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Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 positive cells are located in the reproductive organs of an adult mammal

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Abstract

Endogenous carcinoembryonic antigen-cell adhesion molecule-1 positive totipotent stem cells and stage specific embryonic antigen-4 positive pluripotent stem cells have been isolated from multiple species of adult animals, including humans. This study was undertaken to test the hypothesis that carcinoembryonic antigen-cell adhesion molecule-1 positive cells and/or stage specific embryonic antigen-4 positive cells were located in the reproductive organs of an adult mammal. Adult male and female rats were euthanized following the guidelines of Mercer University's IACUC. Testes, ovaries, and fallopian tubes were harvested and processed for vibratome- or cryostat-sectioning. Tissue sections were incubated with antibodies to carcinoembryonic antigen-cell adhesion molecule-1 and stage specific embryonic antigen-4 cell surface epitopes and stained using an enzyme-linked immunoculture assay (ELICA) coupled with immunocytochemistry. This is the first report of the existence of native populations of both carcinoembryonic antigen-cell adhesion molecule-1 positive cells and stage specific embryonic antigen-4 positive cells in the reproductive organs of an adult mammal.

Keywords: Testes; Ovaries; Fallopian Tubes; CEA-CAM-1; SSEA-4

1. Introduction

Precursor cells are the resident supportive cells within the body. They consist of maintenance progenitor cells and healing stem cells. Maintenance progenitor cells provide the cellular building blocks to replace worn out differentiated cells, thus maintaining the tissues and organs of the body throughout the lifespan of the individual, from conception to death. Healing stem cells provide the cellular building blocks for tissue replacement and repair following injury [1].

Two categories of healing stem cells have been identified in 33 organs and 46 tissues of 15 species of adult animals, including humans (Table 1) [2]. These two healing stem cell populations are endogenous adult totipotent stem cells and endogenous adult pluripotent stem cells.

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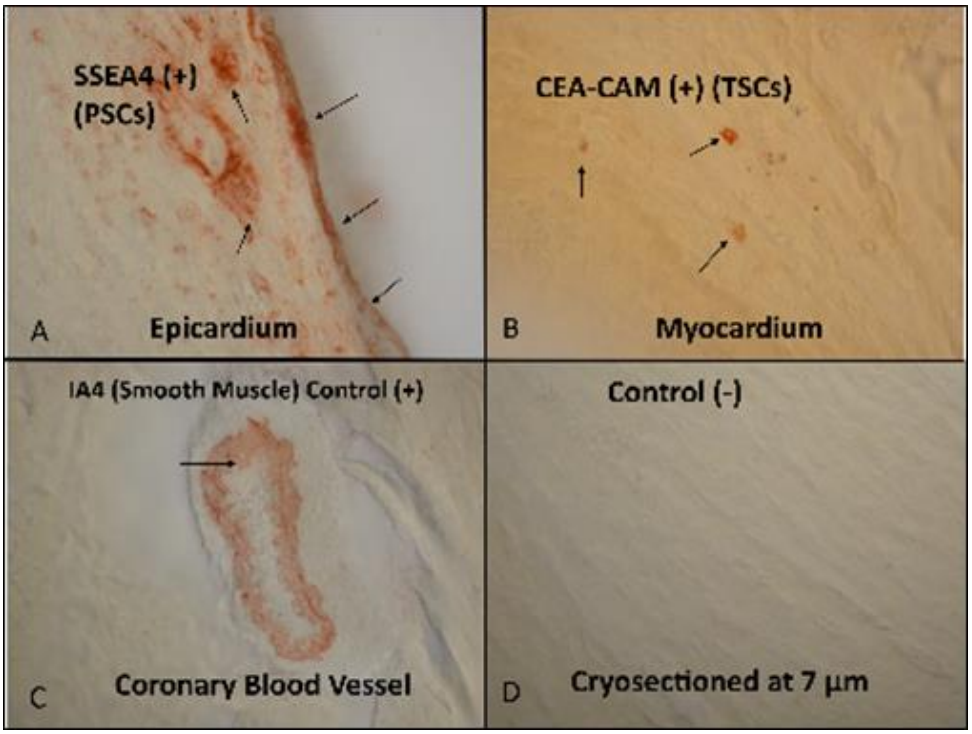
Table 1 Species, Age, and Location of Precursor Healing Stem Cells

