Identification of Putative Fallopian Tube Stem Cells

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Victoria Snegovskikh, MD^{1,2}, Levent Mutlu, MD¹, Effi Massasa, BA¹,
and Hugh S. Taylor, MD¹

Abstract

Stem cells are used to repair and regenerate multiple tissues in the adult. We have previously shown that stem cells play a significant role in mediating endometrial repair and tissue regeneration. We hypothesized that the oviduct may possess a similar population of stem cells that contribute to the maintenance of this tissue. Here we identify label-retaining cells (LRCs) in the murine oviduct which indicate the presence of a stem/progenitor cell population in this tissue as well. Two-day-old CD-1 mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU) or vehicle control. Female animals ($n = 36$ for each group) were killed at 6 weeks post injection. Reproductive tracts were removed, specimens were embedded in paraffin, and 5- μ sections were prepared. Oviduct was identified by hematoxylin and eosin staining and morphology. Immunofluorescence studies were performed on serial sections tissues ($n = 12$ per animal) using antibodies against BrdU. Confocal microscopy was used to identify 4',6-diamidino-2-phenylindole (DAPI)- and BrdU-stained nuclei. In the group of mice exposed to BrdU, we identified a population of LRCs in all specimens and not in controls. The putative stem cells are located at the base of each villi, suggesting the location of the stem cell niche. The number of DAPI-stained nuclei divided by the number of LRCs; LRCs constituted 0.5% of all nucleated cells. The oviduct contains a population of progenitor cells, likely used in the repair and regeneration of fallopian tube. Defective or insufficient stem cell reserve may underlie common tubal diseases, including hydrosalpinx and ectopic pregnancy.

Keywords

stem cell, oviduct, fallopian tube, reproduction, reproductive tract, hydrosalpinx, ectopic pregnancy, ovarian cancer, infertility

Background

Adult somatic stem cells are rare undifferentiated cells with the potential for self-renewal and differentiation. They play a vital role in maintaining the tissue homeostasis by producing a progeny that successfully incorporates into the tissue where they reside. Although they are normally quiescent relative to their surrounding cells, they can become activated to produce progenitor or transient-amplifying cells, which are more committed to produce mature cells.¹ Depending on the tissue needs, the balance between stem cell quiescence and activation is carefully regulated; for example, tissue damage leads to activation of stem cells.

Stem cell identification relies on combinations of cell surface markers, none of which are specifically expressed on all stem cells.² Nevertheless, when defining markers for stem/progenitor cells are unknown, *in vitro* and *in vivo* assays can be used to identify stem/progenitor cells. Identification of "label-retaining cells" (LRCs) is *in vivo* assay for detection of stem cells in tissues. This approach takes the advantage of the relative quiescence of stem/progenitor cells. In this method, Bromodeoxyuridine (BrdU), a thymidine analog, is used as a label. The BrdU is administered for a prolonged time and followed by a chase period. Once administered, BrdU

incorporates into the newly synthesized DNA of replicating cells during the S phase of the cell cycle, substituting for thymidine during DNA replication. Despite their low mitotic index, stem/progenitor cells can be labeled by continuous administration. During the chase period, the cells that divide less frequently retain BrdU, whereas rapidly dividing cells dilute their BrdU signal. As a result, quiescent cells such as stem cells retain their label and are referred to as LRCs.³

Approximately, 3% of the uterine epithelial and 6% of uterine stromal cells were identified as LRCs in murine endometrium.⁴ Epithelial LRCs were primarily located in the luminal epithelium, whereas they were not present in the glands except for occasional LRCs in the neck of a gland. Stromal LRCs were observed adjacent to luminal and glandular epithelium as well

¹ Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University, New Haven, CT, USA

² Department of Obstetrics and Gynecology, Warren Alpert Medical School of Brown University, Providence, RI, USA

Corresponding Author:

Hugh S. Taylor, Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA.
Email: hugh.taylor@yale.edu

as at the endometrial–myometrial junction.⁴ We hypothesized that the oviduct would contain a population of stem cells.

In this study, we identified LRCs in the murine oviduct suggesting the presence of a stem/progenitor cell population in this tissue.

Material and Methods

CD-1 mice were housed in standard conditions with food and water provided ad libitum at a constant light cycle of 12 hours. Ethics approval for this project was granted by Yale University Institutional Animal Care and Use Committee.

Two-day-old mice were injected intraperitoneally with BrdU (Abcam, Cambridge, Massachusetts) at the dose of 50 µg/g body weight, while the control group received normal saline (Baxter Chemical, St Stephenville, Texas).

Female animals (36 in each group) were killed at 6 weeks postinjection.

Reproductive tracts were removed surgically, washed in normal saline for 30 minutes, and placed in 10% formaldehyde solution (Sigma Aldrich Co, St Louis, Missouri). Following that, all specimens were embedded in paraffin and 5-µ sections were prepared. Oviducts were identified by morphology after hematoxyline and eosin (H&E) staining (Sigma Aldrich Co) staining. Three experienced researchers blinded to the experimental group (VS, LM, and EM) performed detailed examination of all the slides.

