cells were immediately isolated from the amniotic fluid by centrifugation at 1000 rpm for 5 min, and then the cell pellet was washed with phosphate-buffered saline (PBS) until the supernatant was clear. Subsequently, the cells were re-suspended in DMEM/F12 (11330, Gibco Life Technology, Grand Island, NY, USA) with 20% fetal bovine serum (FBS, Gibco, Australia) and incubated at 37 °C in a humidified incubator with 5% CO_2 for days until the amniotic fluid-derived cells reached 60–70% confluence. The medium was replaced every 2 days to remove the non-adherent cells.

2.2. Selection and maintenance of AFS

To distinguish hAFS cells from fibroblast-like cells in the primary cell culture, a CD117 MicroBead Kit (130-091-332, Miltenyi Biotec Inc., Bergisch Gladbach, Germany) was used to select CD117-positive AFS cells. When the cells reached confluence, the TrypLETM Express (Gibco, USA) was used to dissociate cells. The magnetic sorting method was used to select CD117-positive AFS cells from heterogeneous primary cultures. The preparation of cells was performed by magnetically labelling the cells, followed by a magnetic separation process following the instruction of the manufacturer of the product (Miltenyi Biotec Inc.). The CD117(+) hAFS cells were cultured in complete growth medium which contains DMEM (Gibco), 20% (FBS, Gibco), 2 mM Glutamine and 0.1 mM (Gibco) non-essential amino acids (NEAA, Gibco).

2.3. The formation of embryoid bodies (EBs)

The hAFS cells were harvested and re-suspended in DMEM/F12 medium plus 10% FBS. The hanging drop method was adopted in this research (Agarwal et al., 2012). Drops of 30 μl containing 250 cells, 500 cells, 1000 cells, 3000 cells, 6000 cells, 12000 cells in suspension were placed on the lid of a 100-mm petri dishes containing PBS, separately. On day 4, all EBs were collected and proceeded to perform the analysis.

2.4. Gene expression analysis

At passage 3–4, the AFS cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of stem cell-related genes and neural development related genes. RNA was extracted using the E.A.N.A TM Total RNA Kit I (R2634-01, OMEGA, USA,). The RT-PCR and quantitative PCR (qPCR) were performed using the PrimeScript RT reagent Kit with gDNA Eraser (RR047A, TAKARA, Japan). PCR reactions were performed using GoTaq-Flexi-DNA polymerase (TAKARA). The primers were used were the following: OCT4-forward: GCAAAGCAGAAACCCTCGTG, OCT4-reverse: GAACCACACTCGGACCACAT; NANOG-forward: CTTCTGCGTCACACCATTGC, NANOG-reverse: CTTCTGCGTCA-CACCATTGC; c-Myc-forward: CTTGTTGCGGAAACGACGAG, c-Myc-reverse: ACTCAGCCAAGGTTGTGAGG; CCND1-forward: CACACGGACTACAGGGGAGTT, CCND1 reverse: CGGCGGATG-GTTTCCACTTC; MMP7-forward: CATGATTGGCTTTGCGCGAG, MMP7-reverse: CTACCATCCGTCCAGCGTTC; MMP14-forward: TGCTGTGTGGAAAACAACGG; MMP14-reverse: TGCAGGGAGT-GAATCAGAAGG; β-ACTIN-forward: CCAACTGGGACGACATGGAG, β-ACTIN-reverse: AGGGATAGCACAGCCTGGAT. The housekeeping gene β-ACTIN was used as the control in all RT-qPCR analyses.

2.5. Protein expression analysis

Immunocytochemistry (ICC) and fluorescence activated cell sorting (FACS) were utilized in this study to monitor the expression of cell specific markers. For ICC, cells were plated on 12-well plates until 60–70% confluence, then the cells were rinsed twice with PBS and fixed with ice-cold 4% paraformaldehyde (PFA) in PBS. Fixation was followed by permeabilization and blocking, and finally stained with antibodies. For FACS analysis, the cells were harvested and fixed with ice-cold 70% ethanol for 5 min, then blocked for 1 h in blocking solution (10% FBS, 0.1% Tween 20, PBS). Next, cells were incubated with primary antibodies for 1 h at room temperature, followed by secondary antibodies staining for 30 min–1 h at room temperature. Fluorescent images were captured using a Leica fluorescence microscope (Leica DMI3000B, Germany). A BD FACSCantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the percentage of positive cells in cultures. The antibodies used were the following: anti-Human CD73/CD90/CD34/CD45/CD31 FITC (BD Biosciences), monoclonal Anti-POU5F1 (OCT4, 1:1000, SIGMA-ALDRICH, Co. Saint Louis, MO, USA), anti-Nestin (1:50, Beyotime, China), neuronal class III β-tubulin (1:250, Beyotime), anti-GFAP (MXB, China), anti-β-catenin (1:50-500, SANTA CRUZ Biotech. Inc., Santa Cruz, CA, USA), anti-Ki67 (MXB), anti-Vimentin (MXB), anti-Cytokeratin (MXB), Alexa Fluor 488 goat-anti mouse IgG2α (1:1000, Life Technologies), Alexa Fluor 488 goat-anti mouse IgG (H + L) (1:1000, Life Technologies) and FITC-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (1:50-200, Jackson, USA). The 4,6 diamidino-2-phenylindole (DAPI) was used as a nucleus staining.

The number of NESTIN, class III β-tubulin and GFAP expressing cells were quantified as the percentage of positive cells by ImageJ Cell Counter software. The percentage of positive cells were quantified by selecting 5 different fields under the microscope.