Am J Transl Res 2017;9(2):611-619
www.ajtr.org /ISSN:1943-8141/AJTR0036071

Original Article

Which has more stem-cell characteristics: Müller cells or Müller cells derived from in vivo culture in neurospheres?

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Received July 18, 2016; Accepted January 27, 2017; Epub February 15, 2017; Published February 28, 2017

Abstract: Objective: Müller cells can be acquired from in vitro culture or a neurosphere culture system. Both culture methods yield cells with progenitor-cell characteristics that can differentiate into mature nervous cells. We compared the progenitor-cell traits of Müller cells acquired from each method. Methods: Primary murine Müller cells were isolated in serum culture media and used to generate Müller cells derived from neurospheres in serum-free culture conditions. Gene expression of neural progenitor cell markers was examined by Q-PCR in the two groups. Expression of rhodopsin and the cone-rod homeobox protein CRX were assessed after induction with 1 μM all-trans retinoic acid (RA) for 7 days. Results: After more than four passages, many cells were large, flattened, and difficult to passage. A spontaneously immortalized Müller cell line was not established. Three-passage neurospheres yielded few new spheres. Genes coding for Nestin, Sox2, Chx10, and Vimentin were downregulated in cells derived from neurospheres compared to the cells from standard culture, while *Pax6* was upregulated. Müller cells from both culture methods were induced into rod photoreceptors, but expression of rhodopsin and CRX was greater in the Müller cells from the standard culture. Conclusion: Both culture methods yielded cells with stem-cell characteristics that can be induced into rod photoreceptor neurons by RA. Serum had no influence on the "stemness" of the cells. Cells from standard culture had greater "stemness" than cells derived from neurospheres. The standard Müller cells would seem to be the best choice for transplantation in cell replacement therapy for photoreceptor degeneration.

Keywords: Stemness, neurospheres, Müller cells

Introduction

Radial glia serve as the primary progenitor cells of the developing vertebrate central nervous system, and are capable of generating neurons and neuroglia. In the retina, these cells are referred to as Müller cells and are the most common glial cell type there. Müller cells are important in development of the retina and are currently under active study for their role in neural regeneration. Of particular interest are Müller cells of teleost fish, such as zebra fish, which are capable of regenerating a damaged retina [1, 2]. Some regenerative capacity of Müller cells has also been detected in chickens [3] and mammals, including rats [4, 5], mice [6, 7], and even humans [8].

When the retina of a zebrafish is damaged, the Müller cells can re-enter the cell cycle and generate nearly all retinal cell types [1, 2]. The cells can be induced to differentiate into either photoreceptors [9, 10] or ganglion cells [11-14] when supplied extrinsic factors and transfection genes. Cells can be grown in a monolayer culture and directly induced to differentiate into neurons by external factors [15]. Alternately, they may first be de-differentiated into neurospheres that acquire a three-dimension structure in serum-free culture. The resulting neurospheres subsequently differentiate into neurons and glial cells [11-13, 16]. The Müller cells that differentiated in the first protocol (denoted the standard protocol here) cannot be considered to be true stem or progenitor cells, while in

the second method, after the cells dedifferentiate, they acquire the properties of stem or progenitor cells [17-19]. Despite this difference, it appears that the same type of neurons can be induced by either method.

The true progenitor nature of the cells that are acquired in the neurosphere method is appealing, but there are concerns regarding the method. It is costly due to the supplements required for the culture, and it is more time consuming than the first (direct) protocol. The ideal procedure for generating neurospheres, such as the correct number of passages, seeding density, and the equipment employed, has not been established and the uncertainty of the procedures has yielded low viability, leading to widespread lack of confidence in this protocol [20]. Nonetheless, it is not certain which method is superior.

In the present study, we assessed and compared Müller cells derived from neurospheres with Müller cells grown in standard culture. We compared gene expression of the markers of stem or progenitor cells in both cell types. More stem-cell markers were upregulated in the standard cells compared to the neurosphere-acquired cells, except for expression of *Pax6,* an embryonic transcription factor. When the two groups of cells were induced to differentiate into rod photoreceptors by all-trans retinoic acid (RA), expression of rhodopsin was greater in standard Müller cells than cells derived from neurospheres. Our study suggests that standard Müller cells are the proper choice for seed cells for endeavors such as cell replacement for photoreceptor degeneration.

