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Rat full term amniotic fluid harbors highly potent stem cells

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ABSTRACT

Amniotic fluid stem cells (AFSCs) are commonly isolated from mid-term amniotic fluid (AF) of animals and human collected *via* an invasive technique, amniocentesis. Alternatively, AFSCs could be collected at full-term. However, it is unclear whether AFSCs are present in the AF at full term. Here, we aimed to isolate and characterize stem cells isolated from AF of full term pregnant rats. Three stem cell lines have been established following immuno-selection against the stem cell marker, c-kit. Two of the new lines expressed multiple markers of pluripotency until more than passage 90. Further, they spontaneously differentiated into derivatives of the three primary germ layers through the formation of good quality embryoid bodies (EBs), and can be directly differentiated into neural lineage. Their strong stemness and potent neurogenic properties highlight the presence of highly potent stem cells in AF of full-term pregnancies, which could serve as a potential source of stem cells for regenerative medicine.

1. Introduction

Amniotic fluid stem cells (AFSCs) have been successfully isolated in 2007 from mid-term amniotic fluid (AF) of both humans and rodents (De Coppi et al., 2007). These broad multipotent stem cells are characterized based on their expression of the surface antigen, c-kit (CD117, type III tyrosine kinase receptor for stem cell factor), which distinguishes them from amniotic fluid mesenchymal stem cells (AF-MSCs), that do not express c-kit (reviewed in Cananzi and De Coppi, 2012). These AFSCs express *Oct4*, a marker for pluripotent cells (Karlmark et al., 2005; Prusa, 2003), and *Tert*, a component of telomerase associated with immortality (Kim et al., 2007; Mosquera et al., 1999). The cells have a 36 h doubling time and can be cultured up to 300 passages (De Coppi et al., 2007; Phermthai et al., 2010). These cells with high differentiation capacities are able to give rise to tissues derivatives of the three primary germ layers; ecto-, meso- and endoderm lineages (De Coppi et al., 2007). They can be spontaneously differentiated through the formation of embryoid bodies (EBs; Valli et al., 2010), and can also be directed to differentiate under appropriate culture conditions (De Coppi et al., 2007).

Previously described AFSCs also exhibit intermediate characteristics between embryonic and adult stem cells, and are devoid of ethical controversies and are likely to be safer in clinical applications as they do not form tumors upon transplantation (Chen et al., 2011; De Coppi et al., 2007; Pozzobon et al., 2010). The expression of stemness and pluripotency markers, such as Oct4, Nanog, Sox2 and tert, has allowed them to be classified as pluripotent stem cells (De Coppi et al., 2007), however, their inability to form tumors upon transplantation has positioned them as broad multipotent (reviewed in Cananzi and De Coppi, 2012) or potentially pluripotent stem cells (reviewed in Gao et al., 2013). It is therefore obvious that these cells may have good prospects in cell therapy. Their discovery not only identified an alternative source for stem cells, but also marked a potentially significant advancement in regenerative medicine (Da Sacco et al., 2010).

Since then, AFSCs have been isolated from various species including dogs, pigs, horses and buffaloes, targeting mid-term AF collected *via* an invasive procedure, amniocentesis (Chen et al., 2011; Choi et al., 2013; De Coppi et al., 2007; Dev et al., 2012a,b; Filioli Uranio et al., 2011; Iacono et al., 2012; Yadav et al., 2011). Nonetheless, there are concerns about the potential risks posed by the invasive procedure used to collect mid-term AF samples, such as complications associated with infection

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of the amnion sac from the needle, leakage of the sac, and most seriously, miscarriage (Kong et al., 2006; Leschot et al., 1985). Thus, AF from full-term pregnancy could be a better source for isolating AFSCs, as it is usually discarded after birth and collection of the fluid is not harmful to the fetus. A small number of studies have reported the isolation of stem cells from full-term pregnancies. Among these studies, mainly mesenchymal-like cells with limited differentiation potential have been isolated, specifically from two species, humans and dogs (Fernandes et al., 2012; You et al., 2008, 2009; Zheng et al., 2010). Therefore, it is not clear whether AF of full-term pregnancies contains stem cells of high potency, higher than that of AF-MSCs.