Loca[1]	**Sal** [2]	**Re**[3]	**Av** [4]	**Mo**[5]	**Rt** [6]	**Rb**[7]	**Fe** [8]	**Ca** [9]	**Ov** [10]	**Cp** [11]	**Pr** [12]	**Bo** [13]	**SB** [14]	**Eq** [15]	**Hu** [16]
Pre[17]			+ [18]												+
Mor[19]															SEM[20]
Psn[21]	+ H[22]	+ Is[23]	+ Is	+ Is	+ Is H Hc [24]	+	+ Im[25]	+ Im	+ Im	+ Im	+ Is Im	+ Is	+ Is	+ Is	+ Is Hc
Nb[26]				Is	Is										Is
Ad[27]				Is	Is										Is
SM[28]	H Hc			Is	Is	Is	Is	Is	Is	Is	Is	Is	Is	Is	Is
Ge[29]		Is	Is	Is								Is	Is	Is	Is
SkM[30]	H Hc		Is	Is	Is Im	Is	Is	Is	Is	Is	Is	Is	Is	Is	Is
Der[31]	H Hc		Is,	Is Im	Is Im						Is Im				
Hrt[32]			Is, Im	Is	Is Im						Is Im				
GT[33]					Is						Im				
Pos[34]	H Hc		Is	Is	Is Im						Im				Is
Pch[35]	H Hc		Is	Is	Is Im						Im				Is
Ns[36]	H Hc		Is	Is	Is Im						Im				Is
Adip[37]	H Hc		Is	Is	Is Im						Im				Is
Lig[38]	H Hc		Is	Is	Is Im						Im				Is
Ten[39]	H Hc		Is	Is	Is Im						Im				Is
Bv[40]	H Hc		Is	Is	Is Im						Im				Is
BoM[41]	H Hc		Is	Is Im	Is Im						Im				Is
Bld[42]		Is	Is	Is	Is	Is	Is	Is	Is	Is	Is			Is	Is
Tra[43]					Is Im						Im				
Lng[44]					Is Im						Im				
Eso[45]					Is Im						Im				
Stm[46]					Is Im						Im				
Liv[47]					Is Im						Im				
SmI[48]					Is Im						Im				
LgI[49]					Is Im						Im				
Spl[50]					Is Im						Im				
Brn[51]					Is Im						Im				
Men[52]					Is Im						Im				
SpC[54]											Im				
PanEn[55]					Is Im						Im				
PanEx[56]					Is Im						Im				
Kid[57]					IsIm						Im				
Ub[58]					Is Im						Im				