Materials and methods

Müller cell culture

Male or female C57BL/6J mice (5-7 days old) were obtained from the Animal Experiments of Central South University, Changsha, China, according to the approval of the Animal Research Committee, Xiangya School of Medicine, Central South University, Changsha, China. Animals were rapidly killed by cervical dislocation under anesthesia with isoflurane. Briefly, the eyes were enucleated under sterile conditions and placed in ice-cold phosphate buffer solution (PBS). The cornea and lens were removed, and neuroretina were dissected 0.5 mm from the oraserrata to avoid contamination by retinal stem cells at the ciliary margin, taking care not to disrupt the underlying retinal pigmented epithelia. The neuroretinas were dissociated into small aggregates, digested by Trypsin-EDTA (0.25% trypsin, 2% EDTA [trypsin-EDTA], Gibco, USA) for 5 minutes, and then pipetted vigorously. The trypsin-EDTA was then neutralized in Dulbecco's modified Eagle's medium F12 (DMEM/F12, Hycolon, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, USA), filtered through a stainless-steel sieve, and centrifuged at 1000 rpm for 5 minutes. The cell pellets were re-suspended in DMEM/F-12 containing 15% FBS and seeded on 25 cm^2 culture flasks at 37°C in an incubator under 5% CO_2. The cells were passaged via trypsinization every 4-5 days. Cells from passage 2-3 were used in the experiments, and will be referred to here as standard Müller cells.

Neurosphere culture

We generated neurospheres according to the well-established standard technique that is frequently used to isolate and propagate neural stem cells [21]. Müller cells from the second passage (see above) were dissociated with trypsin-EDTA and cultured in a serum-free de-differentiation media containing DMEM/F12 supplemented with N-2 and twice the usual Vitamin B27 without Vitamin A (all from Gibco, USA), 20 ng/mL murine epidermal growth factor (EGF, Peprotech, USA), 10 ng/mL murine basic fibroblast growth factor (bFGF2, Peprotech, USA), and 2 mM L-glutamine (HyClone, USA) at a density of 1×10^5 cells/cm^2. Half of the de-differentiation medium was changed every other day. For passaging, neurospheres were collected, centrifuged, and digested with Accutase (Invitrogen, USA) for 8 minutes at 37°C and mechanically triturated. Accutase was selected because it could achieve higher viabilities and fast recovery after dissociation than other enzymatic methods [22]. The cells were pelleted at 1000 × g for 5 minutes at room temperature and resuspended in the same serum-free de-differentiation medium.

Differentiation

Cells prepared by both methods were collected and transferred to 6-well plates precoated with poly-L-lysine (Sigma, USA). After 24 hours, the medium was changed to DMEM/F12 medium

Table 1. List of antibodies

Antibody	Dilution (IF)	Dilution (WB)	Species	Source
GS	1:100		Rabbit	Abcam ab73593
Vimentin	1:50		Mouse	Abcam ab8978
Sox2	1:150		Rabbit	Abcam ab92494
Pax6	1:100		Rabbit	Abcam ab5790
Nestin	1:100		Mouse	Abcam ab6320
Rhodospin		1:500	Mouse	Abcam ab5417
Crx		1:10000	Rabbit	Santa Cruz sc-30150

IF: Immunofluorence; WB: western blot.

Table 2. List of primers

Gene	Forward	Reverse
GS	aggcaccagtaccacattcg	ggccgacggtcttcaaagta
Vimentin	gatcagctcaccaacgacaa	ctttcggcttcctctctctg
Nestin	agcaactggcacacctcaag	ccaagagaagcctgggaact
Sox2	agggctgggagaaagaagag	acttggcggagaatagttgg
Chx10	aagcccactacccagatgtc	tctccctcttcctccacttg
Pax6	agtgaatgggcggagttatg	tacgcaaaggtcctggtttc
β-actin	gtggggcgccccaggcacca	ctccttaatgtcacgcacgatttc

containing 1% FCS, 2 mM L-glutamine, and RA (Sigma, USA) diluted in DMSO to a concentration of 1 μM for 7 days. Half of the medium was changed every other day.