In this study, we aimed to isolate highly potent AFSCs from amniotic fluid of rat full-term pregnancies. Cells positive for c-kit were isolated using magnetic activated cell sorting (MACS) on primary full-term amniotic fluid cells that had been expanded in culture. The c-kit positive cells were characterized for cell doubling time, expression of surface markers for mesenchymal, haematopoietic, and endothelial lineages, in addition to intracellular markers for pluripotency. An *in vitro* functional pluripotency test was also carried out by testing the ability of the c-kit positive cells to form good quality embryoid bodies (EBs) and spontaneously differentiate into cell derivatives of the three primary germ layers. Finally, we explored the directed differentiation potential of AFSCs specifically into the neural lineages. Our results strongly suggest the presence of highly potent stem cells in rat full-term amniotic fluid with high differentiation capacity (broadly multipotent). The finding also strongly suggests that it may be possible to isolate AFSCs from AF of full-term pregnancy or during delivery of more complex animals.

2. Materials and methods

2.1. Collection and culture of rat full-term AF cells

Amniotic fluid (AF) samples were collected from time-mated Sprague Dawley rats (n = 3), on the 20th day of pregnancy (full-term). The rats were sacrificed by cervical dislocation before the amnion sacs were removed from the uterus and washed with PBS and placed in a petri dish. AF was then drawn from each sac and pooled under sterile conditions. The pooled AF was then centrifuged at 160 ×*g* for 10 min prior to culture in Amniomax medium (Gibco-Invitrogen) and incubated at 37 °C in 5% CO_2. The primary culture was expanded before attaining the sufficient amount of cells for the isolation of c-kit using miniMACS (Miltenyi Biotec). Animal sampling procedures were approved and performed according to the guidelines established by Animal Care and Use Committee (ACUC) of Universiti Putra Malaysia (UPM) [project no: UPM/FPSK/PADS/BR-UUH/00204].

2.2. Isolation and culture of c-kit positive cells

Immuno-selection of c-kit positive cells was performed using miniMACS kit (MiltenyiBiotec) according to the manufacturer's suggestions. Primary incubation was done with degassed buffer and the primary antibody, c-kit (Santa Cruz, cat no#sc-5535,) at a concentration of 0.02 μg/ml for 5 min at 4 °C. Thereafter, the cells were suspended in 1 ml of cold degassed buffer (Supplementary 1) prior to spinning it down at 103 ×*g* for 5 min. The supernatant was discarded. The pellet was then incubated with 20 μl of goat anti rabbit IgG micro beads (Miltenyi Biotech; cat no #130-048-602) and 80 μl of degassed buffer (1:5 dilution) for 15 min at 4 °C. After the incubation, the cells were washed with the degassed buffer to elute the negative cells. Then the column was removed from the magnetic field and washed with cold degassed buffer in order to elute the c-kit positive cells. The isolated c-kit positive cells were then cultured in ES medium [2.3% sodium bicarbonate, 1 mM L-glutamine, 0.5 mM sodium pyruvate, 1× non-essential amino acids and 0.1 mM β-mercaptoethanol into 1× GMEM (Gibco-Invitrogen), with 15% fetal bovine serum (FBS) (Gibco-Invitrogen), and 10 ng/ml of rat LIF (Chemicon)] in a gelatin-coated flask and subcultured by mild trypsinization.

2.3. Population doubling time

For the calculation of cell doubling time, cells were randomly chosen from P20–50 and grown in a T-25 flask, with initial cell number of 1×10^6 cells and the final live cell counted over 48 h. Cell were then stained with Trypan Blue and calculated using a hemocytometer. Each passage was repeated with at least duplicates. The mean doubling time ± SEM was calculated using an online formula for PDT (www.doubling-time.com).

