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Research Paper

Neural fate commitment of rat full-term amniotic fluid stem cells *via* three-dimensional embryoid bodies and neurospheres formation

Nuratiqah Azmi [a], Siti Sarah Mustaffa Al Bakri [a], Winnie Khor [a], Siti Nurusaadah Hamzah [a], Nurfarhana Ferdaos [a,1], King-Hwa Ling [a,b,*], Norshariza Nordin [a,b,*]

[a] Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[b] Genetics and Regenerative Medicine (ReGEN) Research Group, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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ABSTRACT

Full-term amniotic fluid stem cell (AFSC) is an underexplored reserve of broadly multipotent stem cells with potential applications in cell replacement therapy. One aspect worth exploring is the potential of AFSCs to differentiate into neural lineages. Previously, we have shown that full-term AFSC lines established from term gestation amniotic fluid, known as R3 and R2, differentiated into neural lineage through the monolayer adherent method suggesting their neurogenic potential. The neural commitment of the cells through the formation of multicellular aggregates has never been shown before. Here, we explored the ability of R3 to commit to neural fate *via* the formation of three-dimensional multicellular aggregates, namely embryoid bodies (EBs) and neurospheres, exhibiting distinct characteristics resembling EBs and neurospheres as obtained from other published pluripotent and neural stem cells (NSCs), respectively. Different cell seeding densities of the cells cultured in their respective induction medium generated two distinct types of aggregates with the appropriate sizes for EBs (300–350 μm) and neurospheres (50–100 μm). The neurospheres expressed a significantly high level of Nestin than EBs. However, EBs stained positive for TUJ1, suggesting the presence of early post-mitotic neurons representing the ectodermal lineage. In contrast, the presence of the NSC population in neurosphere culture was validated with positive expression of Sox1. Notably, dissociated cells from both aggregates differentiated into MAP2-positive neural cells, highlighting the ability of both types of multicellular aggregates to commit to the neural fate. In conclusion, this study highlights the first evidence of neurosphere formation from full-term AFSCs in addition to neural fate commitment *via* EBs formation. Findings from this study allow researchers to select the suitable approach for neural cell generation and expansion according to research needs.

1. Introduction

One of the most potent applications of stem cells is the generation of cells and tissues for cell-based replacement therapy. The limited availability of donated organs and host immune rejection have hindered the use of organs or tissues for transplantation (Beyar, 2011; Deroos et al., 2019). On the other hand, stem cells could provide different cell types for a wide range of organ-specific cell replacement therapy. In neural transplantation, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have become popular sources for differentiating into neural stem/progenitor cells. However, hESC sourcing is ethically and politically controversial, and many countries have banned the production of hESC lines that require the destruction of embryos (Matthews and Moralí, 2020). On the other hand, the hiPSCs application is limited by the inefficiency of the reprogramming method. The difficulty in controlling the expression of transcription factors, Oct4, Sox2, Klf4, and c-Myc (OSKM) in genome-integrating delivery approaches may increase the risk of disrupting the existing genome of the adult cells (Medvedev et al., 2010). Aside from that, neural cell lines have also been derived from tumours such as neuroblastomas and glioblastomas for neural transplantation (JH et al., 1987). However, these are considered abnormal cells and do not produce real phenotypes of normal neural cells; hence not entirely suitable for application in neural transplantation (Gottlieb, 2002). Few studies have isolated

* Corresponding authors at: Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
E-mail addresses: lkh@upm.edu.my (K.-H. Ling), shariza@upm.edu.my (N. Nordin).
[1] Current address: Department of Pharmacology and Chemistry, Faculty of Pharmacy Kampus Puncak Alam, 42300 UiTM Cawangan Selangor, Malaysia.

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neural stem cells (NSCs) from rat or mouse embryonic/neonatal brains, which involved sacrificing the animals (Iwamaru et al., 2013). Hence, neural cells derived from a sustainable source and devoid of ethical conflicts are desired for better application in cell replacement therapy.

In recent years, some attention has been directed towards amniotic fluid stem cells (AFSCs) as the alternative source of stem cells for tissue regeneration. AFSCs are broadly multipotent and can differentiate into all three embryonic germ lineages (De Coppi et al., 2007). Aside from their high self-renewal and broad differentiation potential, stem cells from amniotic fluid do not produce teratomas when transplanted into a host and have low antigenicity (De Coppi et al., 2007) hence the cells are not categorised as pluripotent even though they can generate derivatives of the three primary germ layers. The early studies of AFSCs have involved amniotic fluid obtained from mid-term pregnancies (De Coppi et al., 2007; Vlahova et al., 2019). The procedure to get mid-term amniotic fluid involves an invasive technique termed amniocentesis. It has been reported that the risk of early foetal loss in twins undergoing amniocentesis appears to be higher than that of exposed singletons or unexposed twins (Yukobowich et al., 2001), although some studies have concluded that the risks are negligible (Beta et al., 2018; Salomon et al., 2019). Alternatively, amniotic fluid obtained from full-term pregnancies during delivery could eliminate the associated risks since no invasive procedure would be involved. It is also great to use full-term AFSCs as an alternative to ESCs due to the absence of ethical concerns.

Several experiments conducted on animal models have demonstrated the wide-range regenerative application of AFSCs in *in vivo* scenarios such as in regeneration of bone tissue in calvarial bone defect (Basile et al., 2020), regeneration of neural tissue in spinal cord injury (Ataei et al., 2021), wound healing with reduced fibrotic scarring (Zhang et al., 2021), and cartilage repair in osteoarthritis (Zavatti et al., 2020). In a study, human amniotic fluid stem cells (hAFSCs) transplanted into mouse successfully recruited host osteoprogenitor cells into the site of calvarial bone defect, thus promoting regeneration of bone tissue (Basile et al., 2019). Injection of hAFSCs into the lesion site of spinal cord injury promoted regeneration and restoration of the damaged neural tissue (Ataei et al., 2021). hAFSc-derived exosomes accelerated wound healing rate and improved regeneration of hair follicles, nerves, and vessels, as well as increased proliferation of cutaneous cells and the natural distribution of collagen during wound healing, and suppressed the excessive aggregation of myofibroblasts and the extracellular matrix (Zhang et al. 2021).

