Research
ISSN 2413-0516

Isolation and localization of cells expressing Sca-1 in the adult mouse ovary: An evidence for presence of mesenchymal stem cells

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(*Submitted: 18 December 2017 – Revised version received: 10 January 2018 – Accepted:15 February 2018– Published online: 26 June 2018*)

Objective Recently growing evident declared that 'neo-oogenesis' continues in mature female life span and simultaneously another studies confirmed the presence of spermatogonial stem cells (SSCs). Even though there is an agreement between scientist about SSCs population in male gender but on the other side ovarian stem cells have received raising challenges regarding the existence in the surface epithelium of ovary. Mesenchymal stem cells (MSCs) are the most applicable source of stem cells and the common marker of MSCs is Sca-1 so that the purpose of this study was to clarify the incidence of stem cells in the surface epithelium of ovary.
Methods Forty C57BL6 mice were sacrificed and the ovary carefully excised from its surrounding fat tissue, after mechanical and enzymatic digestion cells were stained with Sca-1 to estimate the incidence of positive stem cells (SCs) population fluorescence activated cell sorting (FACS). Part of digested cells used for RT-PCR, also histological section prepared for immunohistochemical staining of Sca-1 in ovarian surface epithelium FACS.
Results The gene expression of Sca-1 was confirmed in the ovarian tissue. As well, localization of Sca-1$^+$ cells was detected in the germinal layer of ovary and epithelial granular layer of primordial follicles. Moreover, we could successfully isolate the Sca-1$^+$ cells through
Conclusion The present work findings confirmed an inclusive stem cell population in the ovary which can be a strong evident for regeneration of ovarian tissue in either purpose of ovulation scar and neo-oogenesis.
Keywords ovary, stem cell, Sca-1, surface epithelium

Introduction

The ovary is an organ which is responsible to prepare competent and mature oocytes in reproductive procedure. Moreover, it involves in signaling pathways of oogenesis and folliculogenesis through secreting the different types of growth factors (GFs), hormones and cytokines.[1] According to the principle (dogma), before or at birth, a finite number of oocytes develop and reserve in the ovary of female mammals which they can be determined as primordial follicles established during the neonatal period with suitable number and quality. Recently, neo-oogenesis has been introduced as a restricted supply of oocytes by existence of renewable germ line stem cells (GSCs) in the gonads of postnatal female mammals.[2,3]

Based on neo-oogenesis dogma, the GSCs of the peripheral blood, bone marrow (BM) or ovarian surface epithelium can differentiate into various types of cells in the ovary such as fibroblast-like cells, oocyte and cells with granulosa phenotype, under suitable stimulation and *in vitro*.[4,5] As well, studies have identified existence of GSCs in models of non-mammalian organisms like Drosophila.[6] However, there was enough evidence to confirm the presence of GSCs in mammalian ovaries.[2] In the literature, the presence of stem cell markers including Oct4[7] and c-kit[8] in the cellular subpopulations have been reported in the aged ovary.

Additionally, the other kinds of stem cells have been identified in the adult ovary, such as very small embryonic-like stem cells[9] and a subpopulation of granulosa cells up-regulate some stemness genes related to bone marrow mesenchymal stem cells (MSCs).[10] In other way, more recently, stem cell antigen 1 (Sca-1) have been introduced as a selective marker for MSCs in mouse model.[11]

In this study, we tried to characterize the MSCs in the ovary of mouse model by detecting the cells expressing Sca-1 as a distanced marker of MSCs using different techniques including fluorescence activated cell sorting (FACS) analysis, immunohistochemistry (IHC) and RT-PCR.

Materials and Methods

Animals

This experimental study was performed on 40 adult females C57BL/6 mice 6 weeks of age. They were placed in individual cages during the study period, with 12 h of darkness and 12 h of light available. Mice had access to water and food and ad libitum. The housing temperature range was kept between 25 and 30°C. The mice were kept at the animal facility center for 2 weeks to adapt to the new environment prior to the study.