Immunocytochemical analysis

Immunocytochemical analysis was performed as previously described [12]. Briefly, cells on slides were fixed in 4% paraformaldehyde in PBS for 30 min, and then blocked in PBS containing 5% goat serum and 0.3% TritonX-100 at 37°C for 1 h, followed by incubation with the primary antibodies overnight at 4°C, as listed in **Table 1**. Following PBS washing, the secondary antibodies were added, including coat anti-mouse IgG (ZSGB-BIO, China), and coat anti-rabbit IgG (Multi Sciences, China). Nuclear counterstaining was conducted with 4',6'-diamidino-2'-phenylindole (DAPI; Sigma, USA) for five minutes. Fluorescent images were recorded using confocal microscopy (Leica SP8, Germany) or fluorescent microscopy (Leica DM5000B, Germany).

Edu labeling analysis

To evaluate the proliferation of stem cells, freshly passaged cells were incubated with 1:1,000 5-ethynyl-2'-deoxyuridine (Edu; RiboBio, China) diluted in culture solution overnight at 37°C. After washing several times, the cells were fixed with 4% paraformaldehyde for 30 minutes, incubated with an Apollo buffer for 30 minutes at room temperature in the dark, washed with 0.5% TrixtonX-100 (diluted in PBS) for 10 minutes, and stained with Hoechst 33342 (RiboBio, China) at room temperature for 30 minutes in the dark. Images were captured using confocal microscopy (Leica SP8, Germany) or fluorescent microscopy (Leica DM5000B, Germany).

Quantitative RT-PCR

Total RNA was isolated from the cells using the RNeasy kit (Bioflux, Japan). RNA was reversely transcribed using RT-PCR system (Promega, USA). Quantitative PCR was carried out by All-in-one™ qPCR Mix (GeneCopoeia, USA). The list of primers is shown in **Table 2**.

Western blot analysis

Proteins were extracted from cells using the Radio Immuno Precipitation Assay (RIPA; RiboBio, China) buffer containing a protease inhibition cocktail (Sigma, USA). Alpha-tubulin was used as a loading control in each experiment. Samples of 50 μg were loaded onto 15% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at 280 mA for 55 min. The membranes were blocked with 5% skimmed milk in Tris buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at 37°C, and then incubated with primary antibodies overnight at 4°C. After washing several times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Primary antibodies are listed in **Table 1**. Secondary antibodies included horseradish peroxidase-conjugated donkey anti-rabbit IgG, HRP-coat anti-mouse IgG and HRP-coat anti-goat IgG (all from Multi Science, China). The bands were semi-quantified by densitometry using Bio-Rad imaging software.