There are many ways to differentiate the broadly multipotent AFSCs into multiple cell types. The most efficient method could be achieved by forming three-dimensional multicellular aggregates known as embroid bodies (EBs). EBs formation is the 'golden standard' for *in vitro* pluripotency test., as only pluripotent stem cells can form EBs. There are at least three main methods to create EBs; cell suspension culture in the petri dish, hanging drop method, and microwell/capsule method (Liyang et al., 2014). However, the simplest way to form EBs is by cell suspension culture in the bacteriological grade petri dish, which involves the withdrawal of leukaemia inhibitory factor (LIF) from the culture medium to trigger the formation of EBs. Upon aggregation, a cavity will also form in the middle of the EBs caused by cell death, corresponding to the cavitation process during early embryonic development. Bain et al. (1996) used retinoic acid (RA) for four days on mature EBs after growing immature EBs for four days to promote neural lineage. Hence the protocol is known as the 4-/4+ protocol (Bain et al., 1996). An EB contains cells of the three primary germ layers; therefore, generating EBs will be beneficial in producing many organ-specific cells in many types of disorders and diseases.

Neurosphere (NS) is another form of free-floating multicellular aggregates, but in contrast to EBs, the aggregates are made of dividing NSCs. The neurosphere culture can provide neural stem and progenitor cells for neural replacement therapies. Generally, neurospheres are smaller than EBs. Researchers have attempted to achieve the ideal size of neurospheres to generate good-quality neural stem cells. The size of neurospheres is essential to the viability and metabolism of neural stem/progenitor cells (NSPCs) as the transfer of nutrients gradually reduces along with the increasing size of neurospheres (Moeller and Dimitrijevich, 2004). Previous works have reported the size of neurospheres to be between 50 and 250 μm (Mori et al., 2006; Dan Ge, 2012). In one study, neurospheres cultured in flat-bottom 96-well plates formed small sizes (less than 50 μm), possibly resulting from clonal cell division and spontaneous spheres aggregation at a low-frequency U-bottomed wells, more and larger neurospheres formed. They reached a diameter of approximately 250 μm after only 24 h of culture (Mori et al., 2006). On the other hand, a study by Dan Ge (2012) discovered that day-3 neurospheres with diameter within 50–100 μm show a high proliferative ability and increasing growth rate compared to the neurospheres that have larger diameter (more than 100 μm). These studies highlight the importance of neurosphere size on the metabolic activity and viability of NSPCs in neurospheres. As neurosphere size increased, food supply and metabolic waste removal became restricted, resulting in significant and rapid death of neurospheres (Dan Ge, 2012). In contrast to the EB formation assay, neurospheres are only formed in the medium supplemented with epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) (Gottlieb, 2002). The generation of neurospheres will be specifically beneficial for producing NSCs and neural lineage cells for therapeutic applications in various neurological diseases. Additionally, the neurosphere culture system represents a valuable *in vitro* model for studying neurogenesis and neural development.

Previously our group has successfully isolated and characterised two full-term rat amniotic fluid stem cell lines, termed R2 and R3, which have neurogenic potential through monolayer differentiation protocol (Mun-fun et al., 2015). In this study, we aimed to discover the neural fate potential of R3 *via* three-dimensional multicellular aggregates; EBs and neurospheres. We found that different cell seeding densities and appropriate culture conditions promoted the formation of good quality of these multicellular aggregates with neurogenic potential to generate neurons.

2. Methodology

2.1. Maintaining and culturing of rat amniotic fluid stem cell line

R3 cell line was established in-house from amniotic fluid of full-term pregnancy rats (day 20 post-coitum) (Mun-Fun et al., 2015). Passages 35–45 of R3 were used throughout the experiments. Cells were maintained in a complete medium consisting of Glasgow Minimum Essential Medium (GMEM) supplemented with 2.3 % sodium bicarbonate, 0.5 mM sodium pyruvate, 1 × non-essential amino acid (NEAA), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 15 % foetal bovine serum (FBS), all from Gibco Invitrogen, on gelatinised tissue culture-grade flasks or plates (TPP) with a seeding density of 3–4 × 10^4 per cm^2. Additionally, 20 ng/ml of recombinant rat Leukaemia Inhibitory Factor (LIF) protein (Merck Millipore) was added to the medium (Fig. 1). The culture was incubated for 48 h at 37 °C with 5 % CO_2.

2.2. Embryoid bodies formation assay

Embryoid body formation was carried out by dissociating R3 cells into single cells with 0.25 % trypsin with 1 mM EDTA (Gibco Invitrogen). The cells were then cultured in suspension, in a medium consisting of GMEM (Gibco Invitrogen) supplemented with 2.3 % sodium bicarbonate, 0.5 mM sodium pyruvate, 1 × non-essential amino acid (NEAA), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 10 % FBS (Gibco) on 100 mm non-coated bacteriological-grade culture dish (Fig. 1) at a seeding density of 6.0 × 10^4 cells per cm^2 (5.0 × 10^6 cells in 10 ml of medium). The culture was incubated at 37 °C with 5 % CO_2. On day 2, the medium was changed to a fresh medium. After four days, the morphology of the EBs was observed, and the diameter of the EBs was measured.