Isolation of SCs from mouse ovaries

For isolation of SCs, ovaries were harvested and the fat pad, bursa and oviduct were carefully excised. Ovaries were then minced into very small pieces. Ovarian tissue was digested in 0.83 mg/ml collagenase type 2 (Worthington Biochemical Corporation NJ, USA; #LS004174) in sterile Hank's Balanced Salt Solution (HBSS; diluted from 10× stock, containing no calcium, no magnesium, no phenol red; Life Technologies CA, USA; #14185-052) containing 3% bovine serum albumin (BSA), 1.23 mM calcium chloride, 1.03 mM magnesium chloride and 0.83 mM zinc chloride for 15 min in a 37°C shaking

water bath (160–180 rpm), and interrupted incubation with vigorously shaking by hand for 10–20 s after 10 min of incubation. The supernatant was separated from the pellet by centrifugation at 400 *g* for 3 min. At last, the ovarian homogenate was re-suspended in 3 ml HBSS, 3% BSA wash buffer and filtered through sterile 40 µm (BD Biosciences CA, USA; #352340) nylon mesh filters before antibody staining.

Cell staining and FACS

Digested ovarian homogenate which stained for Sca-1 stem cell marker were sorted and characterized by FACS analysis, LY-6A/E PE/CY5 (Sca-1) (Biolegend#108109) (1:500) stained the positive population. Antibody were diluted according to the manufacturer's recommendations in wash buffer and the ovary pellet was re-suspended in antibody staining solution and placed on ice in the dark for 30 min. An excess of wash buffer was then added and the stained cell suspension was centrifuged at 300 *g* for 3 min. The supernatant was carefully removed and the pellet was re-suspended in wash buffer and subsequently filtered through a 40-µm nylon filter prior to FACS. Isotype-matched negative controls were used to define background staining. Cell sorting was performed using BD FACS Aria II software (BD Biosciences).

Immunohistochemical staining of tissue sections

To confirm the presence of Sca-1+ population in the ovary, ovaries were dissected, fixed in 4% PFA, and paraffin blocks were prepared and cut using a microtome into 6 µm sections. Sections were deparaffinized by heating to 60°C for 1 hour, followed by three passages in xylenes for 10 min and subsequent rehydration with a graded alcohol series and finally into PBS. This was followed by boiling sections for 10 min in sodium citrate (pH = 6) for antigen retrieval. Endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 in 50% methanol for 10 min at room temperature. Sections were blocked using 10% horse serum and rabbit serum (Vector Laboratories). Slides were then incubated overnight with primary antibody (rabbit anti Sca-1 ab; 109211, diluted 1:50), at 4°C. The next day 3 PBS washes were followed by incubation for 1 hour at room temperature in biotinylated horse-anti-rabbit and rabbit-anti-goat IgG (1:200; Vector Laboratories), and colorimetric detection was performed using ABC Vectastain Elite reagents with DAB (Vector Laboratories). Sections were counterstained with hematoxylin (Sigma-Aldrich). Images of stained sections were captured using an Olympus BX-51 microscope (Olympus).

Ovarian stem cell culture

After sorting, the cells were re-suspended in primary culture medium consisting of 40 ml DMEM-F12 (Gibco#11330-032) supplemented with 10% fetal bovine serum (FBS; Gibco#10437028) and 10^3 U/ml leukaemia inhibitory factor (LIF; amsbio#AMS-263) were plated in 12 well plates at a density of 2×10^5 cells per well. Plates were incubated at 37°C in an atmosphere of 5% CO_2 in air.

Results

Gene expression of Sca-1 in the ovary using RT-PCR

To find out gene expression of Sca-1 in the ovarian homogenate, RT-PCR was performed. According to Figure 1, the expression of Sca-1 gene was confirmed. Expression of β-actin was considered as a housekeeping gene.

Localization of Sca-1-expressing cells in ovary tissue using IHC analysis

To determine the location of ovarian MSCs, we analyzed paraffin sections of normal ovary in normal mice by IHC staining with Sca-1 antibody. In the analysis, we could detect the Sca-1+ cells in the germinal layer of ovary and epithelial granular layer of primordial follicles (Figure 2).

