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Adult Telomerase Positive Stem Cells: Introduction and Location

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Abstract

Endogenous adult telomerase positive stem cells (aTPSCs) are naturally occurring and are located throughout the body. Three categories of aTPSCs were discovered: totipotent stem cells (TSCs, 0.1-2.0-microns) that will form all somatic cells of the body, gametes, placental cells, and the nucleus pulposis of the intervertebral disc (the only adult-derivative of the notochord; pluripotent stem cells, including halo-like stem cels (HLSCs, >2-4-microns), corona-like stem cells (CLSCs, >4-<6-microns), and pluripotent stem cells (PSCs, 6-8 microns), that will form all somatic cells of the body; and individual populations of multipotent germ layer lineage stem cells, e.g., ectodermal stem cells (EctoSCs,10-12-microns), mesodermal stem cells (MesoSCs, 10-12-microns) and endodermal stem cells (EndoSCs,10-12-microns), that will only form cells within their defined embryonic germ layer lineage. The proposal was to identify the location of aTPSCs, isolate, plate, propagate, segregate, sort, genomically label and test. Biopsy specimens of TSCs and PSCs were obtained from 11 species of animals. The biopsy specimens were fixed, sectioned, and stained with antibodies for carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4, examined histologically for various cells and histochemically for extracellular matrix components. When attempting to isolate the cells problems were encountered. Problems also occurred with every other step in the proposed plan. This begins a series of studies examining the rarity of aTPSCs and unique difficulties encountered when attempting to determine their functionality in regenerative medicine.

Keywords: Differentiated Cells; Progenitor Cells; Stem Cells; Location; Telomerase Positive; Adult

1. Introduction

An extremely rare group of adult telomerase positive stem cells (aTPSCs) was discovered in 1975 while studying limb regeneration in the adult terrestrial salamanders, *Ambystoma annulatum, Ambystoma maculatum, Ambystoma tigranum,* and *Ambystoma texanum* [1-9]. Following metamorphosis from the juvenile aquatic form, the adult terrestrial form was purported to have lost the ability to regenerate a limb. This was based on studies from juvenile aquatic Ambystoma and juvenile and adult newts. Those studies reported maintaining the aquatic animals in 4^{o}C water and feeding them raw beef liver during daylight hours. After amputation, the investigators examined the regrowing limbs every five days, both grossly and histologically, until complete regeneration occurred, ~30-45 days, equating to 6-9 time points. The popular belief was based on their examinations, that once an appendage was amputated the transected tissues would dedifferentiate into more primitive stem cells and then transdifferentiate into all of the missing tissues of the appendage

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for its subsequent outgrowth. This dedifferentiation and transdifferentiation of transected tissue had been reported in aquatic species undergoing limb regeneration [10-15].

The studies reporting that no regeneration occurred in the adult terrestrial salamanders, kept the adult terrestrial adult salamanders under the same environmental conditions as their juvenile and adult aquatic counterparts, e.g., in 4°C water and fed beef liver during daylight hours [10,13]. However, it was noted that once the correct environmental conditions and food sources were matched for the nocturnally active adult terrestrial salamanders, e.g., an ambient temperature, terrestrial environment, nocturnal food source (nightcrawlers and cockroaches), and self-feeding during the night time hours [3], that adult terrestrial salamanders would completely regenerate a limb. The time for complete regeneration to occur ranged from 324-370 days, dependent on the species analyzed. Using the same five-day intervals to examine the regenerating limb, both grossly and histologically, equated to using 64 -74 time points, a different scenario was noted [1-9].