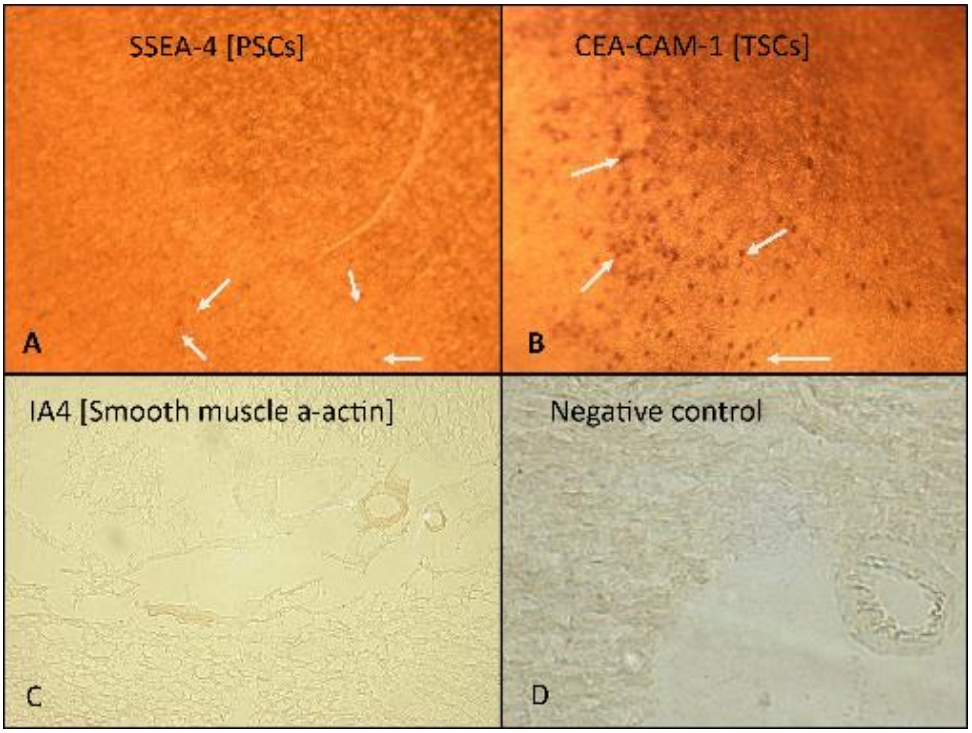
Thy[59]					Is Im						Im				
Tng[60]					Is Im						Im				
Kar[61]				Dip 40[62]	Dip 42						Dip 38			Dip64	Dip 46

Table 1. Loca[1], Location; Sal[2], adult terrestrial salamanders: *Ambystoma annulatum, maculatum, texanum, tigranum;* Re[3], reptile – Komodo Dragon; Av[4], Avians – chicken, Wadel Crane; Mo[5], mice; Rt[6], rat; Rb[7], rabbits; Fe[8], felines; Ca[9], canines; Ov[10], ovine; Cp[11], caprines; Pr[12], porcine; Bo[13], bovine; SB[14], Spectacled Bear; Eq[15], equine; Hu[16], humans (male and female); Pre[17], prenatal; +[18], presence; Mor[19], morula; SEM[20], scanning electron microscopy; Psn[21], post-natal (adult); H[22], Histology; Is[23], isolation; Hc[24], histochemistry; Im[25], immunocytochemistry; Nb[26], newborn; Ad[27], adolescent; SM[28], sexually mature; Ge[29], geriatric; SkM[30], skeletal muscle; Der[31], dermis; Hrt[32], heart (epicardium, myocardium); GT[33], granulation tissue; Pos[34], periosteum; Pch[35], perichondrium; Ns[36], nerves; Adip[37], adipose tissue (white fat); Lig[38], ligaments; Ten[39], tendons; Bv[40], blood vessels; BoM[41], bone marrow (hematopoietic cells, stromal cells); Bld[42], blood; Tra[43], trachea; Lng[44], lung; Eso[45], esophagus (lamina propria, submucosa, adventitia); Stm[46], stomach (submucosa, serosa); Liv[47], liver; SmI[48], small intestine (lamina propria, submucosa, serosa); LgI[49], large intestine (lamina propria, submucosa, mesocolon); Spl[50], spleen (capsule, trabeculae, interstitial tissue); Brn[51], brain (white mater, gray matter); Men[52], meninges (dura mater, arachnoid mater, pia mater); SpC[53], spinal cord (white mater, gray matter); PanEn[54], endocrine portion of pancreas (pancreatic islets); PanEx[55], exocrine portion of pancreas; Kid[57], kidney (capsule and interstitium); Ub[58], urinary bladder; Thy[59], thyroid gland; Tng[60], tongue; Kar[61], karyotypic analysis; Dip[62], diploid number of chromosomes correct for respective species [2]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014.