Potency of individual cells to form cells with spherical morphology

To evaluate the potency of individual cells to form cells with spherical morphology, the sorted cells were cultured in media and the Sca-1+ showed the potency of colony formation after 1 week of culture (Figure 3).

Discussion

The existence and confirmation of OSCs are of importance for the field of reproductive science; as a result, some scientists focused more concern to figure out whether or not oogenesis occurs from these localized stem cells in the adult mammalian ovary *in vivo*. The current study has shown that adult mouse ovary contains a population of Sca-1+ cells with stem cell characteristics. However, because of being challenging field as well

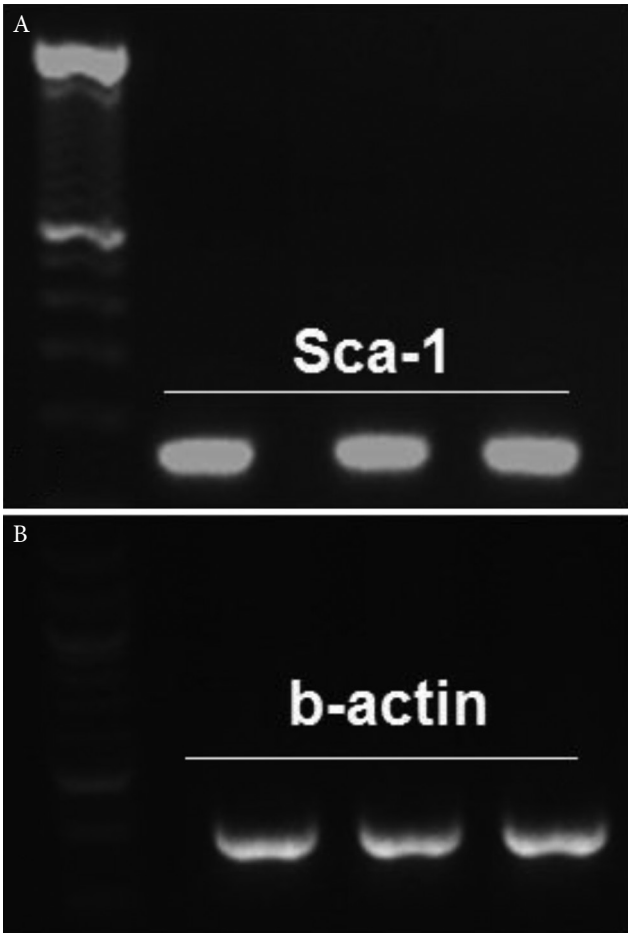

Fig. 1 **1 Stem cell gene marker expression of (A) Sca-1 in ovarian homogenate. (B) Expression of *β*-actin was considered as a housekeeping gene.**