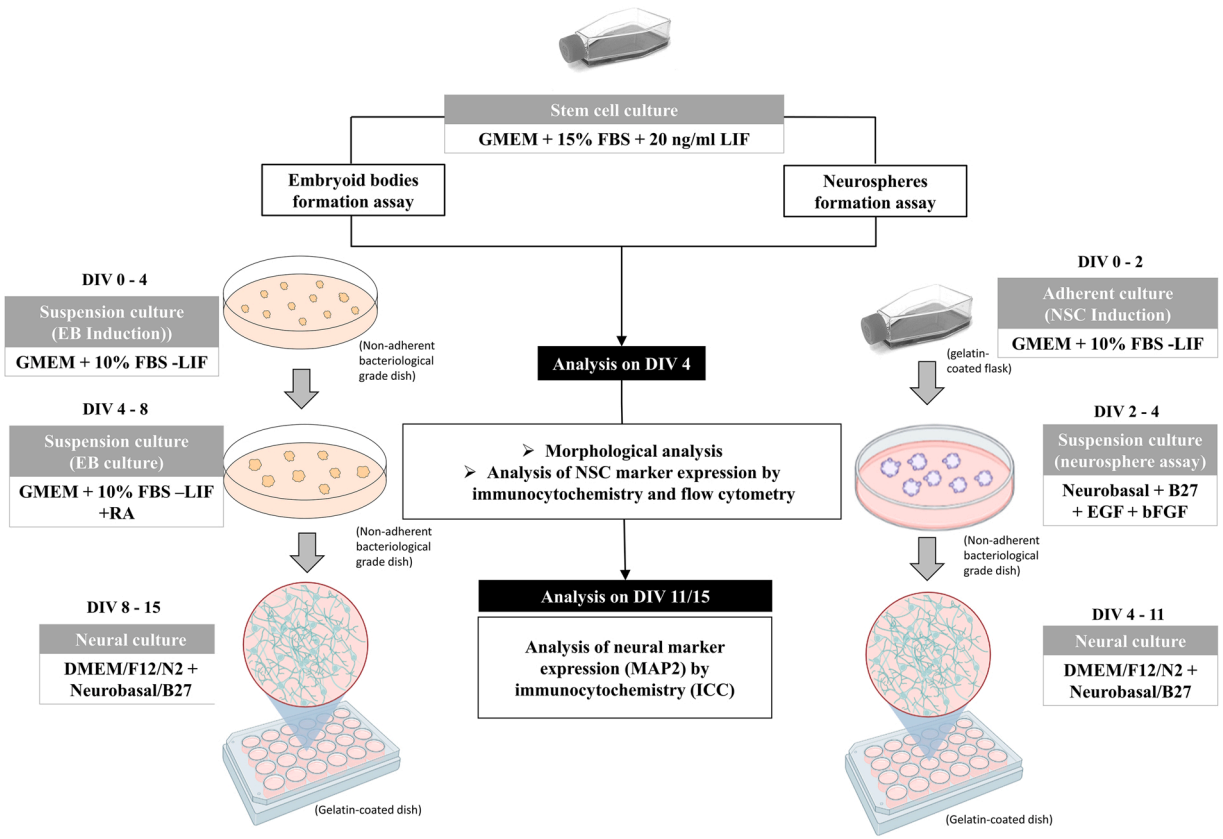

Fig. 1. Summary of the workflow of the study. FBS, foetal bovine serum; LIF, leukaemia inhibitory factor; DIV, day *in vitro*; RA, retinoic acid; NSC, neural stem cell.

On DIV4, the EBs were treated with all-trans-retinoic acid (RA) for four days until DIV8 to promote neural lineage induction before dissociating them into single cells in 4X trypsin (4X trypsin-EDTA, 4 % chicken serum in 1XPBS). The DIV8 EBs were stained for Nestin (Sigma Aldrich) and TuJ1 (Sigma Aldrich).

2.3. Neurosphere formation assay

Our neurosphere formation assay was a two-step method, where firstly, the undifferentiated R3 cells were induced towards NSC lineage by monolayer adherent culture (Fig. 1). The medium consists of GMEM (Gibco Invitrogen) supplemented with 2.3 % sodium bicarbonate, 0.5 mM sodium pyruvate, 1x non-essential amino acid (NEAA), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 10 % foetal bovine serum (FBS) all from Gibco in T25 flask at cell seeding density of 3.0×10^4 cells per cm^2. The culture was incubated for 48 h at 37 °C with 5 % CO_2. The cultured cells were dissociated into single cells in the second step by 0.25 % trypsin with 1 mM EDTA (Gibco Invitrogen). The cells were then cultured in Neurobasal (Gibco) medium supplemented with B27 (Gibco), with the addition of 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (bFGF) on 100 mm non-coated bacteriological-grade culture dish at a concentration of 3.0×10^4 cells per cm^2. The culture was incubated for 48 h in a 37 °C, 5 % CO2 incubator. After 48 h (DIV4), the morphology of the neurospheres was observed, and the diameter was measured, followed by the detection of specific neural stem cell markers, Nestin (Sigma Aldrich) and Sox1 (Sigma Aldrich).

2.4. Neural differentiation

To accelerate the cell dissociation, both types of multicellular aggregates were treated with 0.25 % trypsin-EDTA in a 37 °C water bath for 5 min. Dissociated cells from neurospheres were induced to generate neural cells from DIV4 until DIV11, followed by immunocytochemistry (ICC) detection of MAP2. On the other hand, dissociated cells from EBs were induced to generate neural cells from DIV8 until DIV15 before detecting MAP2 (Sigma Aldrich) (Fig. 1). Single cells were replated into 24-well plates with a seeding density of $2.0–3.0 \times 10^4$ cells per cm^2 in DMEM/F12 medium for seven days in 37 °C, 5 % CO_2 incubator. Half of the medium was changed every two days.

2.5. Morphological analysis

The morphology of the aggregates (EBs and neurospheres) was viewed using a fluorescence microscope (Olympus IX51), and the diameter was measured by CellSens software on DIV4.

2.6. Immunocytochemistry analysis

The ICC was carried out by first fixing the cells in the fixative solution containing 4% paraformaldehyde, 1 M sodium hydroxide, and 1 × phosphate buffer solution (PBS) for 30 min at room temperature. Next, the cells were permeabilised with a solution consisting of 0.1 % Triton-X, 1 % tween 20, and 1 × PBS for 15 min. After permeabilisation, the cells were blocked using a blocking solution composed of 1 % goat serum albumin, 1 % bovine serum albumin, and 1 % Tween 20 PBS for 30 min. The cells were then incubated in primary antibodies at the ratio of 1:200 overnight at 4 °C. Secondary antibodies were added and incubated for 2 h in the dark at room temperature. Then, 100 μL of 4′,6-diamidino-2-phenylindole (DAPI) blue staining solution or propidium iodide (PI) was added to counterstain the nuclei for 10 min in the dark at room temperature. The fluorescence images were viewed using a microscope (Olympus IX51) and analysed using CellSens software. Table 1 describes the antibodies used.