A, epicardium, SSEA-4+ PSCs; B, myocardium CEA-CAM-1+ TSCs; C, muscular coronary blood vessel, IA4+ for smooth muscle alpha-actin in tunica media of blood vessel as positive procedural control, and D, absent primary antibody as unstained negative procedural control [28]. Reprinted with permission from Stout et al. Discovery of pluripotent and totipotent stem cells in the heart of the adult rat. Amer Surg. 2007; 73: S63

Figure 1 Cryosectioned adult rat heart stained with antibodies



A, SSEA for PSCs (white arrowheads); B, CEA-CAM-1 for TSCs, dark brown spots with some noted with arrowheads; C IA4 for smooth muscle in wall of blood vessels as positive procedural control; and D, and absence of primary antibody as the negative procedural control [29]. Reprinted with permission from Young HE, Lochner F. Telomerase positive totipotent stem cells in the adult brain. I. cerebral cortex. Regen Med Biol Res 2021; 2(1):1-16

Figure 2 Adult rat brain stained with antibodies

Previous studies, using immunocytochemical staining of cryosectioned mammalian tissues demonstrated the presence of CEA-CAM-1 positive (totipotent stem cells) and SSEA-4 positive (pluripotent stem cells) in the adult rat heart (Fig. 1) [3,4] and in the adult rat cerebral cortex (Fig. 2) [3,5]. The current study was undertaken to test the hypothesis that CEA-CAM-1 positive cells and SSEA-4 positive cells were located within the reproductive organs of an adult mammal.

To test the hypothesis, sectioned tissues from male and female adult rats were stained immunocytochemically with antibodies to the cell surface markers CEA-CAM-1 and SSEA-4 following the methodologies established in previous studies, using the enzyme-linked immunoculture assay (ELICA) coupled with immunocytochemistry [3-5].

2. Material and methods

Animal Use: The use of animals in this study complied with the guidelines of Mercer University's IACUC. These guidelines reflect the criteria for humane animal care of the National Research Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health [6].

Tissue Harvest: Postnatal male and female Sprague-Dawley rats (200-300 g) were euthanized using a lethal dose of a Ketamine/Xylazine mixture (Ketamine 300-360 mg/Kg and Xylazine 30-40 mg/Kg). In males, the scrotum was washed with Betadine solution and each testis with attached epididymis within the tunica vaginalis testis was excised from the scrotal sac. A longitudinal incision was made in the tunica vaginalis testes. The testis was separated from the epididymis and removed under aseptic conditions and placed into respective containers for fixation and vibratome sectioning. In females, the abdominal musculature was excised, the ovaries removed with attached fallopian tubes and placed into respective containers for fixation and cryosectioning.

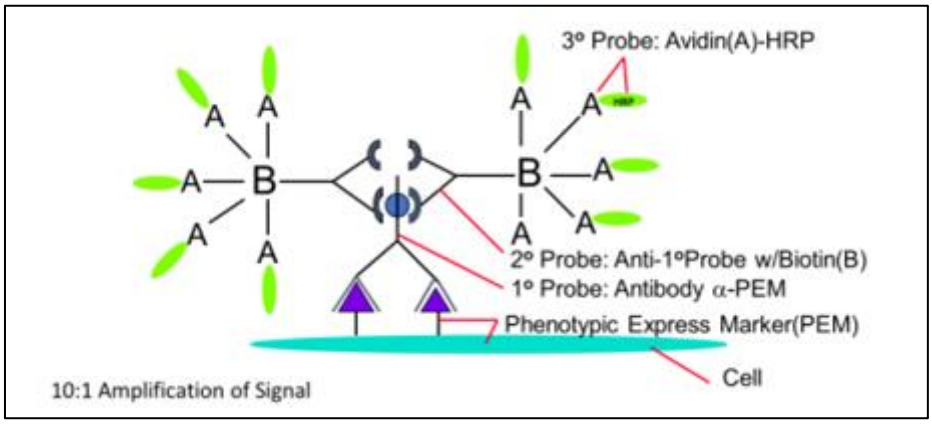
Tissue Fixation: The excised testes, ovaries and fallopian tubes destined for immunocytochemistry, were placed into ELICA fixative [3-5] for 2 days and then rinsed with and stored in Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO / Invitrogen Corporation, Carlsbad, CA). The ELICA fixative was composed of 45.4-ml of DPBS, 30.0-ml of 5% w/v sodium azide (Sigma-Aldrige, St Louis, MO), 12.5-ml of 37% w/v paraformaldehyde (Fisher Scientific Co, Norcross, GA), 2.0 ml 50% v/v glutaraldehyde (Sigma), 0.9-ml beta mercaptoethanol (#1273943, GIBCO), pH to 7.4, then D-glucose

(Sigma, tissue culture grade) was added until an osmolarity of 1.0 was reached. The fixative was stored at room temperature until used.