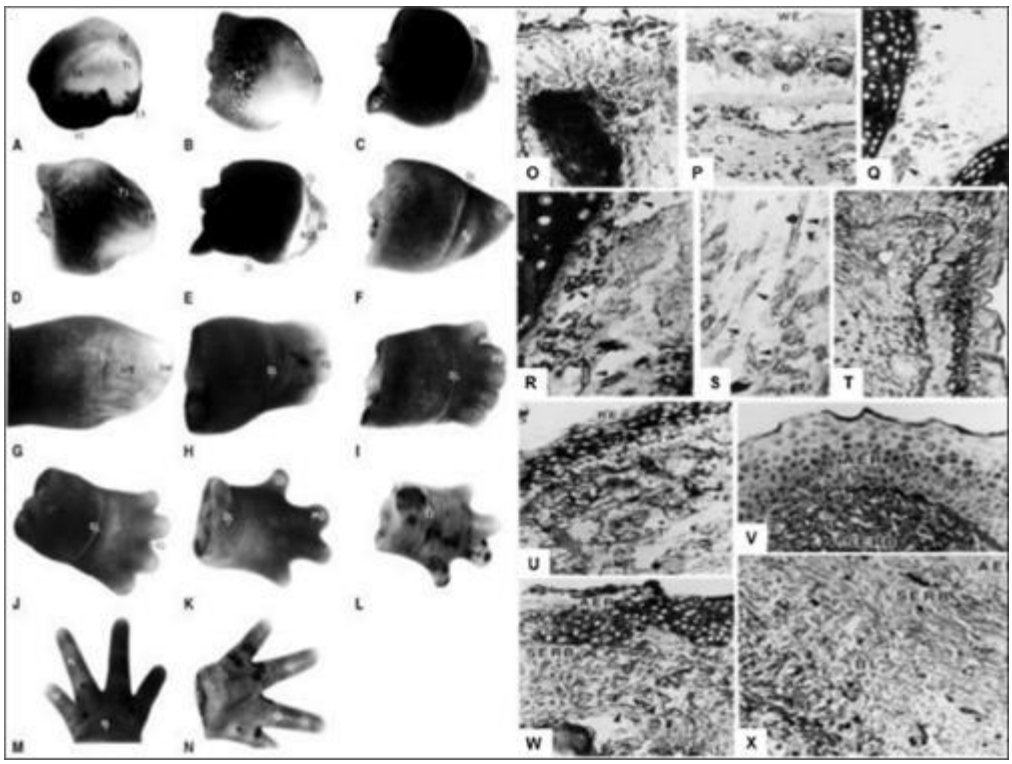

Figure 1 Wound healing stage, dorsal oblique view, shows a slight swelling in the regenerate epidermis at the distal tip and the first evidence for the bud outgrowth, *Ambystoma annulatum*, Mag. ×100. B. Early bud stage, ventral view, shows the rounded outgrowth of the blastema with the epidermal ridge at its distal tip, *A. annulatum*, Mag. ×70. C. Middle bud stage, dorsal view, shows a demarcation line separating the stump tissues from a symmetrical cone-shaped blastemal outgrowth, *A. annulatum*, Mag. ×94. D. Middle bud stage, ventral view of the same bud as in C. Tension lines are visible from the epidermal ridge to the stump tissues, *A. annulatum*, Mag. ×90. E. Late bud stage, dorsal view of a flattened cone-shaped outgrowth. The demarcation line continues to separate the blastemal outgrowth from the remaining portion of the limb, *A. annulatum*, Mag. ×60. F. Early palette stage, dorsal view of a paddle-shaped outgrowth that can be segregated into two regions: region 1 comprises the area of the future digits; region 2 comprises the area of the future "hand," *A. annulatum*, Mag. ×70. G. Middle palette stage, dorsal view of an elongated palette-shaped outgrowth. The apical palette epidermis covers the outgrowth, and a demarcation line separates the stump tissues from regenerate outgrowth tissues. Along the distal ridge of the outgrowth, slight swellings begin to replace the epidermal ridge, *A. annulatum*, Mag. ×70. H. Late palette stage, dorsal view of an elongated palette-shaped outgrowth. A demarcation line separates proximal stump tissues from the distal regenerate tissues. Distinct swellings are visible along the distal border and are indicative of forming digits, *A. annulatum*, Mag. ×90. I. Early digit stage, dorsal view of the regenerate structure that has the distinct appearance of a forming "hand" containing digits. The forming digits are beginning to separate from each other, *A. annulatum*, Mag. ×60. J. Middle digit stage, dorsal view of a definitive "hand"-like regenerate structure consisting of separated digital regions, *A. annulatum*, Mag. ×70. K. Late digit stage, a dorsal view showing continued separation of the digits, *A. annulatum*, Mag. ×70. L. Late digit stage, a ventral view of the same regenerate as seen in K. Patches of chromatophores (melanocytes) are appearing along this surface. Transverse crease lines overlie future joint areas, such as those for the interphalangeal joints, *A. annulatum*, Mag. ×70. M. Complete limb regenerate stage, a dorsal view of all