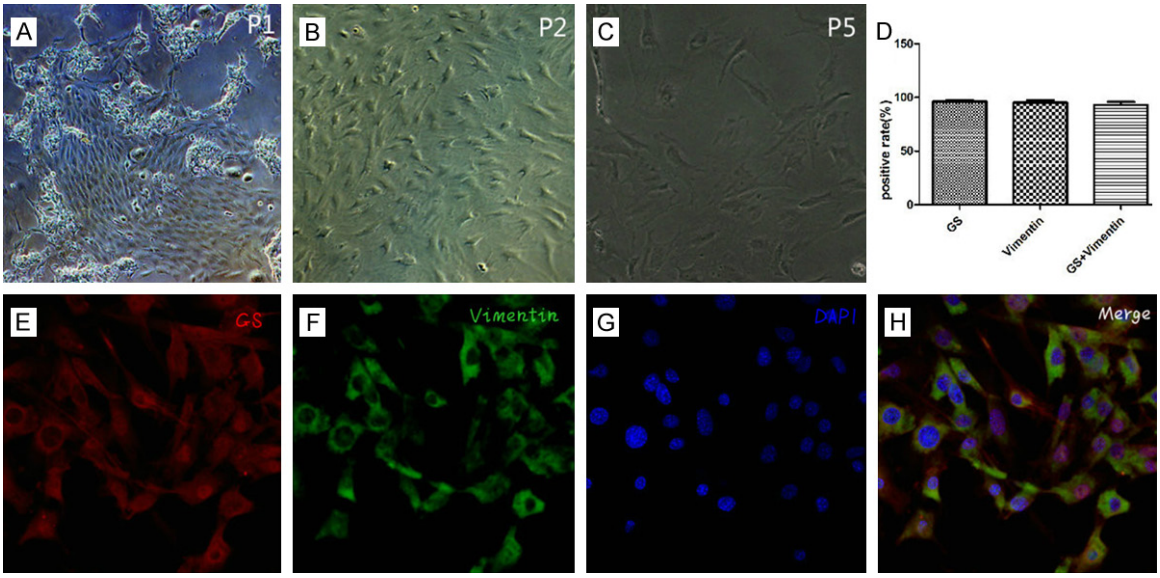

Figure 1. Primary muller cells are cultured in vitro and expresse GS and Vimentin. (A-C) Different passages of muller cells. P2 (passage 2) müller cells have uniform size and shape, with abundant cytoplasm and well-defined membranes. Scale bar: 50 μm (B). Many cells of P5 become larger and flattened morphology (C). (E-H) Many of cells expresse GS (red) and Vimentin (green), the markers of mature muller cells. Scale bar: 20 μm. (D) Statistical analysis shows P2-3 cells are nearly pure retinal muller cells.

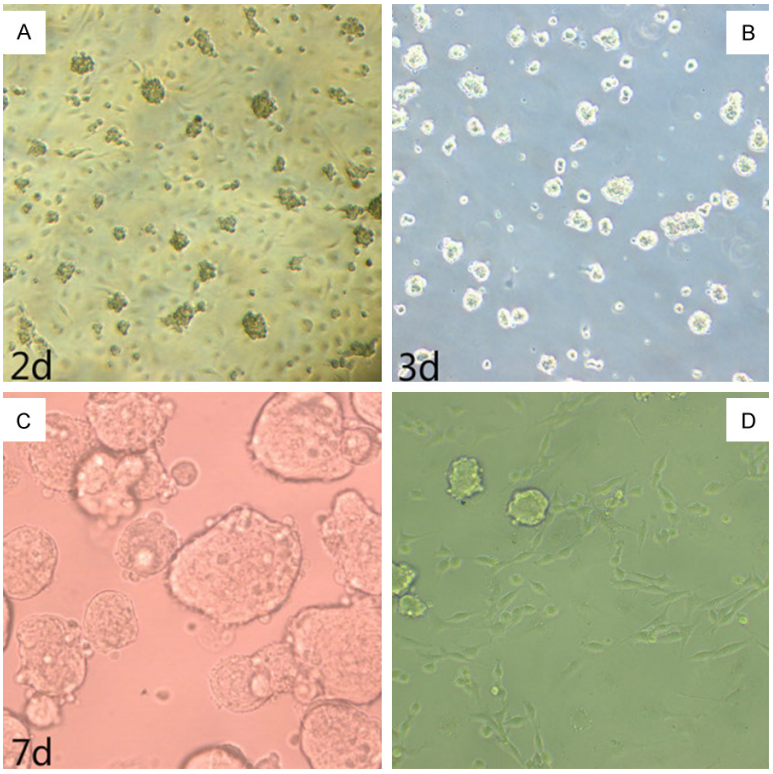

Figure 2. Muller cells-derived neurospheres. (A-C) P2 müller cells are cultured in stem cell conditioned medium for 2 days (A), 4 days (B), 7 days (C). Neurospheres constitutively increase in both number and size with days. (D) The third spheres are cultured for 7 days. Spheres are less number and smaller size.

Statistical analysis

Gene expression results were analyzed using the paired two-tailed Student's t test. Protein expression was analyzed by one-way analysis of variance. All data were expressed as the mean ± SD. Statistical analyses and creation of graphs were carried out using Graph pad Prism 6.0 software. Differences were considered to be significant when $P<0.05$.