Vibratome sectioning: The testes were embedded in 8% w/v gelatin (Sigma) in DPBS and then stored at 4°C until sectioned. The tissue was sectioned on a Vibratome Series 1000 Sectioning System (Technical Products International, St. Louis, MO) to a thickness of 25 microns. The sections were applied to positively charged slides (Mercedes Medical, Sarasota, FL) and stored at -20°C until stained.

Cryosectioning: The ovaries and fallopian tubes were placed in 500-ml wide-mouth tissue culture jars (Corning, Corning, NY) containing 400-ml of cold (4°C) ELICA fixative for 24-48 hours. After fixation, the tissue was transferred and stored in DPBS at pH 7.4 and ambient temperature. The ovaries and fallopian tubes were removed, placed into OTC embedding medium and frozen at -20°C. The frozen pieces were cryostat sectioned at seven microns in thickness, placed on positively charged slides (Mercedes Medical) and refrigerated at -20°C until stained. Prior to staining, the sections were incubated with 95% ethanol (Sigma) to remove the OTC cryostat embedding medium and then washed under running water for five minutes. Immunocytochemical staining was performed following established procedures for ELICA analyses [3-5].

ELICA Procedure: The ELICA procedure was designed to measure biological activity, the quantity of phenotypic expression markers/per quantity of DNA (quantity PEM/quantity DNA) for bioactive factors, human recombinant proteins, and for the identification and purification of exosomes in serum-free defined conditioned media. Included in the ELICA was an antibody amplification procedure (Fig. 3). This allowed for a 10:1 enhancement of antibody staining to extracellular, cell surface, and intracellular phenotypic expression markers. Utilizing the amplification procedure, within the ELICA, it was possible to measure femtogram to microgram quantities of phenotypic expression markers per microgram to milligram quantities of DNA [3,7].



The ELICA is a high throughput immunocytochemical procedure based on an ELISA (enzyme-linked immunosorbent assay). But instead of absorbing substrate to a nylon membrane for subsequent analysis, it was developed to amplify signal expression to quantify antibody binding using a soluble HRP substrate, followed by visualizing antibody binding using an insoluble HRP substrate (AEC or DAB), followed by quantification of DNA, all within the same well of a 96-well plate or on a single tissue section [3]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14.

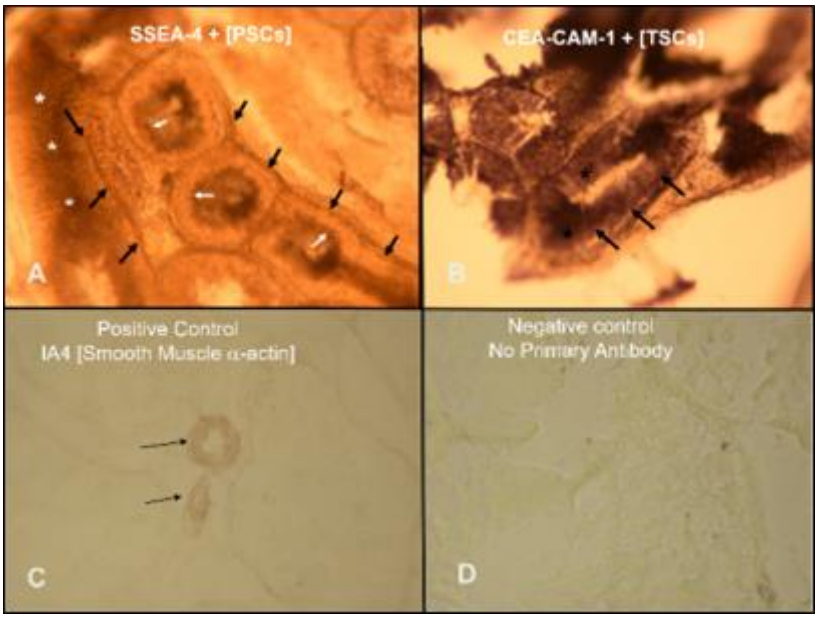
Figure 3 Enzyme-Linked Immuno-Culture Assay (ELICA)