digits separated from each other, with the regenerate forelimb indistinguishable from either the original or sham-operated control limbs. Within the regenerated limb, the demarcation line remains and marks the spot of the original amputation site, *A. annulatum*, Mag. ×60. N. Complete regenerate stage, a ventral view of M, Mag. ×60. At the beginning of this stage, patches of chromatophores appear along the ventral surface, but by the end of this stage, the pigmentation pattern assumes that of its respective species (i.e., annular rings for *A. annulatum*, spots for *A. maculatum*, a mottled appearance for *A. texanum*, and stripes for *A. tigranum*), *A. annulatum*, ×60. Definitions: APE, apical palette epidermis; C, chromatophores; CL, crease lines; DE, dorsal regenerative epidermis; DL, demarcation line; ER, epidermal ridge; FD, forming digits; FL, finger-like digits; IG, interdigital grooves; IW, interdigital wedge; LD, lateral digits; MD, middle digits; R1, distal one-third of outgrowth, region 1; R2, proximal two-thirds of outgrowth, region 2; RGE, regenerate epidermis; SE, stump epidermis; SW, swellings; TH, thumb-like digit; TL, tension lines; VE, ventral regenerate epidermis. O. Limb tissues transected by amputation 18 to 20 h after amputation. Arrowheads denote degenerating tissues along amputation surface, Mag. ×450. P. Postaxial border of stump tissue area just proximal to amputation site, 18 to 20 h after amputation. Stump epidermis adjacent to amputation site demonstrates intracellular staining for hyaluronic acid and glycoproteins. Dermis consists of dense fibrous connective tissue. Inflammatory cells (macrophages and neutrophils) located within and around blood vessel periphery and interspersed within connective tissues, Mag. ×900. Q. Proximal area of stump tissues between two cartilage masses 5 d after amputation. Arrowheads demonstrate cells with intracellular heparan sulfate glycosaminoglycans, pericellular heparan sulfate glycosaminoglycans and glycoproteins, and extracellular hyaluronic acid. This figure reveals small totipotent-like cells associated with the perichondrium covering the cartilage and within the connective tissues between the cartilage masses, Mag. ×600. R. Area of the stump tissues proximal to the amputation site 6 d after amputation. Arrowheads denote cells with intracellular heparan sulfate glycosaminoglycans, pericellular heparan sulfate glycosaminoglycans and glycoproteins, and extracellular hyaluronic acid. These cells are a mixture of putative adult telomerase positive stem cells that are associated with the perichondrium and associated connective tissues adjacent to the cartilage mass, Mag.×1200. S. Area of the connective tissues located between skeletal muscle and adjacent cartilage 6 d after amputation. This area is within the tissues proximal to the amputation site. Putative adult telomerase positive stem cells containing intracellular heparan sulfate glycosaminoglycans, pericellular heparan sulfate glycosaminoglycans and glycoproteins, and extracellular hyaluronic acid (arrowheads) interspersed within the connective tissue, Mag. ×1200. T. Histological view of wound closure by regenerate epidermal cuff across wound surface of stump tissues 10 d after amputation. Intracellular staining for hyaluronic acid and glycoproteins occurs within middle layers of regenerate epidermis (double arrowheads), absent in basal layers of regenerate epidermis (B) and present extracellularly within adjacent underlying wound tissues (single arrowhead), Mag. ×1200. U. Regenerate epidermis overlying wound surface 22 d after amputation. An increase in intracellular staining for both hyaluronic acid and glycoproteins occur in all layers of the regenerate epidermis. Increased extracellular staining for both hyaluronic acid and glycoproteins are located within underlying stump tissue matrices, Mag. ×550. V. Distal regenerate portion of the stump-regenerate complex, sectioned perpendicular to the preaxial/postaxial axis 25 days after amputation. Regenerate epidermis begins to thicken, forming a “cap” of epidermis covering the wound site. This structure is designated as the apical epidermal cap. The “pioneering” cells and the “trailing” cells become the cell population beneath the forming apical epidermal cap. This region is designated as the sub-regenerate epidermal blastema. Hyaluronic acid is present intracellularly within all layers of the apical epidermal cap and extracellularly within the sub-regenerate epidermal blastema, Mag. ×600. W. Stump-regenerate complex 30 d after amputation. All layers of the apical epidermal cap stain intracellularly for hyaluronic acid and glycoproteins, whereas the sub-regenerate blastema stains extracellularly for the same material. Putative adult telomerase positive stem cells (arrowheads) are visible near the sub-regenerate epidermal blastema. These cells stain intracellularly for heparan sulfate glycosaminoglycans, peripherally for heparan sulfate glycosaminoglycans and glycoproteins, and extracellularly for hyaluronic acid, Mag. ×550. X. Regenerate portion of stump-regenerate complex 35 days after amputation. The individual putative adult telomerase positive stem cells have congregated into a large mass of cells, designated the *core blastema*, which lies deep (internal) to the sub-regenerate epidermal blastema, and the apical epidermal cap, Mag. ×600. Definitions: AEC, apical epidermal cap; B, basal layers of regenerate epidermis; C, cartilage; CB, core blastema; CT, loose connective tissues; D, dermis; E, epithelial-like cells; F, free cell population; FM, fine filamentous material; G/GM, granulated wound surface matrix; I, inflammatory cells (macrophages and neutrophils); N, nerve; RE, regenerate epidermis; SREB, sub-regenerate epidermal blastema; ST, stump tissues; TP, trailing cells; WE, wound epidermis. Fig. 1A-M Reprinted with permission from Young HE, Bailey CF, Dalley BK. Gross morphological analysis of limb regeneration in postmetamorphic adult Ambystoma. Anat Rec. 1983; 206: 295–306. Fig. 1, O-X Reprinted with permission from Young HE, Dalley BK, Markwald RR. Glycoconjugates in normal wound tissue matrices during the initiation phase of limb regeneration in adult Ambystoma. Anat Rec.1989; 223, 231–241

In adult terrestrial salamanders following amputation of a limb, it was discovered that macrophages would appear at the wound site and remove all the dead and damaged cells. Then the blastemal-like cellular mass that formed for the regrowth of the limb arose from cells hidden within the more proximal differentiated connective tissues of the limb. These cells consisted of extremely small (totipotent-like) cells contained within an extracellular matrix (ECM). The ECM

consisted of hyaluronic acid, non-sulfated chondroitin proteoglycans, heparan sulfate proteoglycans, and glycoproteins [8,9]. Concurrent with the appearance of the primitive cells, wound closure occurred forming the apical epidermal ridge. The primitive cells would then begin to proliferate and set up a gradient of cells in different stages of differentiation from proximal tissues to the blastema beneath the forming apical epidermal ridge (AER). This gradient consisted of proximal differentiated tissues, to tissue-defined progenitor cells (e.g., osteoblasts, chondroblasts, fibroblasts, neuroblasts, and endothelioblasts), to non-descript progenitor cells (ectodermal stem cells and mesodermal stem cells), to intermediate-sized cells (pluripotent stem cells), to small cells (corona-like stem cells and halo-like stem cells), to very small proliferating (totipotent stem) cells directly beneath the AER. Concurrently, the AER would be secreting periodic-acid Schiff positive glycoproteins (putative exosomes) into the mass of very small cells during this entire process. Once the differentiated cells had reached the AER, the AER would cease secretion of glycoproteins, regress, and form normal epidermal structures [1-9].

I would propose that the differences seen between juvenile aquatic *Ambystoma* and juvenile and adult newts versus adult terrestrial *Ambystoma*, e.g., dedifferentiation and transdifferentiation of adult tissues, versus the appearance of a range of cells from more differentiated (proximally located) cells to completely undifferentiated (distally located) totipotent stem cells, was due primarily to the time period between observations. If aquatic species were viewed an equivalent number of times as terrestrial salamanders, (e.g., 64-74 times) during their 30 to 45-day time period, that would equate to viewing the regenerating limbs every 9.7 to 16.8 hours rather than every 120 hours. With the shortened time frame between observations, it is conceivable that what was actually occurring during limb regeneration in aquatic species would become apparent.

1.1. Adult Telomerase Positive Stem Cell

The observation in the adult terrestrial salamander that multiple populations of primitive stem cells (e.g., putative adult telomerase positive stem cells) were located in the connective tissues of regenerating limbs was the basis to look for similar cell populations of cells in the connective tissues of other species.

Hypothesis to be tested

"Adult telomerase positive stem cells are a conserved population of cells located within the connective tissues of many organs in multiple animals".