Immunofluorescence

Serial sections (5 µm) of paraffin-embedded tissues were labeled accordingly. Sections were deparaffinized and dehydrated through a series of xylene and ethanol washes. Each specimen was stained with H&E for histological evaluation. For immunofluorescence (IF) analysis, after a 5-minute rinse in distilled water, an antigen-presenting step was performed by steaming the slides in 0.01 mol/L sodium citrate buffer for 20 minutes, followed by cooling for 20 minutes. Slides were rinsed for 5 minutes in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST), and sections were circumscribed with a hydrophobic pen. Endogenous peroxidase was inactivated by incubation in 3% hydrogen peroxide for 5 minutes, followed by a 5-minute PBST wash. Using M.O.M. kit (Vector Laboratories, Burlingame, California), sections were incubated with primary mouse monoclonal anti-BrdU antibody (B-8434, 1:100, Sigma Aldrich Co) in a humidified chamber for 1 hour at 4°C. Negative control sections were processed in an identical manner but substituting primary antibodies with normal goat immunoglobulin G. Sections were then washed 2 times for 2 minutes in PBS, and Fluorescein Avidin DCS (Vector Labs, Burlingame, California) prepared per protocol was added to the slides and sections were incubated for 5 minutes.

Then all the slides were equilibrated briefly with PBS. 4',6-diamidino-2-phenylindole (DAPI) stock solution (Invitrogen, United Kingdom) was diluted to 300 nmol/L in PBS, and 200 µL of this dilute DAPI staining solution was added to

the coverslip preparation, making certain that the tissue is completely covered. Sections were incubated for 5 minutes and rinsed several times in PBS. Excess buffer was drained from the coverslip, and slides were mounted using medium with Pro-Long Gold antifade reagent (Invitrogen). Following that, all the samples were immediately viewed using a fluorescence microscope with appropriate filters.

All negative control sections showed no IF signal. The number of stained nuclei was counted separately in 4 random high-power fields on each of the 12 slides from 72 animals by 3 independent observers (576 HPFs [High Powered Field] total per investigator) and averaged for each experimental animal.

Confocal microscopy was used to identify DAPI- and BrdU-stained nuclei. Quantitative analysis of each tissue section was performed using AxioVision and the corresponding digital image processing software (Carl Zeiss Micro imaging, Inc, Thornwood, New York). A computer-generated H-score (in relative light units) was assigned for a representative tissue section and corrected for background light intensity. Results are reported as mean $\pm$ standard error of mean from a minimum of 3 separate readings from 2 separate tissue sections per animal.

Results

The oviduct was normally developed after BrdU exposure, and Figure 1 shows a representative H&E-stained serial section for orientation. In the group of female mice exposed to BrdU (N = 36), we identified a population of LRCs in all specimens. These cells were generally localized at the base of the fallopian tube villi near the serosa. Some (approximately 10%) were located within the villi and then typically in a central location (Figure 2). In almost all sections, BrdU was observed to follow the same pattern as detailed in Figure 2. The pattern was consistent throughout all segments of the tube.

Two distinct levels of BrdU were detected, a brighter and a dimmer fluorescing cell type were interspersed. The brighter population consisted of approximately 20% of the total number of BrdU-containing cells. The LRCs in the fallopian tubes were typically located between the fourth and the seventh position from the bottom of the muscular layer, which is similar to the stem cell progression followed by the progenitor and transient-amplifying cells, respectively, in the luminal epithelium of the intestinal crypts.

The number of DAPI-stained nuclei was divided by the number of LRCs and LRCs constituted $0.5\% \pm 0.05\%$ of all nuclei, suggesting that the stem cell population makes up a small minority of the total cells of the oviduct. The control saline-treated animals examined with the primary antibody (N = 36) were all consistently negative. Similarly, BrdU-treated animals examined without primary antibody showed no fluorescence.

Discussion

In this study, we demonstrate the localization and distribution of candidate stem cells in the epithelial and smooth muscle

Figure 1. Hematoxylin and eosin (H&E)-stained serial section.

layer of the murine oviduct using the LRC approach. Like the endometrium that is known for its remarkable regenerative capacity as it undergoes dynamic changes each estrous cycle, the fallopian tube also has a pool of stem cells available for the processes of repair and regeneration.

After 6 weeks, BrdU intensity was heterogeneous among the cells, indicating the presence of cells with diverse turnover times. Stem cells have longer cellular turnover times than other cells in the tissue, thereby retaining their BrdU content. Additionally, the widely accepted immortal strand model suggests that regardless of the number of divisions, stem cells undergo asymmetrical division and retain the template DNA.⁵ Therefore, the BrdU-bright population in fallopian tubes presumably corresponds to the actual stem cells. Progenitor and transient-amplifying cells have shorter cellular turnover times and undergo symmetrical cellular divisions, thereby losing their labels in time. In agreement with this model, we observed a BrdU-bright and BrdU-dim population, indicating the presence of both stem cells and progenitor and transient-amplifying cells in the oviduct.