The ELICA procedure utilizes multiple wash and blocking steps to prevent non-specific binding of reagents to cultured cells and/or tissue sections. Reagents used are a primary (1°) probe, which is an antibody to a cell-specific phenotypic expression marker (PEM); a secondary (2°) probe, which is an antibody directed against the species of the 1° probe with an attached biotin; a tertiary (3°) probe, consisting of an avidin molecule with an attached horseradish peroxidase (HRP) enzyme; and HRP substrates. Hydrogen peroxide and sodium azide were used in the initial blocking steps prior to antibody binding because all cells contain an endogenous peroxidase that would react with the exogenous HRP substrate, giving a false positive. Positive and negative controls were run simultaneously to verify that the staining was real and not an artifact of technique. For sectioned tissues, the positive procedural control consisted of using IA4, an antibody to smooth muscle alpha-actin, which is present in the muscular wall of blood vessel(s) within the tissue section. Five negative procedural controls were included in each staining run of sectioned tissues using the ELICA fixative with the ELICA procedure. The negative controls consisted of 1. HRP substrate only; 2. no primary probe, with all other steps remaining intact; 3. no secondary probe, with all other steps remaining intact; 4. no tertiary probe, with all other steps remaining intact; and 5. no substrate, with all other steps remaining intact [3-5].

Immunocytochemical Staining of Sectioned Tissues: Vibratome testes sections were 25 microns in thickness. Cryostat sections were 7 microns in thickness. The vibratome and cryostat tissue sections were incubated with 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in DPBS for 60 minutes to irreversibly inhibit endogenous peroxidases. They were washed in running water for five minutes, and then incubated with 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes, also to irreversibly inhibit endogenous peroxidases. Tissue sections were rinsed with running water for five minutes and incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in DPBS. The blocking agent was removed and the sections rinsed with running water for five minutes. The tissue sections were then incubated with primary antibody for 60 minutes. The primary antibodies consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1) in DPBS; 1-microgram per ml stage-specific embryonic antigen-4 (SSEA-4, Developmental Studies Hybridoma Bank, Iowa City, IA) in DPBS; and IA4 (Developmental Studies Hybridoma Bank) in DPBS for smooth muscle alpha-actin present within adjacent blood vessels as the positive procedural control. The primary antibody was removed. The sections were rinsed with running water for five minutes, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat adsorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector Laboratories) in DPBS. The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure, Curtain Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector Laboratories) in DPBS. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with AEC substrate (Sigma) for 60 minutes. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with running water for 10 minutes and then cover-slipped with Aqua-mount (Vector Laboratories) [3-5,7-11].