2. Material and methods

2.1. Materials

Assay Reagents

2.1.1. Species-Specific Buffers:

- Amphibians and Reptiles – 10% Holtfreter's solution [46]
- Avians – Tyrode's balanced salt solution, #T-2145 (Sigma)
- Non-human mammals – Phosphate Buffered Saline, PBS (Sigma)
- Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)

2.1.2. ELICA Fixative Reagents

- Species-Specific Buffer (Avians, Mice, Rats, Cat, Dogs, Sheep, Goats, Pigs, Cows, Equines, Humans)
- Paraformaldehyde, #P6146, Sigma
- Glutaraldehyde, #G5882, Sigma
- Sodium Azide, #S2002, Sigma
- D-Glucose, # G-6138, Sigma

2.1.3. Trypan blue, Kodak

- Positively-charged glass slides, Mercedes Medical
- Inhibition and Exhaustion of Endogenous Peroxidases
  - 5% Sodium Azide, #S2002 Sigma
  - 30% Hydrogen Peroxide, #H-1009, Sigma

2.1.4. Blocking Agents for ELICA

- Horse serum, #H7889, Sigma
- Goat serum, #200-6210-AG, DSHB
- Porcine Serum, #P9783 Sigma
- Bovine serum albumin, #A-7906, Sigma
- Fetal Calf Serum, #F0392, Sigma
- Human Serum, #H4522, Sigma
- Tween 20 (polyoxyethylenesorbitan), ChemPure, Curtain Matheson Scientific, Houston, TX
- Nonidet P-40, P-40, #56007, BDH
- Triton-X-100, #T-6878 (Sigma)
- Powdered non-fat dry milk, Local Grocery Store Brand
- Gelatin (cold water fish soluble collagen), #935425, Sigma

2.1.5. Positive Standard

IA4, antibody for smooth muscle alpha-actin present in tunica media of adjacent blood vessels, #A5228, Sigma

2.1.6. Primary antibodies

- Carcino-embryonic antigen-cell adhesion molecule-1 (CEA-CAM-1), gift from D. Hixson
- Stage-specific embryonic antigen-4 (SSEA-4 / MC-813), Developmental Studies Hybridoma Bank

2.1.7. Secondary antibody

Biotin, goat anti-mouse IgG (fab specific), #B-7151, Sigma

2.1.8. Tertiary Probes

- Streptavidin-peroxidase, #JSA-030084, Accurate
- Avidin-peroxidase, #JSA-030083, Accurate
- Monoclonal anti-goat IgG clone GT-34 biotin, #B-3148, Sigma
- Horseradish peroxidase conjugated Avidin-D, #A-2004, Vector
- Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector Laboratories

2.1.9. Visualize Tertiary Probes

- 3-amino-9-ethylcarbazole (AEC), #A5754, Sigma
- 3,5-Diaminobenzidine, #D7304, Sigma

2.1.10. Miscellaneous Supplies

- Aqua-Mount (mounting coverslips), Vector Laboratories
- 0.1-micron Bottle-Top filters, Thermo-Fisher
- Nitrile Gloves, Diagger

2.1.11. Suppliers

- Sigma, Sigma Chemical Co., St Louis, MO
- GIBCO, GIBCO, Grand Island, NY
- Mercedes Medical Scientific, Lakewood Ranch, FL
- Kodak, Rochester, NY
- Aldrich, Aldrich Chemical Co, Milwaukee, WI
- DSHB, Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biological Sciences, Iowa City, IA
- ChemPure, Curtain Matheson Scientific, Houston, TX
- Vector, Vector Laboratories, Burlingame, CA
- DH, Douglas Hixson, Department of Medicine, Brown University, Providence, RI
- Accurate, Accurate Chemical and Scientific Corporation, Westbury, NY
- Non-Fat Dry Milk, Local Grocery Store
- Diagger Scientific, Vernon Hills, IL
- Thermo-Fisher Scientific, Waltham, MA

2.2. Methods

2.2.1. Generating Reagents

ELICA Fixative

The ELICA fixative is composed of 55-ml of species-specific buffer [16] (Sigma), 12.5-ml of 37% w/v powdered paraformaldehyde (Fisher Scientific) in species-specific buffer, 2.0-ml of 50% v/v glutaraldehyde (Sigma), 30.0-ml of 5% w/v sodium azide (Sigma), and sufficient amount of tissue culture grade D-glucose (Sigma) to reduce osmolarity of 1.0, then pH to 7.4. The fixative is stored at room temperature when not in use.

0.4% Trypan blue in Species-Specific Buffer

Weigh 0.4 g of powdered Trypan blue dye (Kodak) and add w/v to 100-ml of species-specific buffer, stir with stir bar on stir plate until dissolved. Sterilize-filter with a 0.1-micron bottle-top filter into either 100-ml dark brown bottle or 100-ml bottle covered in aluminum foil. Store at ambient temperature. Use inside sterile containment area, e.g., HEPA-filtered Class-2 BioSafety Cabinet.

2.2.2. Procedures

Harvest from Solid Tissues

With IACUC-approval, adult animals were euthanized, and biopsy specimens harvested.

With IRB-approval human biopsy specimens were obtained. The biopsy specimens from both animals and humans were preserved in the ELICA fixative [16,17], frozen in liquid nitrogen, cryosectioned, tissue sections placed on electrostatically-charged glass slides (Mercedes Medical), and prepared for immunocytochemistry (ELICA procedure) with cell surface markers for totipotent stem cells (TSCs, CEA-CAM-1) and pluripotent stem cells (PSCs, SSEA-4) [18,19].