Figure 2. Identification of label-retaining cells in the murine oviduct. Top panel: 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (Blue). Center panel: Immunofluorescence (IF) identification of 5-bromo-2-deoxyuridine (BrdU)-retaining cells (Green). Bottom panel: merge. (The color version of this figure is available at rs.sagepub.com.)

The spatial organization of the BrdU-bright stem cells and their BrdU-dim progeny seems to be substantially different in oviduct compared to the endometrial LRCs. Uterine epithelial LRCs were localized mainly on the luminal epithelium.⁶ Further, the epithelial LRCs in the intestinal crypts have a distinct localization and almost always reside near the fourth position from the bottom of the crypt.^{7,8} The cellular organization in intestinal crypts is progressive, where the LRCs are typically located between the fourth and the seventh position from the

bottom of the crypt; this corresponds to the stem cell population followed by the progenitor and the transient-amplifying cells toward the luminal epithelium.^{7,8} Oviduct epithelial LRCs are located in the center of the villi and near the serosa rather than in the epithelium. Oviduct stem cells likely have a distinct niche from either endometrial or gastrointestinal stem cells. Moreover, the BrdU-bright cells and BrdU-dim cells in oviduct did not demonstrate a consistent spatial relationship and likely coexist within the same or closely linked niche. Finally, in parallel with the uterine LRC studies, we observed LRCs in the stroma and smooth muscle layer.⁹ However, the exact roles of LRCs in the stroma and smooth muscle layer remain to be elucidated.

The percentage of the LRCs in oviduct was much lower than the percentage of uterine LRCs. Although this finding may be explained by the difference in BrdU-labeling intervals and duration between studies, it may also be explained by the need of the relatively high number of stem cells needed for endometrial regeneration. Unlike endometrium, fallopian tubes do not undergo monthly regeneration and breakdown cycles. Therefore, the stem cell reserve in the fallopian tubes may be relatively lower compared to the endometrium. This may also make the tubes more susceptible to damage after injury due to lack of a large reservoir of reparative cells.

Many studies have shown the vast differentiation capacity of endometrial stem/progenitor cells.^{4,8,10-18} Human endometrium-derived cells show remarkable differentiation capability and have been differentiated to chondrocytes, insulin-producing cells, dopamine producing neuron-like cells, and cardiomyocyte-like cells. Few studies have shown the differentiation potential of LRCs in the fallopian tubes. In one study using H2B-GFP (Histone 2B- Green Fluorescent Protein) LRC approach, LRCs formed spheroids that are capable of self-renewal and differentiation.¹⁹ These cells were located predominantly in the distal oviduct, whereas the cells identified here were dispersed throughout the length of the tube. We suspect that the findings in our study differed due to the age of the mice at the time of the pulse. We administered BrdU to 2-day-old mice, at a time when the reproductive tract is still developing; the study by Wang et al used adult mice. They likely identified progenitor cells that are involved in the rapid turnover of adult tissues during the estrous cycle, while we believe that we have identified an earlier stem cell capable of giving rise to multiple cells of the oviduct. Another study found multipotent stem cells in the human fallopian tube; while the spatial location of these cells was not identified, the findings demonstrate the existence of fallopian tube stem cells in humans as well as mice.²⁰ Taken together, these findings showed that the LRCs are stem cells in the fallopian tubes and that there are likely several population of distinct types of stem cells in this tissue.

Extensive tissue damage of the fallopian tube epithelium results in scarring and infertility.²¹ In the endometrium and other tissues, ischemic injury and inflammation recruit stem cells to the damaged organ.⁹ It is likely that in some fallopian tube pathologies, the fallopian tube LRCs are lost and the tissue is incompletely regenerated, which ultimately leads to disease that may include hydrosalpinx, infertility, and ectopic pregnancy.

Tubal epithelial cells have been implicated in ovarian cancer. Paik et al have identified a population of stem-like epithelial cells that lack markers of differentiated epithelium in the distal human fallopian tube.²² They demonstrated that the stem-like epithelial cells in the distal fallopian tube are expanded in precancerous lesions of the tube and in the tubes of women with ovarian cancer. Oviduct stem cells identified here are not entirely epithelial, and therefore not the same as those identified in ovarian cancer. However, the stem cells identified here may serve as the precursors of the cells identified by Paik et al.

In conclusion, we have shown the presence and distribution of LRCs in the oviduct. Defective repair of the fallopian tubes is a common medical problem, leading to hydrosalpinx, infertility, and ectopic pregnancy. Stem cells likely aid in the repair of tubal damage, a defect or insufficiency in stem cell function likely contributes to these common diseases of the fallopian tubes. As some ovarian cancer appears to originate in the fallopian tubes, fallopian tube stem cells may also contribute to ovarian cancer as well. We expect that the identification of tubal stem cells may improve our understanding of tubal disease and potentially provide novel therapies.

Declaration of Conflicting Interests

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