Table 1
The list of antibodies.

Antibodies	Source	Catalogue No.	Dilution
Rabbit polyclonal to Oct4 IgG	Abcam	AB18976	1:300 (Flow cytometry)
Rabbit polyclonal to Nanog IgG	Santa Cruz Biotechnology	SC-33760	1:300 (Flow cytometry)
Rabbit polyclonal to Sox2 IgG	Abcam	AB79351	1:300 (Flow cytometry)
Rabbit polyclonal to Nestin IgG	Sigma-Aldrich	N5413	1:200 (ICC) 1:300 (Flow cytometry)
Rabbit polyclonal to Sox1 IgG	Sigma-Aldrich	GR59210	1:200 (ICC) 1:300 (Flow cytometry)
Rabbit polyclonal to TuJ1 IgG	Abcam	AB15568	1:200 (ICC) 1:300 (Flow cytometry)
Mouse monoclonal to MAP2 IgG1	Invitrogen	13–1500	1:200 (ICC)
Alexa Fluor 488 Goat Anti-Rabbit IgG	Invitrogen	A11032	1:200 (ICC) 1:300 (Flow cytometry)
Alexa Fluor 488 Goat Anti-Mouse IgG	Invitrogen	A11029	1:200 (ICC) 1:300 (Flow cytometry)

2.7. Flow cytometry

Cells were trypsinised into single cells using 0.25 % trypsin-EDTA. 2×10^5 cells were counted and loaded into a round-bottom FACS tube (BD Biosciences). The samples underwent an antibody staining process, where ice-cold methanol was used to fix and permeabilise the cells at 4 °C for 30–40 min. The cells were incubated in a blocking solution containing BSA for 30 min. Subsequently, the cells were incubated in primary antibodies at the ratio of 1:200 for one hour in the dark, then washed with PBS before incubation in secondary antibodies for 30 min. Samples were washed with PBS before being analysed in the FACS machine (FACSAria III, BD Biosciences).

2.8. Statistical analysis

An independent sample t-test was conducted to compare the diameter of embryoid bodies and neurospheres formed from R3 cells for statistical significance. Pre-determined p values ≤ 0.001 were considered statistically significant.

3. Results

3.1. Characterisation of R3

A previous report (Mun-fun et al., 2015) has described the characteristics of R3 cells. In this study, the cultured R3 cells showed good morphology of stem cells, such as having a large nucleus to cytoplasm ratio and having more than two nucleoli (Fig. 2**a**). The average population doubling time (PDT) of R3 cells in this study was 28.17 ± 7.2 h (n = 9, Fig. 2**b**, Supplementary for Fig. 2), which was within a good PDT range for murine AFSCs, which is generally from 24 to 72 h (De Coppi et al., 2007). The morphology of the cells resembled the ones in Mun-fun et al. (2015), which was towards a fibroblast-like shape. We performed flow cytometry analysis on the expression of pluripotency-associated markers (Fig. 2**c–f**) and revealed moderate to high percentage of cell within the population expressing Sox2 (83.3 %), Oct4 (55 %) and Nanog (50.3 %), which corresponds to the findings in Mun-fun et al. (2015). In addition, we also monitored the expression of pluripotency-associated markers (Fig. 2g–i) which demonstrated positive staining for Oct4, Nanog and Sox2. The three markers were also detected to be highly expressed in Mun-fun et al. (2015).

3.2. Characterisation of cellular aggregates

We then subjected the cells to two different approaches for neural fate commitment; the embryoid bodies (EBs) and the neurospheres formation assays. In the EB formation assay, the cells became multicellular aggregates within 24 h in suspension culture. Over days of culture, the size of the aggregates increased. On the day *in vitro* 4 (DIV4), the aggregates were observed to have the distinct characteristics of EBs, such as the apparent presence of cavitation (blue arrows) and having smooth boundaries (orange arrows, Fig. 3**a**). The diameter was measured to determine the size of the cellular aggregates. The average diameter of the cellular aggregates formed in the EB formation assay was 311.4 ± 118.7 μm (n = 10, Fig. 3**d**).

In the neurospheres formation assay, after 48 h of adherent culture (DIV2), the cell morphology resembled NSCs (Fig. 3**b**). The dissociated cells, after 24 h (DIV3) cultured with growth factors, EGF and bFGF, started to form multicellular aggregates (neurospheres). On DIV4, the formation of the neurospheres was more apparent with the appearance of microspikes **(**green arrows**,** Fig. 3**c)**. The average diameter of the cellular aggregates formed in the neurospheres formation assay was 68.8 ± 12.8 μm (n = 10) which is significantly smaller than the diameter of the embryoid bodies (Independent t-value 6.42, $p < 0.001$, Fig. 3**d**, Supplementary for Fig. 3).

3.3. Neurogenic potential

We next assessed the neurogenic characteristics of both cellular aggregates on DIV8 for EBs and DIV4 for neurospheres before they were being dissociated for neural assays. The immunocytochemistry analysis indicated that cellular aggregates in the EBs formation assay were negative for the Nestin marker (Fig. 4**a–c**). We expected EBs to express the markers of the three primary germ layers, including ectodermal lineage that gives rise to neural lineage. We then assessed TuJ1 and observed its remarkable expression in the EB culture, confirming the neural lineage potential of the EBs (Fig. 4**e–g**).

Immunocytochemistry analysis indicated that the neurosphere formation assay stained positively for Nestin (Fig. 4**h–j**). In addition, the neurospheres also stained positive for Sox1, confirming the presence of neural stem cells (Fig. 4**l–n**). The expression of both Nestin and Sox1 was observed primarily near the outer layer of the aggregates. We quantified the corrected total cell fluorescence (CTCF) of all markers by using ImageJ software. For EBs, the CTCF value for Nestin and Tuj1 normalised against DAPI was observed to be 1.63 and 4.39, respectively (Fig. 4**d**, Supplementary for Fig. 4). For neurospheres, the CTCF value for Nestin and Sox1 normalised against DAPI was 1.55 and 1.74, respectively (Fig. 4**k**, Supplementary for Fig. 4).