2.4. Flowcytometry

Intracellular markers: Flowcytometry was performed to characterize the expression of cell intracellular markers, Oct4 and Nanog (1:50; Santa Cruz, cat no#sc-5279, cat no#sc-33760) following an in-house established protocol. Briefly, the c-kit positive cells were grown to 80% confluence and detached from the flasks by mild trypsinization prior to fixation and permeabilization with iced cold methanol for 40 min at 4 °C. The cells were spun down at 447 ×*g*, 4 °C, and then incubated with primary antibodies in 3% BSA for 1 h at 4 °C. The cells were then washed with 1X PBS and incubated with a secondary antibody, IgG-APC (BD Biosciences; 1:200).

Extracellular markers: Flowcytometry was performed to characterize the cell surface markers, namely the typical mesenchymal and adhesion molecules markers (CD90-1, CD44, CD29 and CD71; BD Biosciences; 1:100; CD105; Chemicon; 1:50) and hematopoietic and endothelial markers (CD45, CD11b; BD Biosciences; 1:100; CD34; Santa Cruz; 1:50) in 3% BSA for 1 h at 4 °C. The c-kit positive cells were grown to 80% confluence and detached from the flasks by mild trypsinization prior to incubation with an antibody conjugated with FITC/PE (BD Biosciences; 1:50) for 15 min.

Neuronal markers: For neuronal marker expression, namely nestin (1:50,DSHB, cat#rat-401), Pax 6 (1:50, DSHB, cat#PAX6), class III beta tubulin (1:50; Sigma, cat#T8660), tyrosine hydroxylase, TH (1:50; Abcam, cat#ab6211), and glial fibriliary acidic protein, GFAP (1:50, Sigma,cat#G9269), were examined. The derived neurons were harvested on specific days (nestin and Pax6 after 2 h of monolayer differentiation (MD), class III beta tubulin on Day 2, GFAP on Day 6, and TH on Day 8). The cells were detached from the flasks by mild trypsinization prior to fixation and permeabilization with iced cold methanol for 40 min at 4 °C. The cells were spun down at 447 ×*g*, 4 °C, and incubated with primary antibodies in 3% BSA for 1 h. The cells were then washed with 1 X PBS and incubated with a secondary antibody, IgG-APC (BD Biosciences; 1:200)

All the flowcytometry acquisitions were performed using FACS Fortessa flowcytometer and analyzed using FACS Diva software.

2.5. RNA extraction and RT-PCR

RNA was extracted using RNeasy mini plus kit (Qiagen) and quantitated using Nanodrop (NanoVue). cDNA was then synthesized according to the manual of Reverse Transcription system (Promega). RT-PCR was carried out by amplifying the cDNA at initial denaturation at 95 °C (3 min), followed by amplification of 28–35 cycles at 95 °C (1 min), 50–60 °C (1 min), 72 °C (1 min) and final elongation at 72 °C (5 min). PCR products were analyzed on 2% agarose gel electrophoresis using gel documentation imaging system (Alpha Innotec). The primers used in the PCR were *Oct4* (NM_001009178.2; 272 bp; F: GAGGGATGGCATAC TGTGGAC; R: GGTGTACCCCAAGGTGATCC), *Nanog* (NM_001100781.1; 252 bp; F: TATCCCAGCATCCATTGCAG; R: GTCCTCCCCGAAGTTATGGA G), *Sox2* (NM_001109181.1; 478 bp; F: CCAAGACGCTCATGAAGAAGG; R: CTGATCATGTCCCGGAGGTC), *tert* (NM_053423.1; 185 bp; F: GACATG GAGAACAAGCTGTTTGC; R: ACAGGGAAGTTCACCACTGTC), *beta actin*