Blood Harvest

With IACUC-approval (animal) and IRB-approval (human) blood samples were obtained be venipuncture, placed into purple-top EDTA vacuum tubes (BD), hematocrits formed, plasma supernatant removed, and processed for staining with 0.4% Trypan blue dissolved in species-specific buffer and immunocytochemically (ELICA procedure) with antibodies to cell surface markers for TSCs (CEA-CAM-1) and PSCs (SSEA-4) [20,21].

ELICA Procedure

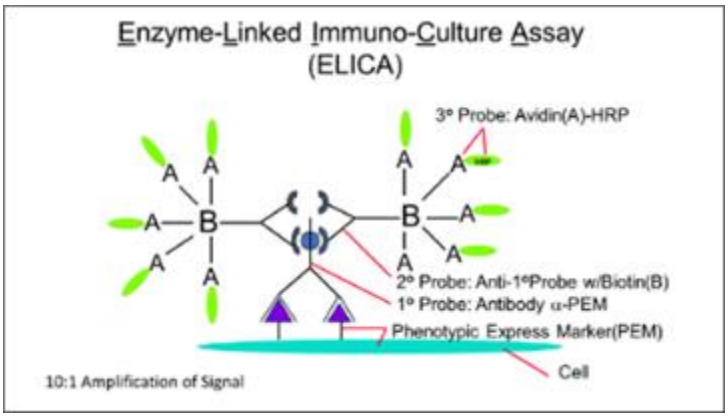

Figure 2 Enzyme-Linked Immuno-Culture Assay (ELICA), a high throughput immunocytochemical procedure was developed to amplify signal expression to quantify antibody binding using a soluble HRP substrate, followed by visualizing antibody binding using an insoluble HRP substrate (AEC or DAB), followed by quantification of DNA, all within the same well of a 96-well plate or on a single tissue section. This procedure utilizes multiple wash and blocking steps to prevent non-specific binding of reagents to cultured cells and/or tissue sections, visualized by brightfield microscopy. Reagents used are a primary (1°) probe, which is an antibody to a 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 because all

cells contain an endogenous peroxidase that would react with the exogenous HRP substrate, giving a false positive. Positive and negative procedural controls were run simultaneously to verify that the staining was real and not an artifact of technique [16,21]. 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; Young HE. A high throughput screening assay to quantify, visualize, and standardize biological activities: Enzyme-Linked Immuno-Culture Assay (ELICA). GSC Advanced Research and Reviews. 2025; 24(02): 091-114

Inhibition and Exhaustion of Endogenous Peroxidases

Five percent aqueous sodium azide v/v in HPLC water (Sigma) and 30% hydrogen peroxide (Sigma) were used in separate initial blocking steps prior to antibody binding. Sodium azide inhibits extracellular and cell surface endogenous peroxidases, while 30% hydrogen peroxide exhausts endogenous intracellular peroxidases in the cells that would react with the exogenous HRP substrate, giving a false positive [16,17].

Positive and Negative Controls

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 (tunica media) of blood vessel(s) within the tissue sections [30,33]. Six negative procedural controls were included in each staining run of cryosectioned or vibratome sectioned tissues with the ELICA procedure. The negative controls consisted of 1. Species-specific buffer only; 2. HRP substrate only; 3. no primary probe, with all other steps remaining intact; 4. no secondary probe, with all other steps remaining intact; 5. no tertiary probe, with all other steps remaining intact; and 6. no substrate, with all other steps remaining intact [18,19,22,23].

2.2.3. Immunocytochemistry of Tissue Sections

Wash tissue sections with species-specific buffer

- Avian cells – Tyrode's buffer, pH 7.4
- Non-Human Mammalian Cells – PBS with 0.2% glucose, pH 7.4
- Human Cells – Dulbecco's Phosphate Buffered Saline, pH 7.

Remove species-specific buffer and incubate with 5% sodium azide (w/v) in species-specific buffer to irreversibly inhibit endogenous peroxidases. Time will vary dependent on the particular content of endogenous peroxidases for each specific cell type. Sodium azide in the ELICA fixative negates peroxidase activity intracellularly, cell surface and in extracellular space. Optimum incubation time with sodium azide is determined empirically by testing a range of times from 0 to 5 min, using 5-sec intervals. *Optimum azide incubation time is the respective time point at which the horseradish peroxidase substrate remains clear, plus 5 sec.

After azide incubation for designated time for each cell type, rinse tissue sections with species-specific buffer.

Remove species-specific buffer and incubate with 30% hydrogen peroxide to irreversibly inhibit endogenous peroxidases. Time will vary dependent on the particular content of endogenous peroxidases for each specific cell type. Optimum incubation time with hydrogen peroxide is determined empirically by testing a range of times from 0 to 5 min, using 5-sec intervals. *Optimum azide incubation time is the respective time point at which the horseradish peroxidase substrate remains clear, plus 5 sec.

Residual azide and hydrogen peroxide can negate activity of exogenously added HRP and therefore must be removed from the system to prevent erroneous results. This is accomplished by rinsing copiously with species-specific buffer containing primary blocking agent. The primary, secondary, and tertiary blocking agents used are dependent on the non-specific binding kinetics of the primary probe (anti-PEM), secondary probe (anti-species of primary), tertiary probe (anti-species of secondary with attached biotin) and therefore must be determined empirically for each probe.

Determine the Non-Specific Binding Kinetics. There are 6 negative controls to determine the non-specific binding kinetics of the particular probes used in the procedure, to ensure sensitivity of the assay.

- species-specific buffer only;
- No substrate, but all other steps intact;
- No primary probe, but all other steps intact;

- No secondary probe, but all other steps intact;
- No tertiary probe, but all other steps intact;
- No exogenous horseradish enzyme, but all other steps intact.