To further evaluate the efficiency of our protocol in producing good-quality multicellular aggregates to generate neural cells, the aggregates were dissociated and subjected to neural differentiation for seven days. On day 7 post-plating, the morphology of dissociated cells from both cultures was observed (DIV11 neurospheres-derived neural culture and DIV15 EBs-derived neural culture). The cells in both cultures were observed to exhibit neural-like morphology with EBs-derived neural cells to have more neurite extension (shown by the arrows, Fig. 5**a**). Both neural cultures showed expression of MAP2 (a marker for mature neurons), suggesting neuronal cell differentiation and commitment from both EBs and neurospheres (Fig. 5**b, e**).

4. Discussion

Fast, scalable, and reproducible neural differentiation of full-term amniotic fluid stem cells is essential to attain their full potential for research and clinical applications. Full-term amniotic fluid stem cells (AFSCs) are neurogenic in *in vitro* culture. We have utilised a full-term AFSC line derived from rats, called R3 and R2 cells, to understand the neural fate commitment of full-term AFSCs. In this study, we have

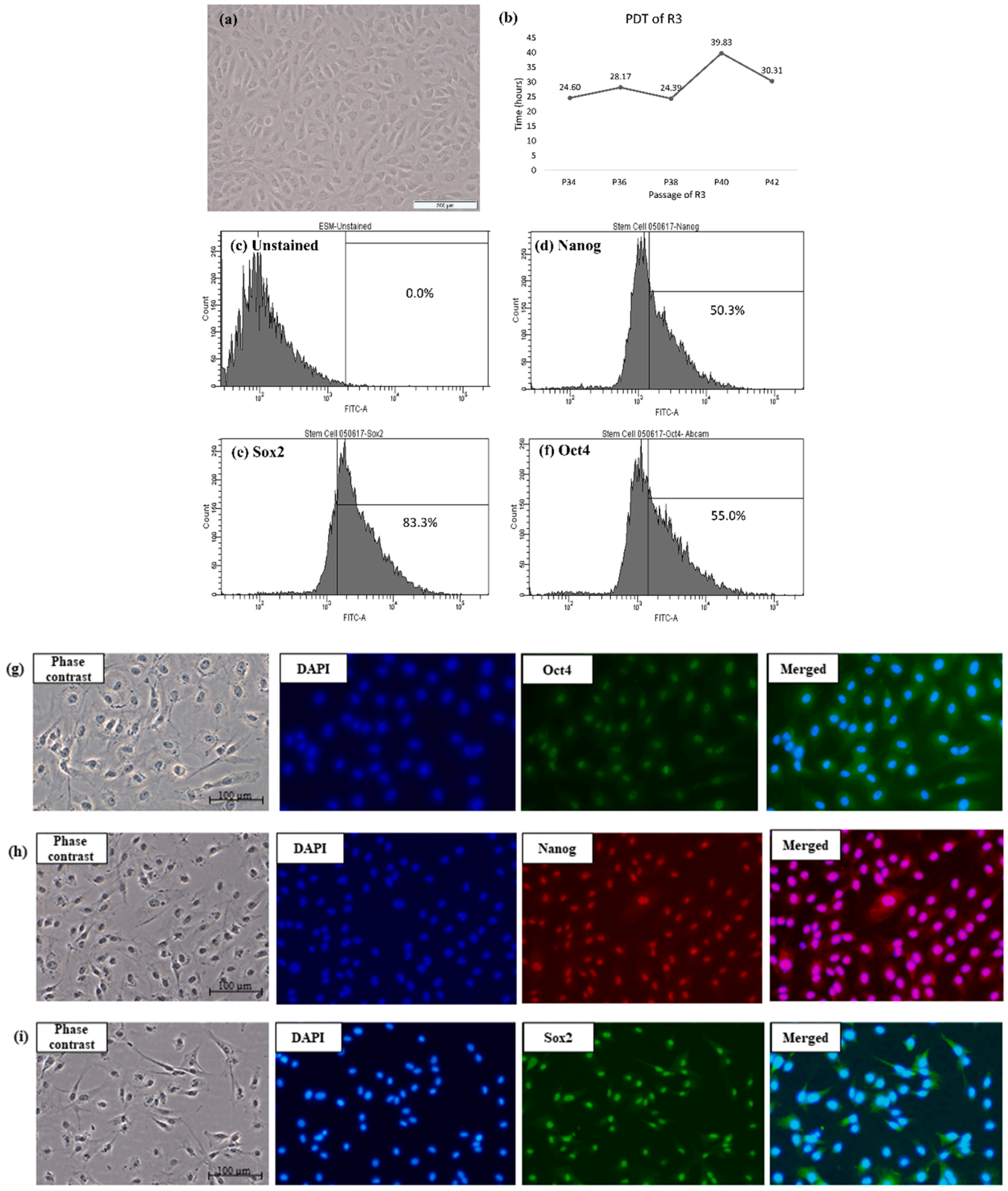

Fig. 2. Characterisation of R3 showing (a) morphology of the undifferentiated R3, (b) R3 population doubling time, (c) Unstained flow cytometry analysis of R3, (d–f) flow cytometry analysis of pluripotency-associated markers; Sox2, Nanog and Oct4, (g-i) immunocytochemistry analysis of pluripotency-associated markers.

managed to show the ability of R3 cells to form into two types of cellular aggregates, EBs and neurospheres. Our EBs formation assay is a single-step protocol that depends on the withdrawal of pluripotency factor (LIF) to induce the formation of multicellular aggregates in suspension cultures in the presence of serum seeded with high cell seeding density. No growth factors were added to the culture. On the other hand, the neurosphere formation assay is a two-step protocol that involves the withdrawal of pluripotency factor (LIF) and serum to induce them into neural stem/progenitor cells (NSPCs). Then two growth factors, EGF and bFGF, were added into a serum-free medium to promote the NSPCs to