Results

Generation of neurospheres derived from Müller cells

After 4-5 days in primary culture (**Figure 1A**), some cloned neurospheres were apparent. Ten to fourteen days later, the cells had formed a complete confluent mono layer. Generations of neurospheres derived from Müller cells from passages 2 and 3 had uni-

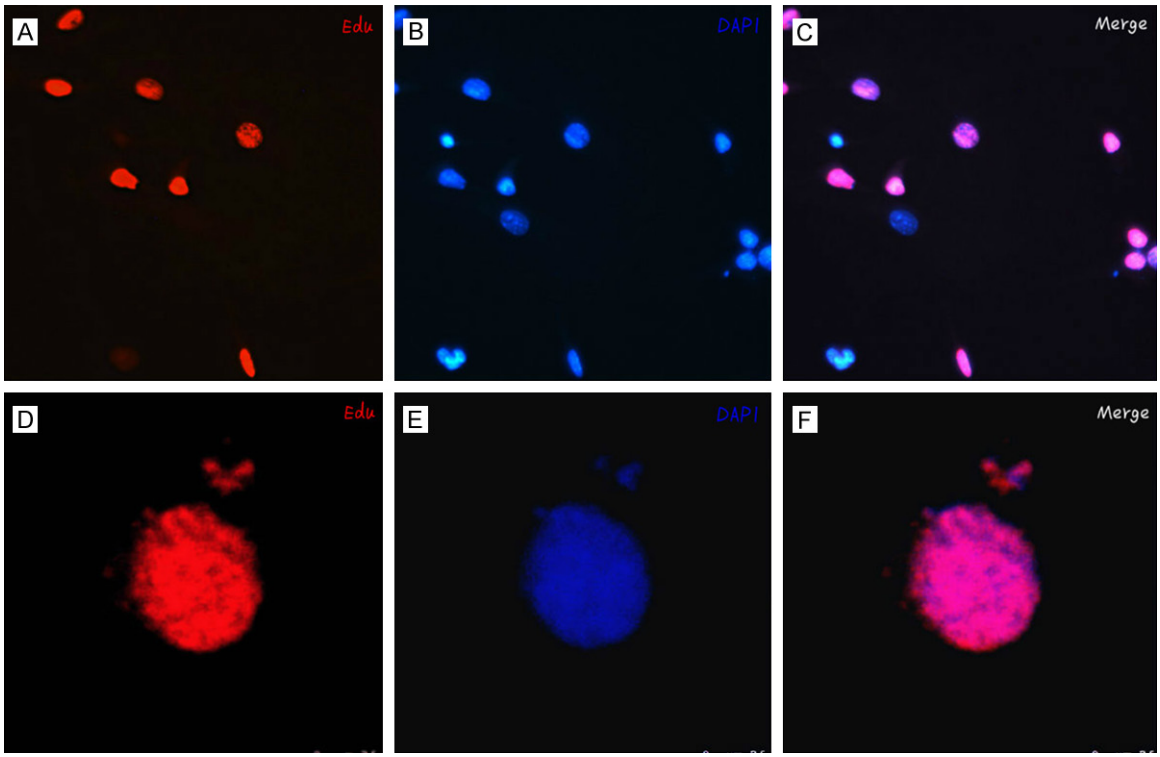

Figure 3. Edu assay showed that many of P2-3 muller cells (A-C) and cells in neurosphere (D-F) had proliferative capacity. *Scale bar*: 50 μm (A-C), 20 μm (D-F).

form size and shape, abundant cytoplasm, and well-defined membranes (**Figure 1B**). More than 90% of the cells were immunoreactive to both Vimentin, glutamine synthetase (GS), and markers of mature Müller cells (**Figure 1D-H**). After more than four passages, however, many cells became larger, morphologically flattened, and difficult to passage (**Figure 1C**). We did not succeed in establishing a spontaneously immortalized Müller cell line as described by Jeons et al. [23]. This may be because mammalian Müller cells have adult progenitor-cell properties rather than embryonic progenitor-cell properties [24]. We concluded that the Müller cells we derived from passages 2 or 3 in the neurosphere assay were nearly pure retinal Müller cells. These were used in the rest of the study.