Remove primary blocker and incubate with primary probe directed against particular phenotypic expression marker (CEA-CAM-1, SSEA-4, or IA4), for 1 hour at 37°C in a humidified environment. The probe is diluted with species-specific buffer (Note: addition of blocking agent at this step will decrease binding efficiency of primary probe). The humidified environment used consisted of a glass baking dish containing water-soaked paper towels, the dish covered with Saran wrap, and placed into a 37°C incubator. One positive (per primary probe) and six negative procedural controls should be run simultaneously with each experiment to ensure there are no mistakes with the procedures

3. Results

Two categories of telomerase positive stem cells (aTPSCs) were identified in the connective tissues of solid tissue biopsy specimens. Totipotent stem cells were identified using a cell surface antibody to carcino-embryonic antigen-cell adhesion molecule-1 (CEA-CAM-1). While pluripotent stem cells were identified using a cell surface antibody to stage-specific embryonic antigen-4 (SSEA-4). Totipotent stem cells, halo-like stem cells, and corona-like stem cells were identified in blood using Trypan blue staining and CEA-CAM-1 staining.

The following are representative figures of immunochemical and Trypan blue stained cells from 11 organs and 56 tissues with cell-surface and cell-specific expression markers (Figs. 3-21).

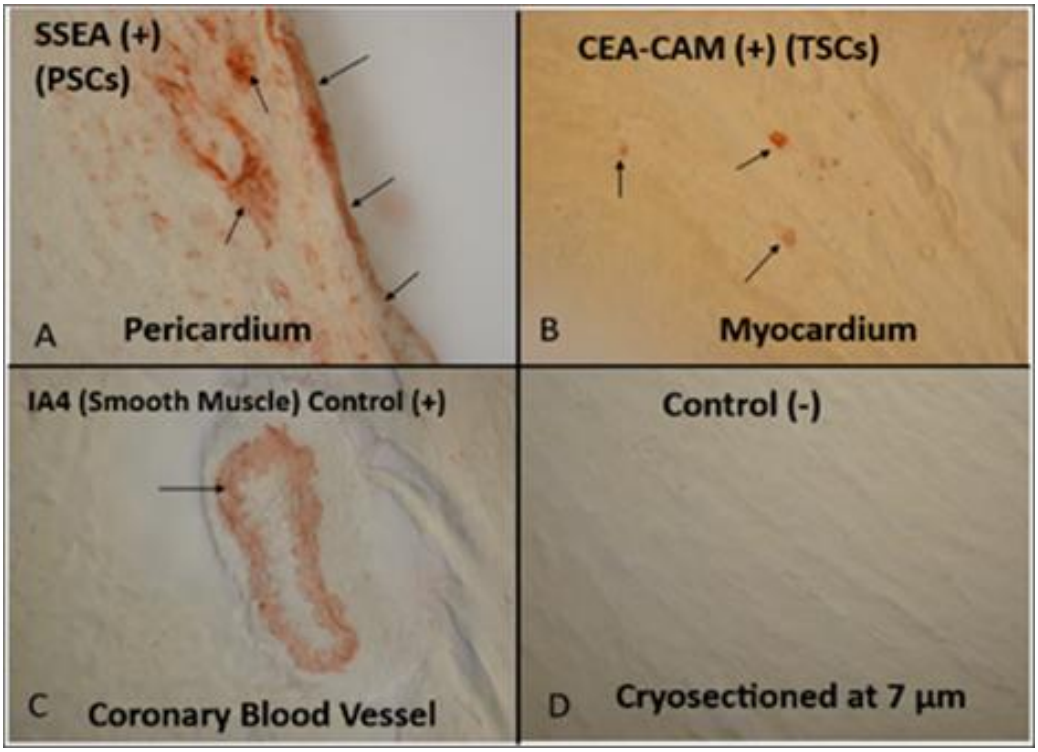

Figure 3 ELICA fixative preserved, frozen, and 7-micron cryosectioned adult rat heart stained with antibodies for: A, PSCs (SSEA4+) were located in the pericardial connective tissues covering the outside of the heart; B, TSCs (CEA-CAM-1+) were located within the endomysial connective tissues of the myocardium of the heart; C, smooth muscle alpha-actin (IA4+) in tunica media of coronary blood vessel as positive procedural staining control, and D, no primary antibody as unstained negative procedural control [23]. Reprinted with permission from Stout CL, McKenzie J, Long G, et al. Discovery of pluripotent and totipotent stem cells in the heart of the adult rat. Amer Surg. 207; 73: S63