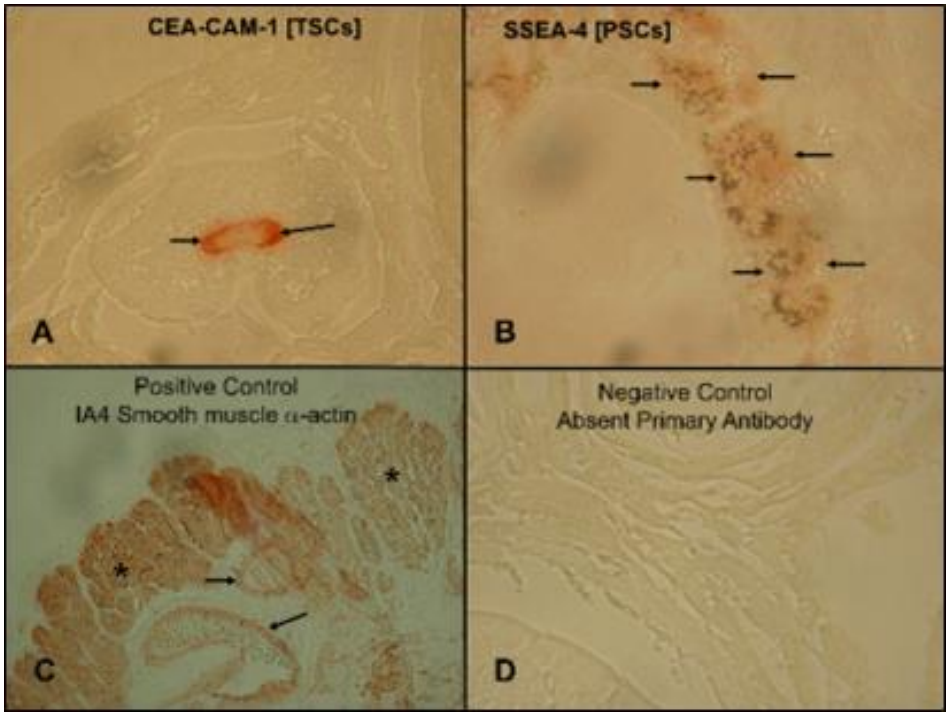
3. Results

Putative precursor stem cells, e.g., CEA-CAM-1 ~~(putative totipotent stem cells)~~ positive cells and SSEA-4 ~~(putative pluripotent stem cells)~~ positive cells, were identified in adult rat reproductive organs, e.g., testis (Fig. 4), ovary (Fig. 5), and fallopian tube (Fig. 6), utilizing immunocytochemical ELICA staining procedures with ~~precursor~~ cell-surface, cell-specific antibodies. Procedural controls were performed simultaneously to ensure validity of the results. The positive control consisted of the antibody IA4 for smooth muscle alpha-actin located in the tunica media of muscular arteries within the sectioned tissues. The negative controls consisted of no primary antibody, with all other steps intact; no secondary antibody, with all other steps intact; no tertiary probe, with all other steps intact; and no substrate, with all other steps intact.



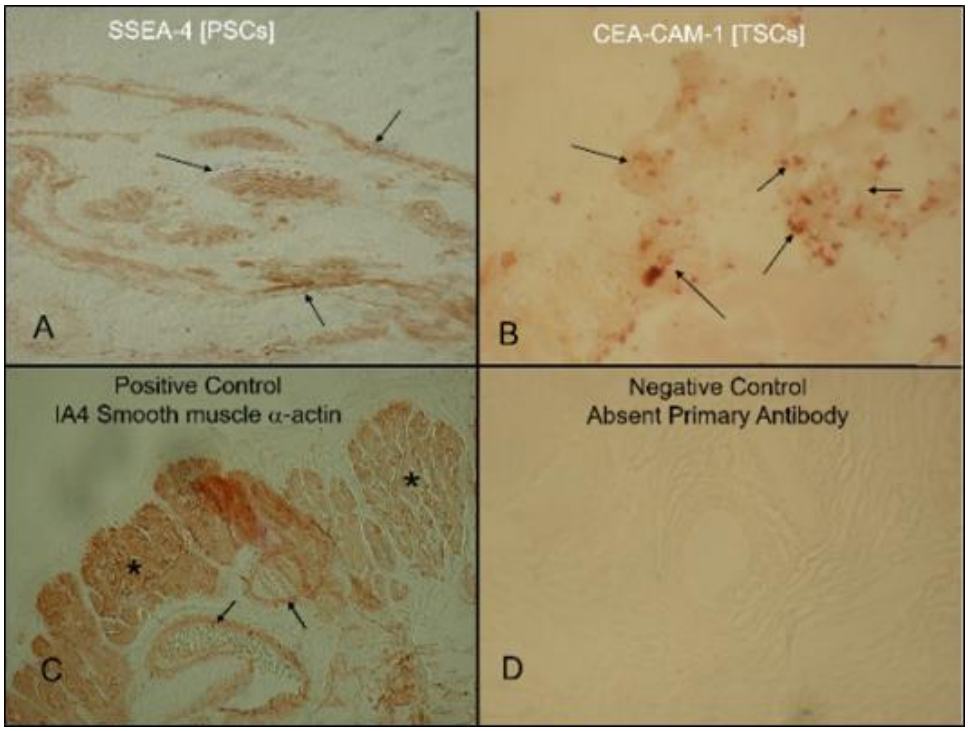
Testes were harvested, fixed in ELICA fixative, vibratome-sectioned at 25 microns, mounted on glass slides, and stained with appropriate antibodies. A. SSEA positive cells (PSCs) surrounding outside of seminiferous tubules (black arrows) as well as within the innermost layer of seminiferous tubules (white arrows and asterisks), 200x. B. CEA-CAM-1 positive staining (TSCs) were present within of seminiferous tubules, with heaviest staining along the inside border of the tubules, 200x. C. IA4 positive staining of smooth muscle alpha actin within the tunica media of blood vessels within the testis, positive procedural control, 400x. D. No primary antibody, negative procedural control, 400x.