Culture of neurospheres is reported to be more difficult than the standard culture, as mentioned above. In our study, Müller cells after passage 2 were cultured in stem-cell-conditioned medium for 2-3 days, and some small spherical or mulberry-shaped cells aggregation appeared (**Figure 2A**). These increased in number and size and had good refraction and well-defined boundaries (**Figure 2B**); we referred to these entities as neurospheres (**Figure 2C**), and they remained stable for more than 7 days. Neurospheres were dissociated into single cells by Accutase, and resuspended in the stem-cell-conditioned medium, leading to the generation of "secondary neurospheres." These secondary neurospheres did not readily give rise to more neurospheres, and more time was required to generation the third generation, which were not numerous and small (**Figure 2D**). As more passages were performed, almost no new neurospheres were generated. Our results are contrary to Florian C et al. [25], who observed that murine Müller cells from older cultures-passages 7 and more-were able to form neurospheres and differentiate into neuronal-like cells, while Müller cells before passage 6 did not form neurospheres, but only fibrous tissues.

Expression of neural stem or progenitor marks by Müller cells and neurospheres

An Edu assay showed that many of the neurosphere-derived cells (**Figure 3A-C**) and neurospheres (**Figure 3D-F**) had proliferative capaci-

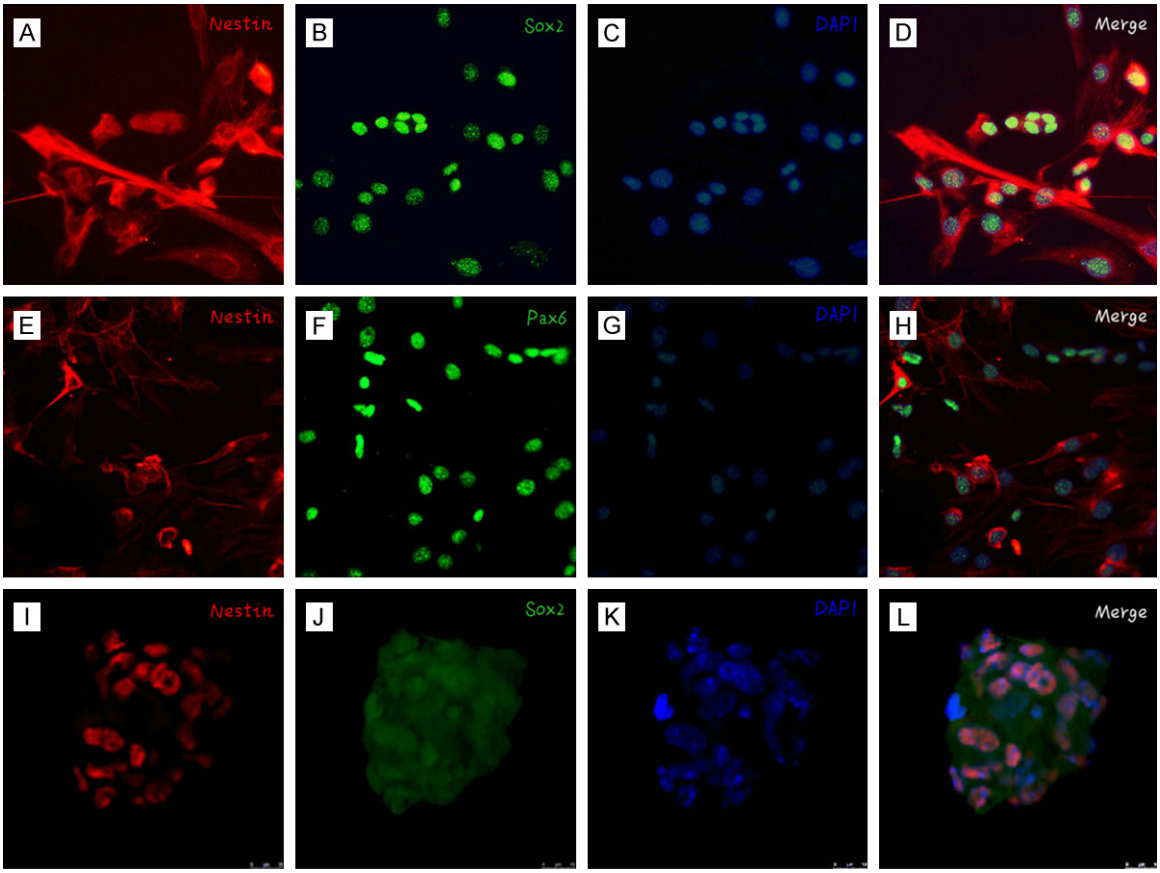

Figure 4. Müller cells and müller cells-derived neuroshperesboths express Pax6, Sox2, Nestin, the markers of stem/progenitor cells. A-D. Müller cells express Nestin (red), Sox2 (green), DAPI (blue). E-H. Müller cells express Nestin (red), Pax6 (green), DAPI (blue). I-L. Muller cell-derived neurospheres express Nestin (red), Sox2 (green), DAPI (blue).