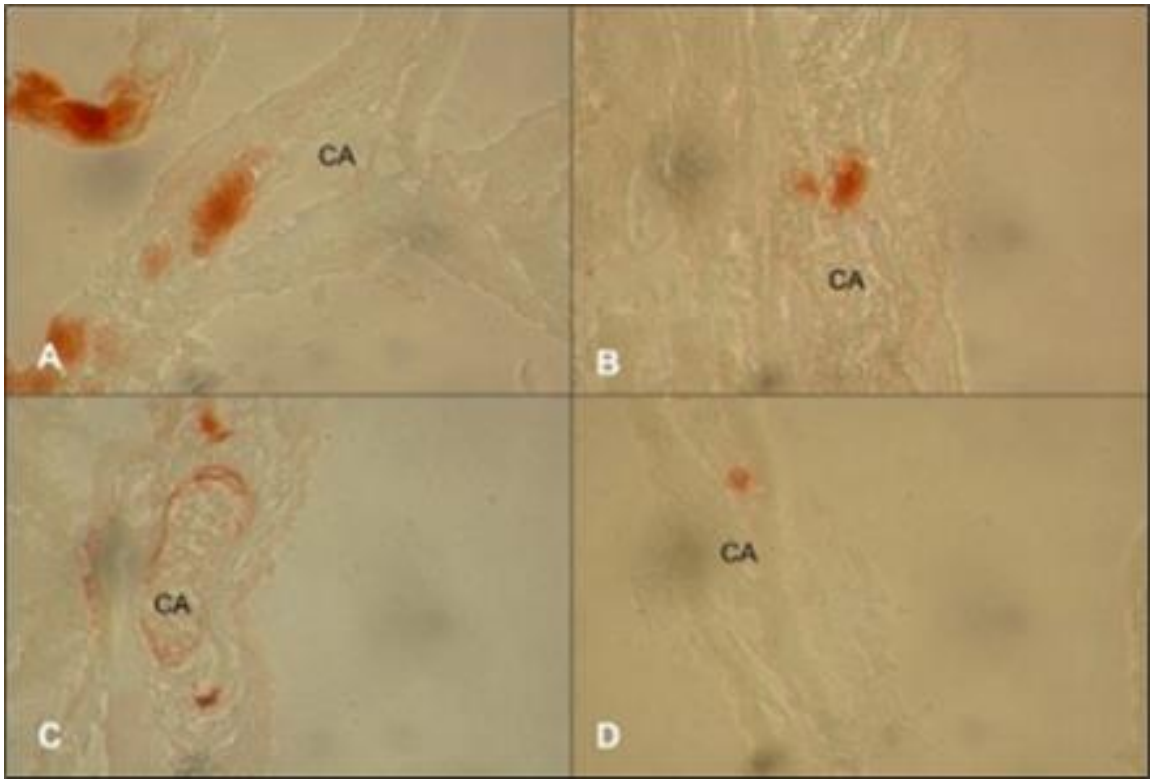

Figure 4 Cryosectioned and immunocytochemically stained (AEC) porcine septal coronary arteries. A. Clusters of cells positive for CEA-CAM-1 were noted next to longitudinal cardiac myofibers. Mag, x100. B. Clusters of cells positive for CEA-CAM-1 were noted within the connective tissue stroma between cardiac myofibers. Mag, x100. C. Groups of cells positive for SSEA-4 were identified within the connective tissues between cardiac myofibers and within the wall structure of a coronary artery. Mag, x100. D. Groups of cells positive for SSEA-4 were identified within the lumen of a coronary artery. Mag, x100. Fig. 3C, smooth muscle alpha-actin (IA4+) in tunica media of coronary blood vessel as positive procedural staining control, and Fig. 3D, no primary antibody as unstained negative procedural control [23]. Reprinted with permission from Stout CL, McKenzie J, Long G, et al. Discovery of pluripotent and totipotent stem cells in the heart of the adult rat. Amer Surg. 207; 73: S63