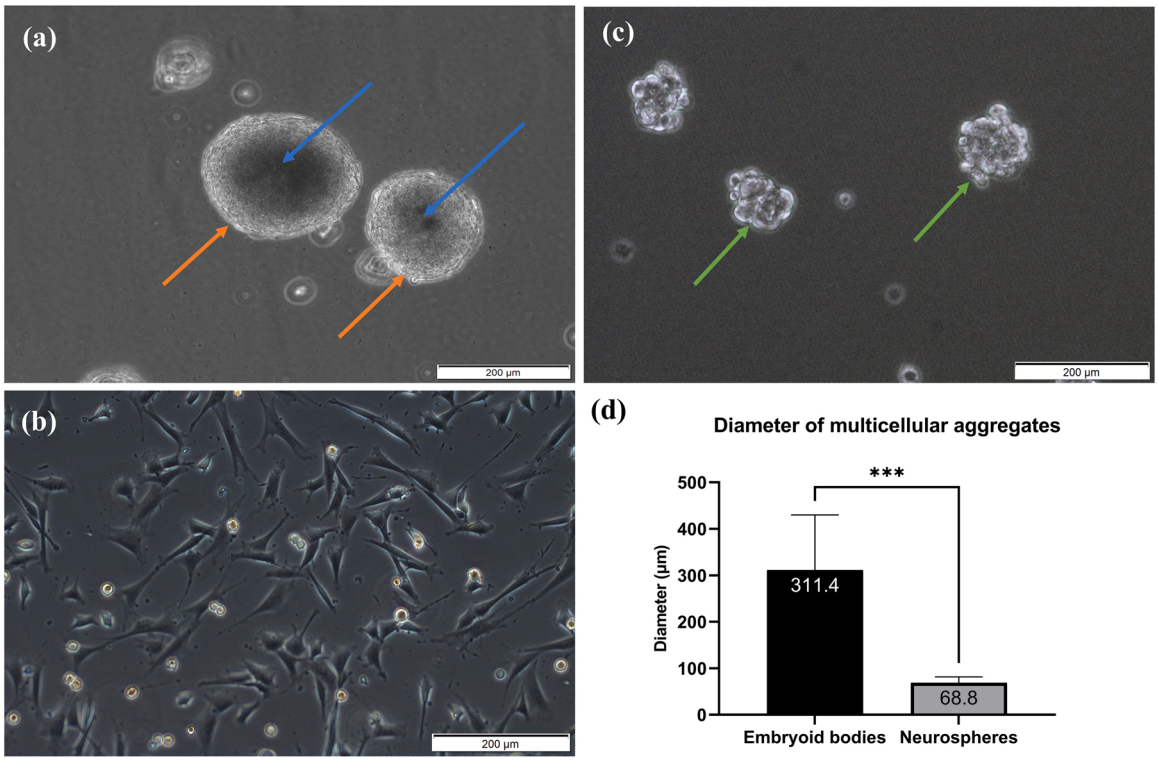

Fig. 3. The formation of multicellular aggregates from R3 on DIV4, showing (a) the morphology of EB, the orange arrows showing the smooth boundaries of EBs and the blue arrows indicating the cavitation process. Cells in the NSC induction medium after 48 h of adherent culture show (b) the morphology of neural stem/progenitor-like cells before the cells were subjected to neurospheres formation as illustrated in (c) showing the appearance of microspikes as pointed by the green arrows. The bar graph illustrates (d) the average diameter of both types of aggregates observed on DIV4. The error bar indicates ± standard deviation (n = 10) with *** indicates significant difference between the EBs and neurospheres with p value < 0.001.

form neurospheres. To our knowledge, no study has attempted to generate neurospheres from full-term AFSCs.

The multicellular aggregates formed in the EBs culture exhibit good quality EBs, such as the apparent presence of cavitation and a smooth boundary. The presence of cavitation in the EBs mimics the event of gastrulation during embryonic development, in which pluripotent epiblast cells are allocated to the three primary germ layers (ectoderm, mesoderm and endoderm) (Loebel et al., 2003). This feature is constant with the finding documented earlier by Mun-fun et al. (2015) with EBs generated from R3 and R2 cells. The aggregates produced in this study are within a good range of EBs size based on previously documented studies, which was between 100 and 400 µm (Valamehr et al., 2008; Liyang et al., 2014). The formation of EBs within the good size range ensures further differentiation into derivatives of the ectodermal, mesodermal, and endodermal lineages. On DIV8 EB culture, the multicellular aggregates formed expressed a very low level of Nestin, signifying the low to the absence of the NSC population. However, there is a clear expression of TuJ1, a marker for post-mitotic neurons, which represents ectodermal lineage. Culturing of dissociated cells from the aggregates formed neural-like morphology with MAP2 expression. Based on the characteristics analysed, it is evidenced that the multicellular aggregates formed in this study were EBs, which can further differentiated into neurons based on MAP2 and Tuj1 expression.

In neurospheres culture, the multicellular aggregates formed have a significantly smaller diameter than EBs. The size of aggregates in the group is within a reasonable range of neurosphere size as reported by other studies, between 50 and 250 µm (Mori et al., 2006; Dan Ge, 2012). The aggregates that are produced in this study can be classified as a good quality neuropsheres, as most of the aggregates in average have diameter within 50–100 µm, in which cells in the neurospheres with diameter around 50–100 µm has shown to have a good proliferative ability and higher growth rate (µ) within 72 h of culture (Dan Ge, 2012). The study showed that the neurospheres that have larger diameter (more than 100 µm) to have reduction in cell viability and metabolic activity after 80 h of culture. The presence of cavitation is not evident, unlike in EBs. The morphology of multicellular aggregates generated in this study resembles neurospheres generated from human bone marrow stromal cells and human adipose-derived stem cells documented in other studies (Suzuki et al., 2004; Peng et al., 2019). Furthermore, there was a remarkable expression of both key markers of NSPCs, Nestin, and Sox1, strongly signifying the presence of the NSC or NSPC population. Culturing of the dissociated cells from the aggregates generated MAP2-positive neural cells. Based on the characteristics analysed, the multicellular aggregates formed in our study were concluded as neurospheres, which can be differentiated into neurons based on the MAP2 expression.