Figure 4 Adult rat testes (RM1021)



Ovary with attached fallopian tube harvested from euthanized rat, fixed in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. CEA-CAM-1 positive staining in area of zona pellucida of ovum inside tertiary follicle (arrows), 400 x. B. SSEA positive cells interspersed among granulosa cells of Graafian follicle (arrows), 400 x. C. IA4 positive staining for smooth muscle alpha-actin in both the muscular wall (asterisks) and tunica media of blood vessel (arrows) of the fallopian tube, 400 x.Figure 5C was used in this staining series as the positive procedural control because of the amount of smooth muscle present in the fallopian tube wall musculature and the vascular supply, 200 x. D. No primary antibody, no staining present within ovary, used as the negative procedural control, 400x

Figure 5 Adult female rats (RF1022)



Fallopian tube harvested from euthanized rat, fixed in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. SSEA-4 positive PSCs within area of ampulla of fallopian tube (arrows), 200x. B. CEA-CAM-1 positive TSCs in the connective tissues of ampulla region of the fallopian tube (arrows), 400x. C. IA4 positive smooth muscle alpha-actin in the smooth muscle wall (asterisks) and blood vessels (arrows) of the fallopian tube, as the positive procedural control, 200x. D. No primary antibody, negative procedural control, 400x

Figure 6 Adult female rat (RF1022)

4. Discussion

Telomerase positive precursor stem cells have been identified in 15 adult animals, including humans, from 33 organs and 44 tissues [2]. This study was undertaken to test the hypothesis that CEA-CAM-1 positive cells and SSEA-4 positive were present in the reproductive organs of an adult mammal (rat). Based on immunocytochemical staining with selected cell surface antibodies to phenotypic expression markers, CEA-CAM-1 positive cells and SSEA-4 positive cells were present within the testis (Fig. 4), ovary (Fig. 5), and fallopian tubes (Fig. 6) of an adult mammal (rat), albeit in separate compartments within these organs. CEA-CAM-1 positive cells was located at the base of the seminiferous tubules of the testis, within the zona pellucida of a developing follicle in the ovary, and dispersed among the epithelial cells lining the fallopian tube ampulla and fimbriae. SSEA-4 cells were located in the interstitial tissues surrounding and outside the seminiferous tubes of the testis, amongst the granulation cells surrounding the antrum in a Graafian follicle, and within the wall structure of the ampulla of the fallopian tube.

5. Conclusion

The original intent of this study was to test the hypothesis that CEA-CAM-1 positive cells and SSEA-4 positive cells were present in the connective tissues of adult mammal reproductive organs, e.g., testes, ovaries, and fallopian tubes. Their potential existence was initially assessed by immunocytochemistry. An additional follow-up study, based on isolation and characterization of the immunocytochemical positively stained cells was performed to either verify or deny that the CEA-CAM-1 positive cells are endogenous naturally-occurring totipotent stem cells and that the SSEA-4 positive cells are endogenous naturally-occurring pluripotent stem cells.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Animal Use: The use of animals in this study complied with the guidelines of Mercer University's IACUC. These guidelines reflect the criteria for humane animal care of the National Research Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health [6].

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