ty. Immunofluorescence staining for the stem or progenitor markers Nestin, Sox2, and Pax6 was positive in these cells and neurospheres (**Figure 4**). Pax6 was not detected because it had been reported [13]. Their positive rates were not calculated and compared with each other, because neurospheres were comprised of hundreds of cells and it was difficult to make correct data of the three dimensional structure.

Q-PCR analysis showed that mRNA coding for Nestin, Sox2, chx10, and Vimentin was downregulated in neurospheres compared with standard Müller cells, while Pax6 was upregulated (**Figure 5A**) compared to neurosphere-derived cells. Sox2 is required for survival of Müller stem cells, maintenance of progenicity in vitro [26]; it is upstream of Pax6 [27]. The downregulation of Sox2 and upregulation of Pax6 in cells derived from neurosphere culture compared to cells in standard culture supports the conclusion that the cells from the standard culture had more "stemness" than cells from the neurospheres culture. The expression of GS and Vimentin supports the conclusion that cells kept the characteristics of their original phenotype.

Müller cells and neurospheres were induced into photoreceptors by RA

There are numerous reports that neurospheres derived from Müller cells cultured in vitro could be differentiated to photoreceptors, but the ratio ranged greatly [15, 28, 29]. In our study, the Müller cells that were acquired from either culture system could be induced into photoreceptors by RA. Western blot analysis demonstrated that expression of rhodopsin protein, a marker of the rod photoreceptor, was increased in both groups of cells, but more so in Müller cells from the standard culture than in cells derived from neurospheres. **Figure 5B**, **5C**

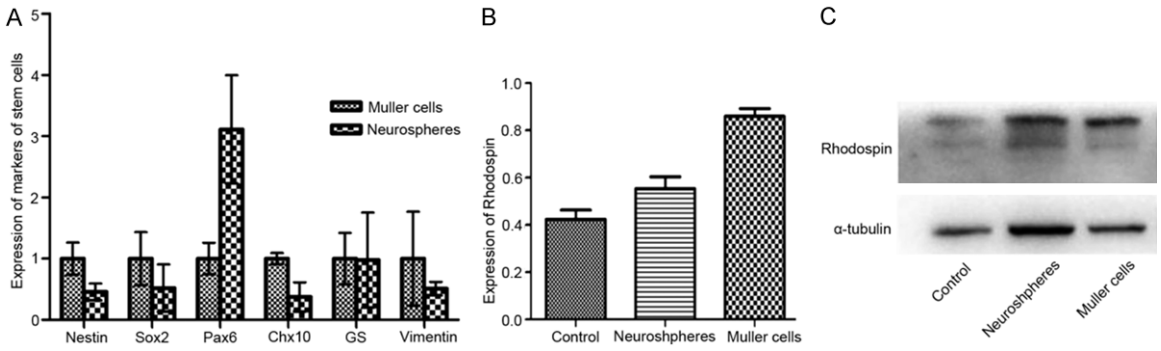

Figure 5. Comparison of gene expression of markers of stem/progenitor cells and rhodopsin protein induced by RA between P2-3 müller cells and müller-derived neurospheres. A. Q-PCR analysis showed that mRNA coding for Nestin, Sox2, chx10, Vimentin were downregulated in neuroshperes compared with müller cells, while Pax6 were upregulated. B. Q-PCR analysis demonstrated that mRNA of Rhodospin induced by RA was upregulated in P2-3 müller cells compared with müller cells. C. Western blot analysis demonstrated that expression of rhodopsin protein induced by RA between P2-3 müller cells and müller-derived neurospheres

shows that expression of rhodopsin was 1.6 times greater in cells from the standard culture than in neurosphere-derived cells.