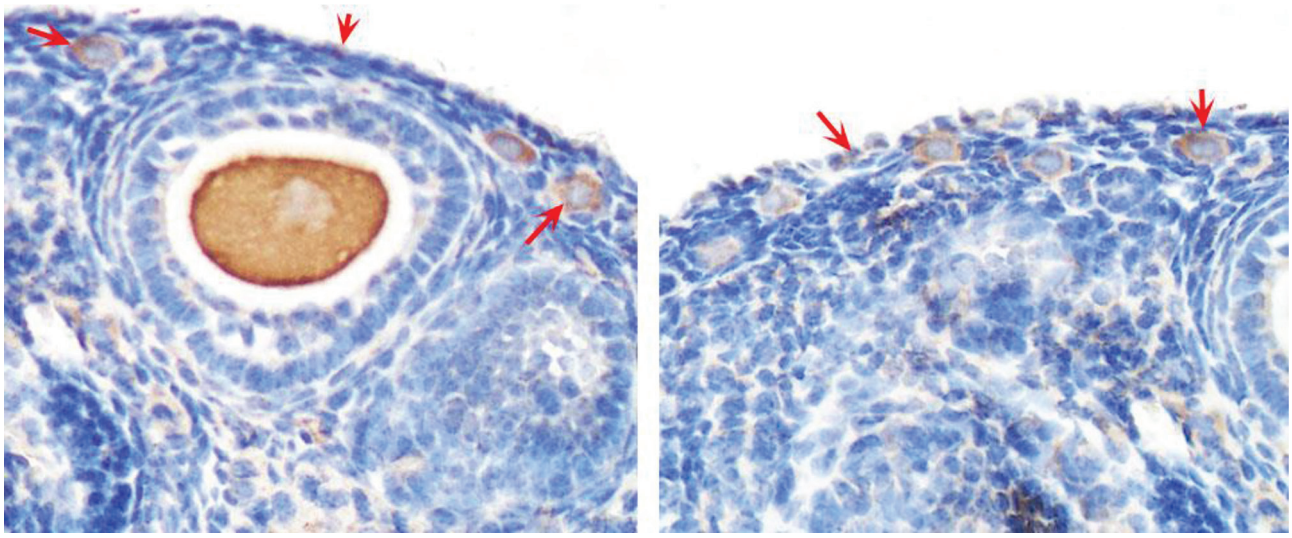

Fig. 2 **Detection of Sca-1 expressing cells (Sca-1+ cells) in adult mice ovary section using IHC staining with anti-Sca-1 antibody. Sca-1 expressing cells are found in the germinal layer of ovary and in the epithelial granular layer of primordial follicles (red arrows), magnification of 100×.**