We could infer that culture condition is the key to direct different cell commitments based on the results. EBs were formed fully in suspension culture with serum (FBS) and without LIF as early as after a day they were cultured on a non-adhesive substratum coated plate (we used a bacteriological-grade dish). The same culture medium was used to generate NSCs, and a treated-cell culture plate is needed to allow adherent culture conditions, and the withdrawal of LIF in adherent culture can induce the generation of NSCs. In contrast to EB formation, neurosphere formation requires growth factors (EGF and bFGF) in a serum-free medium (Neurobasal). In the adult central nervous system, EGF and bFGF are factors that drive the cell fate and maintenance of NSCs (Reynolds et al., 1992; Bithell et al., 2008). The addition of EGF and bFGF into neurospheres forming medium has been commonly included in many previous studies to generate neurospheres from the CNS tissues (Marshall et al., 2008), embryonic stem cells (Azari et al., 2011), and iPSCs (Zhou et al., 2016). Therefore, we showed that the same growth factors are the critical drivers of generating neurospheres from mammalian full-term amniotic fluid stem cells.

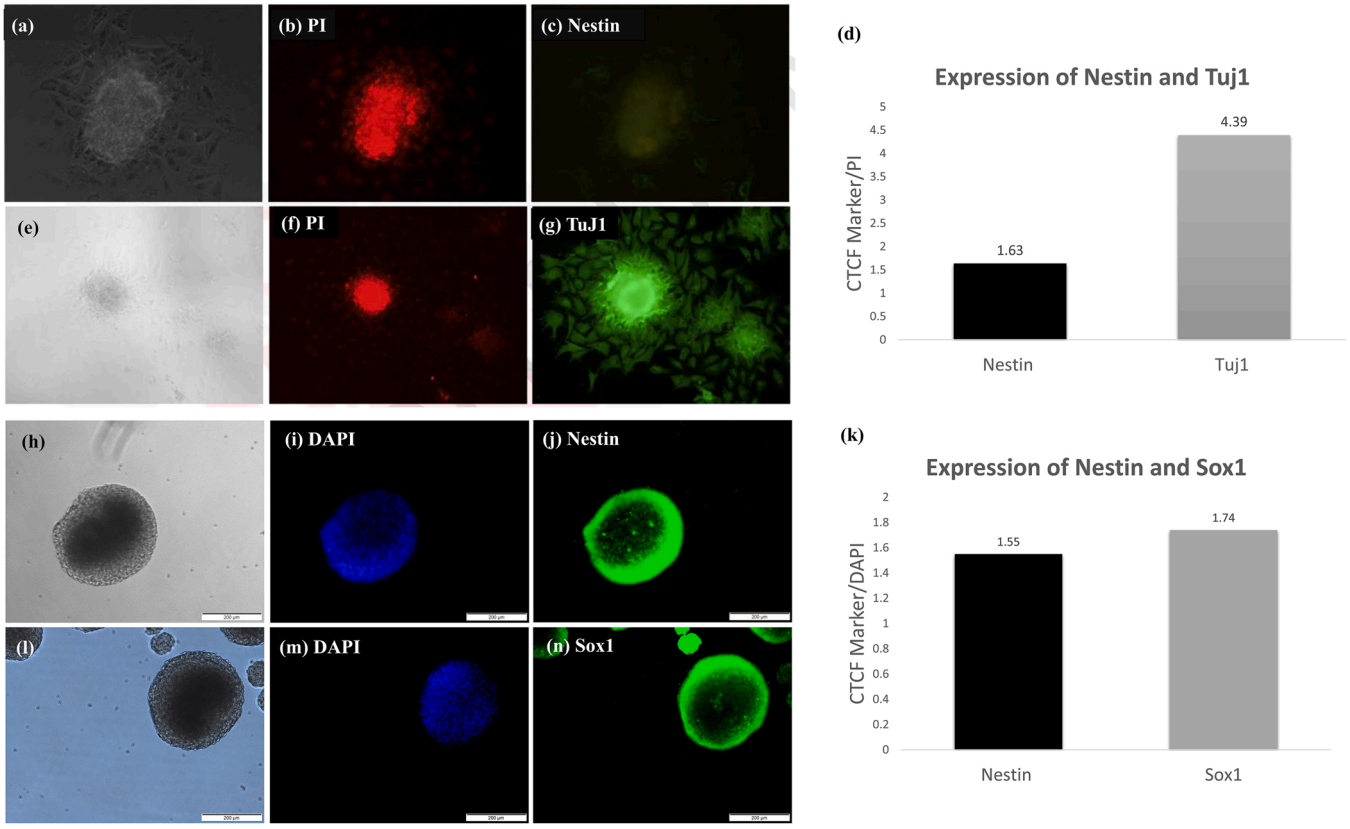

Fig. 4. Protein markers expression by immunocytochemistry on DIV8 EBs and DIV4 neurospheres before the aggregates were dissociated for neural differentiation assay. The aggregates (a,e,h,l) under phase contrast and their respective nuclei counterstaining with propidium iodide (PI) for EB (b,f) and with DAPI for neurospheres (i,m). DIV8 EBs exhibit very low Nestin expression (c,d) with higher expression of TuJ1 (d,g). A higher expression of Nestin (j,k) than Sox1 (n,k) was observed in the DIV4 neurosphere culture. The markers expression was quantified by measuring the CTCF value normalised against DAPI expression using ImageJ (d,k).