Discussion

In the present study, murine Müller cells formed neurospheres in stem-cell-conditioned medium in vitro, and further passage and immunohistochemical analysis for Nestin and Sox2 revealed that pure neurospheres contained these proteins. This leads us to suggest that the cells dedifferentiated and acquired neuronal properties, as has been reported [11-13, 15, 25]. However, some studies have reported that primary-culture Müller cells have stem-cell characteristics and contain Nestin [15, 30]. The Müller cells cultured in our study also expressed Nestin, Pax6, and Sox2. We wished to determine the differences between the standard Müller cells and the neurosphere-derived Müller cells, so we examined gene expression of the markers of stem or progenitor cells by Q-PCR. We were surprised to find that the expression of most markers of stem or progenitor cells, such as Nestin, Sox2, and Chx10, was greater in the standard Müller cells than in the neurosphere-derived cells. We suggest that the cultured murine Müller cells were stem cells, consistent with Lawrence's conclusion [8]. We suppose that dissection and digestion of Müller cells can stimulate them to re-enter the cell cycle and dedifferentiate into stem or progenitor cells, even in the absence of exogenously applied growth factors. The purpose of using serum-free suspension culture is to prevent factors within the serum from inducing differentiation, which could promote stem or progenitor cell proliferation. And sustaining the stem or progenitor characteristics by applied external growth factors. These results confirm those of Lawrence [8] and suggest that serum does not influence stem cell properties of Muller cell.

However, Lawrence [8] also reported that human Müller glial cells with stem characteristics did not proliferate when cultured in stem-cell-conditioned medium, but proliferated when cultured with serum. The presence of FGF2 in the media even induced their differentiation. Since the expression of markers of stem or progenitor cells was greater in Müller cells cultured under standard conditions than in the neurosphere-derived cells, it may be that the passage we consider to be passage 1 (P1) neurospheres came from P2-3 Müller cells, equal to P3-4 Müller cells, so that P2 neurospheres would correspond to P4-5 Müller cells. In vitro, many murine Müller cells of more than four passages became larger and flattened in morphology and were difficult to passage. The Müller cells derived from neurospheres in this study did not give rise to many additional spheres. Few third generation spheres were generated; in addition, they were small and the generation took a long time. The higher-passaged neurospheres yielded almost no new spheres. The results indicate that with many passages, cells' "stemness" declined. There was more Glutamine synthetase and Vimentin expression in the neurosphere-derived cell than in the standard Müller cells, suggesting that cells kept the characteristics of their original phenotype. Nonetheless, it has been reported that Müller cells cultured in vitro had

no or very little Nestin [5, 24] and acquired "stemness" by dedifferentiation in stem-cell-conditioned medium in vitro, inconsistent with our results. Expression of Nestin in Müller cells may differ between species.

The cells in our study were also exposed to 1 μM RA, which is believed to induce embryonic stem cells [31, 32], perhaps similar to the differentiation of Müller stem cells into photoreceptors. Western blot analysis in our study demonstrated that both cell types in our study were induced into photoreceptors, with greater expression of rhodopsin in the standard Muller cells than the neurosphere-derived. The difference is likely due to the two distinct culture systems: the neurospheres are comprised of hundreds of cells, and it is difficult for growth factors, nutrients, and oxygen to be homogenously exposed to all of them, especially in the center of the spheres. Moreover, spheres are motile and merge [20], and their cells are heterogeneous with respect to viability, growth rates, and differentiation state. In contrast, monolayer cultures are homogenously exposed to inducing factors. This consideration may account for the discrepant rhodopsin expression between the two culture systems.

Neurosphere culture system is costly due to its requirement for supplement with various factors, time-consuming, and considered unreliable for many reasons, as we have discussed. The combined results indicate that the standard Müller cells would be more suitable for cell replacement transplantation for photoreceptor degeneration than Müller cells derived from neurospheres.

In conclusion, Müller cells cultured in vitro and Müller cell-derived neurospheres have stem cell characteristics, and both can be induced into photoreceptor neurons by RA. Serum has no influence on the "stemness" of cells. There is more gene expression of markers of stem or progenitor cells in Müller cells cultured in serum media compared with Müller cells derived from neurospheres in stem-cell-conditioned medium, and these would seem to be more suitable for photoreceptor cells degeneration and transplantation, as well as for future studies.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81470653).

Disclosure of conflict of interest

None.

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