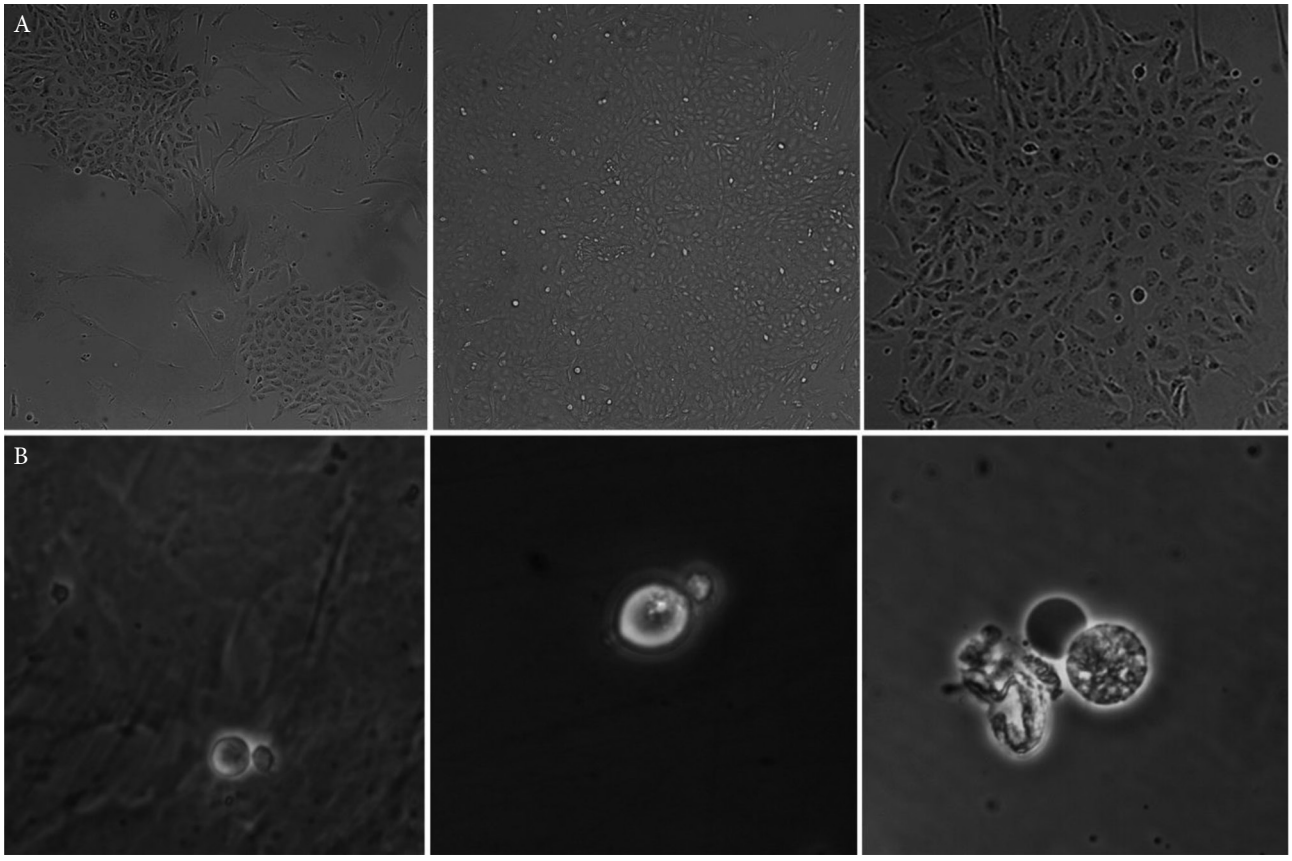

Fig. 3 **(A) The Sca-1+ cells in cell culture media showed the local colonies, (B) in 2nd week the spherical cells were floating in the media.**

as their indefinite character, derivation and function, it cannot be strongly declared that they are "stem cells" with ovarian origin and mostly have been indicated as "putative stem cells" in previous.[8,12,13] It has been proven that two different types of stem cells are located in the mature organs of which the first type is represented as dynamic stem cells and the other type as quiescent stem cells. There was this concept that ovarian stem cells are located in the second category without contribution in "neo-oogenesis".[14–16] Presence of MSCs are almost confirmed in several organs along with bone marrow and white adipose tissue.[17–19] MSCs due to their multipotent characteristic are reliable therapeutic source to support regeneration and repair of injured tissues of mesenchymal basis.[20] Sca-1+ have recently been recognized as selective markers of mouse MSCs.[11] The stem cell niche is a dedicated microenvironment that sustain stem cells' competence to self-renew and differentiate.[21] Here this question raises that why the stem cell niche is inactive in ovary epithelium which can be answered by unknown composition of these stem cells in the ovary. Several studies conducted to isolate stem cell from the ovary with stem cell markers like

SSEA-1,[22,23] Fragilis,[24,25] and DDX4[16] with multiple techniques[26] or based on the cell morphology.[27] Given the limited evidence for distinctive subpopulations of the OSCs cells, in this study we were tried to separate a general fraction of cells with "MSC" characteristics using cell surface expression for Sca-1 with FACS sorting. Additionally, the presence of this fraction in the surface epithelium of ovary were confirmed by IHC staining which demonstrated distinct positive cells in the ovarian histological sections, these characteristics suggest that this cell population is fortified for progenitor cell activity. The Sca-1$^+$ cells have a high colony-forming competence with progenitor cell distinctiveness. These cells have two main characteristics: The capability to self-renewal and differentiation potential. Our findings represent multiple colonies and in long time cell culture these cells progeny were able to differentiate to round and detached cells with different morphology which reflect the number of differentiated cells in culture media.[28]

Since the expression of "MSCs" in the oocyte has not been reported previously this examination raised fascinating possibility of the association between neo-oogenesis and this progenitor cells as indicated before.[13,29,30] To make these cells more applicable for clinical purpose, it is necessary to differentiate the characterized cells with growth factors that they are involved in differentiation. Transplantation of these cells to infertile mice also can be considered as a reliable method for evaluation of these cells function in long term.

In conclusion Sca-1 is a representative marker of MSCs which expressing with low incidence in mouse ovary. Since two types of somatic or germ stem cells defined so far in the ovary, we can assume that these cells are progenitor cells and have role in ovulatory wound healing or they are putative germ stem cells that can be consider for neo-oogenesis. Studies in this area shade a light for the future of infertile patient but further study needed to overcome the challenges of stem cells in the ovary.

Conflict of Interest

None.

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