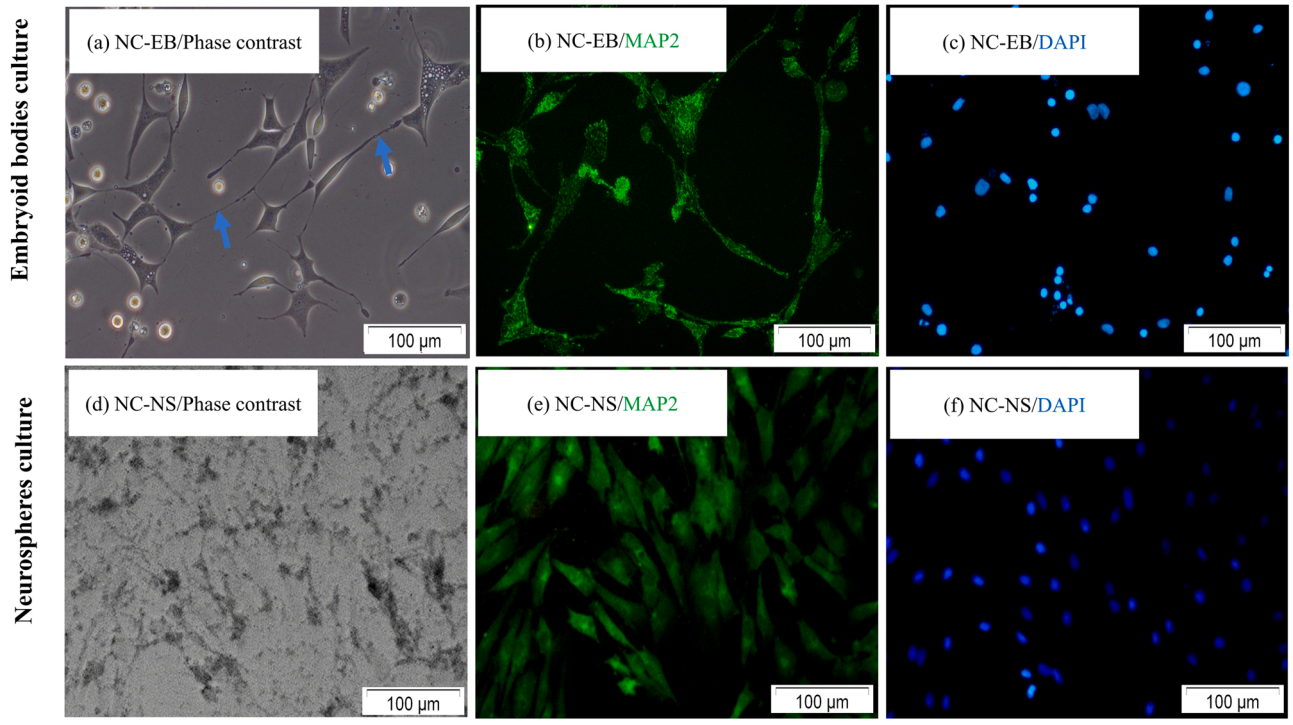

Fig. 5. Expression of MAP2 on day 7 post-plated EBs- and neurospheres-derived neural cultures. DIV15 of EBs-derived neural cultures (NC-EB, a) and DIV11 of neurospheres-derived neural culture (NC-NS, d) under phase contrast with arrows pointing at neurites in NC-EB, showing the expression of MAP2 (b,e), and nuclei counterstaining with DAPI (c,f).

Interestingly, the initial step where the stem cells were cultured in GMEM with 10 % serum as adherent culture was found to be essential to promote the formation or transdifferentiation of AFSCs into NSC-like cells (based on the morphology, Fig. 3b). We had observed that direct application of EGF and bFGF into single cells of AFSCs did not generate neurospheres. It is important to note that the cell seeding density also played an important factor in directing the AFSCs to NSCs and then to form neurospheres. We used 3×10^4 cells/cm^2 for the initial step for NSC induction followed by the same density to generate neurospheres, compared to a higher seeding density, 6×10^4/cm^2, to generate EBs in our study.

5. Conclusion

Our findings reveal the ability of stem cells from a merely discarded fluid, mammalian AFSCs from full-term gestation, to differentiate into neural lineage through two different approaches. Using the slight difference in the protocol, the cells have successfully committed to neural lineage *via* forming two types of multicellular aggregates, the embryoid bodies (EBs) and the neurospheres. We found the cells committed to neural lineage *via* neurospheres formation when the stem cells were seeded at 3×10^4 cells/cm^2 as adherent culture in 10 % serum and GMEM with supplements before being dissociated as single cells to form neurospheres in serum-free medium supplemented with growth factors. On the other hand, the stem cell's commitment to forming EBs was achieved through a high seeding density, 6×10^4 cells/cm^2 in the presence of serum-contained medium cultured on a non-adhesive substratum plate. The neural lineage was enhanced with the addition of RA in a serum-contained medium on mature floating EBs.

The study highlights the successful generation of neural lineage *via* neurospheres, representing the first evidence of amniotic fluid stem cells obtained from amniotic fluid of full-term mammalian gestation to undergo transdifferentiation into NSCs. Both multicellular aggregates formed from R3 stem cells were technically reproducible and warranted further study for biological reproducibility. However, the finding offers further research on full-term amniotic fluid stem cells in regenerative medicine, particularly as the prospective source for neural cells. Although the results presented in this study are obtained from murine stem cells, the rationale lies behind the adaptation of the study to higher-order mammalian organisms, including humans.

CRediT authorship contribution statement

Nuratiqah Azmi: Investigation, Visualization, Writing – original draft. **Siti Sarah Mustaffa Al Bakri:** Investigation, Writing – original draft. **Winnie Khor:** Investigation. **Nurussaadah Hamzah:** Investigation. **Nurfarhana Ferdaos:** Investigation. **King-Hwa Ling:** Supervision, Writing – review & editing. **Norshariza Nordin:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing.

Compliance with ethical standards

The rat full-term amniotic fluid stem cell lines used in this study were obtained from in-house established cells from the amniotic fluid day 20th pregnant rats (Mun-Fun, 2015). The experiment on the establishment was conducted upon approval from the Animal Care and Use Committee of the Faculty of Medicine & Health Sciences, Universiti Putra Malaysia in 2010 (approval number: UPM/FPSK/PADS/BR-UUH/00-204).

This current study used the frozen cells obtained previously. No procurement of new cells was carried out in this study.

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Conflicts of Interest

The authors declare no conflict of interest.

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Contributions

The authors contributed equally to this research.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2023.01.003.

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