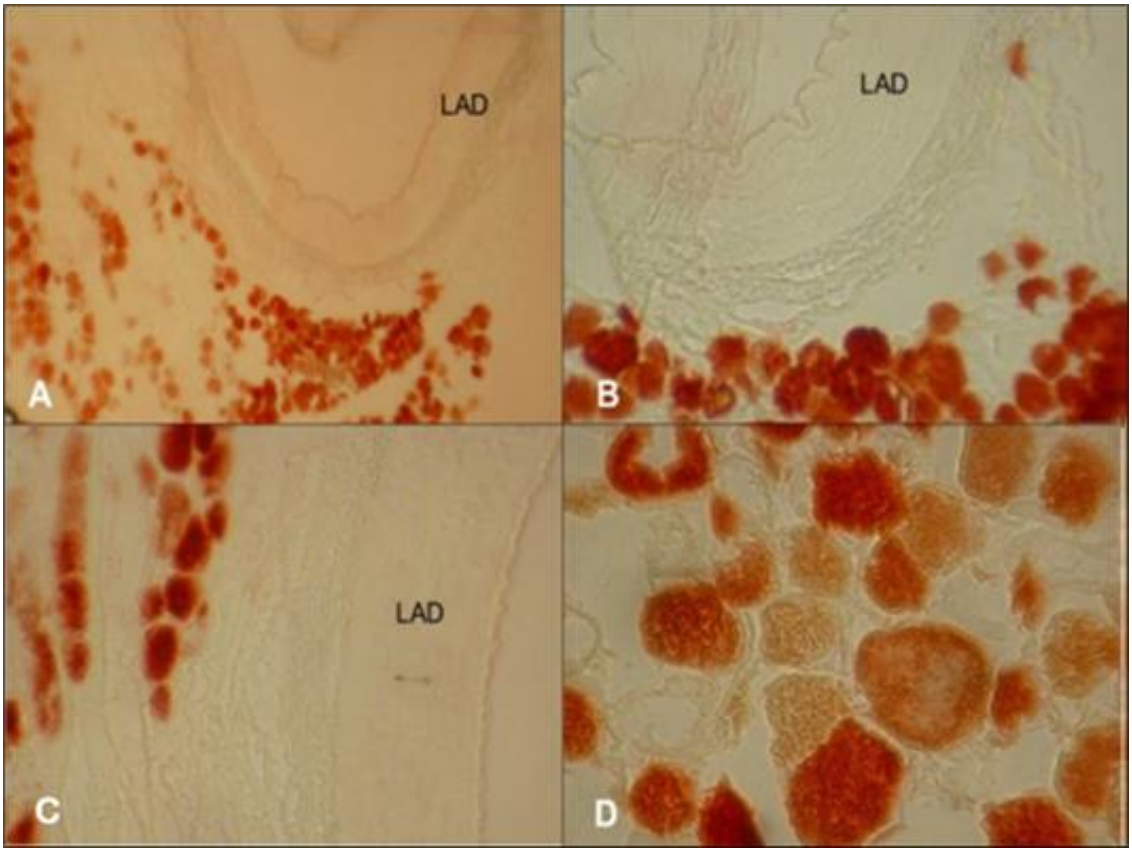

Figure 5 Cryosectioned and immunocytochemically stained (AEC) of spontaneous regeneration of damaged adult porcine heart muscle directly inferior (deep) to left anterior descending coronary artery (LAD). A. Clusters of CEA-CAM-1-positive cells (myofibers, red) located inferior (deep) to LAD. Mag, x100. B. Clusters of CEA-CAM-1-positive cells (myofibers, red) located inferior (deep) to LAD. Mag, x200. C. Clusters of SSEA-4-positive cells (myofibers, red) located inferior (deep) to LAD. Mag, x200. D. Higher power image of clusters of SSEA-4-positive cells (myofibers, red) located inferior (deep) to LAD. Mag, x400. Fig. 3C, smooth muscle alpha-actin (IA4+) in tunica media of coronary blood vessel as positive procedural staining control, and Fig. 3D, no primary antibody as unstained negative procedural control [24]. Reprinted with permission from Kimbrell B, Roberts A, Limnios JI, Lochner F, McCommon G, Samples O, Long GF, Alena C, Krishna V, Woodall MN, Collins JA, Hawkins KC, Hixson D, Bowyer FP III, Black AC Jr, Young HE. Spontaneous Repair of Interventricular Septal Myocardium in the Adult Pig by Primitive Stem Cells. Keystone Symposium on Tumor Suppressors and Stem Cell Biology, Feb, 24-29, 2008, Vancouver, BC

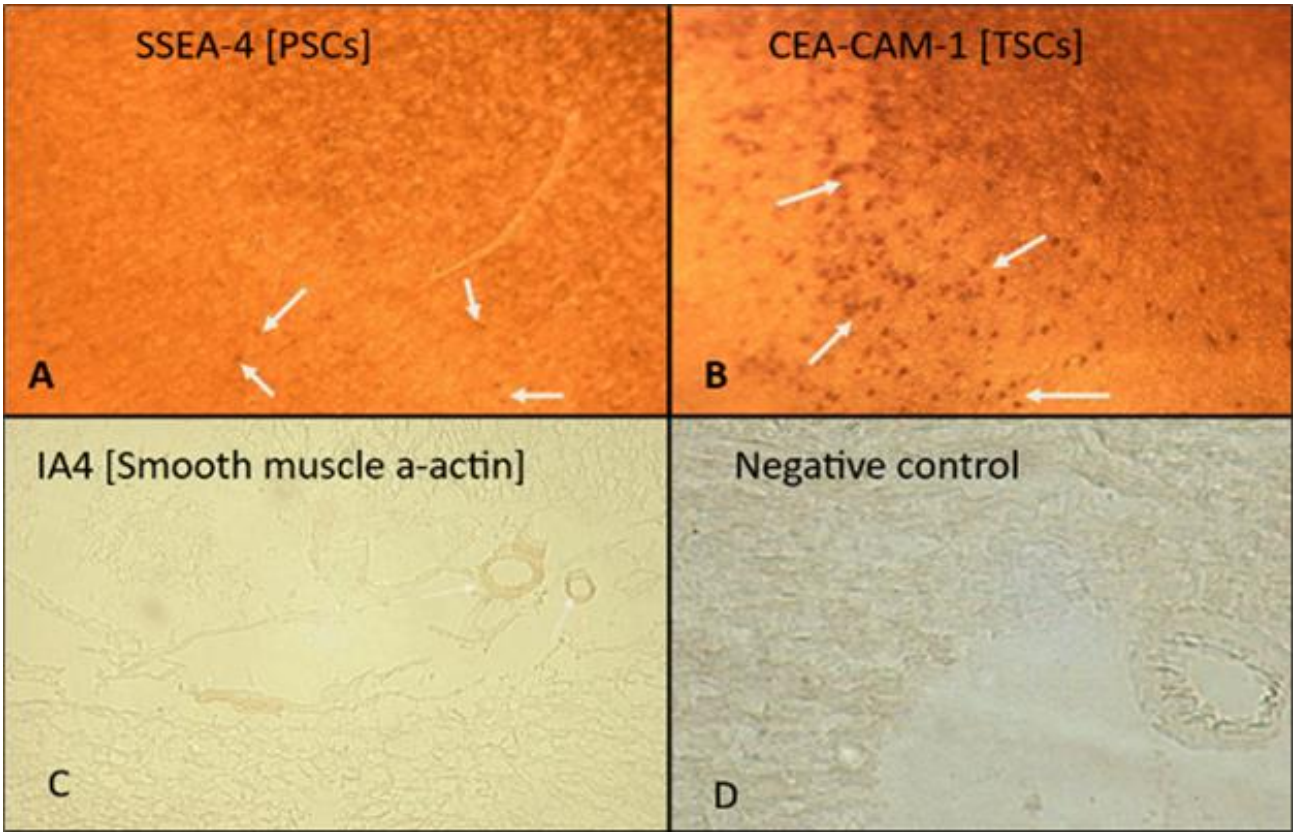

Figure 6 Adult rat brain was immediately removed from its skull and plunged into liquid nitrogen to freeze the tissue. The frozen brain was serially cryosectioned and then sections incubated with antibodies for identification and localization of cell types. SSEA-4 for PSCs, CEA-CAM-1 for TSCS, IA4 for smooth muscle cells in wall of blood vessels. A, SSEA for PSCs (positive cells noted with 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, no primary antibody as negative procedural control [25]. Reprinted with permission from Young HE, Speight MO. Telomerase positive totipotent stem cells in the adult brain. I. cerebral cortex. Regen Med Biol Res 2021; 2(1):1-16

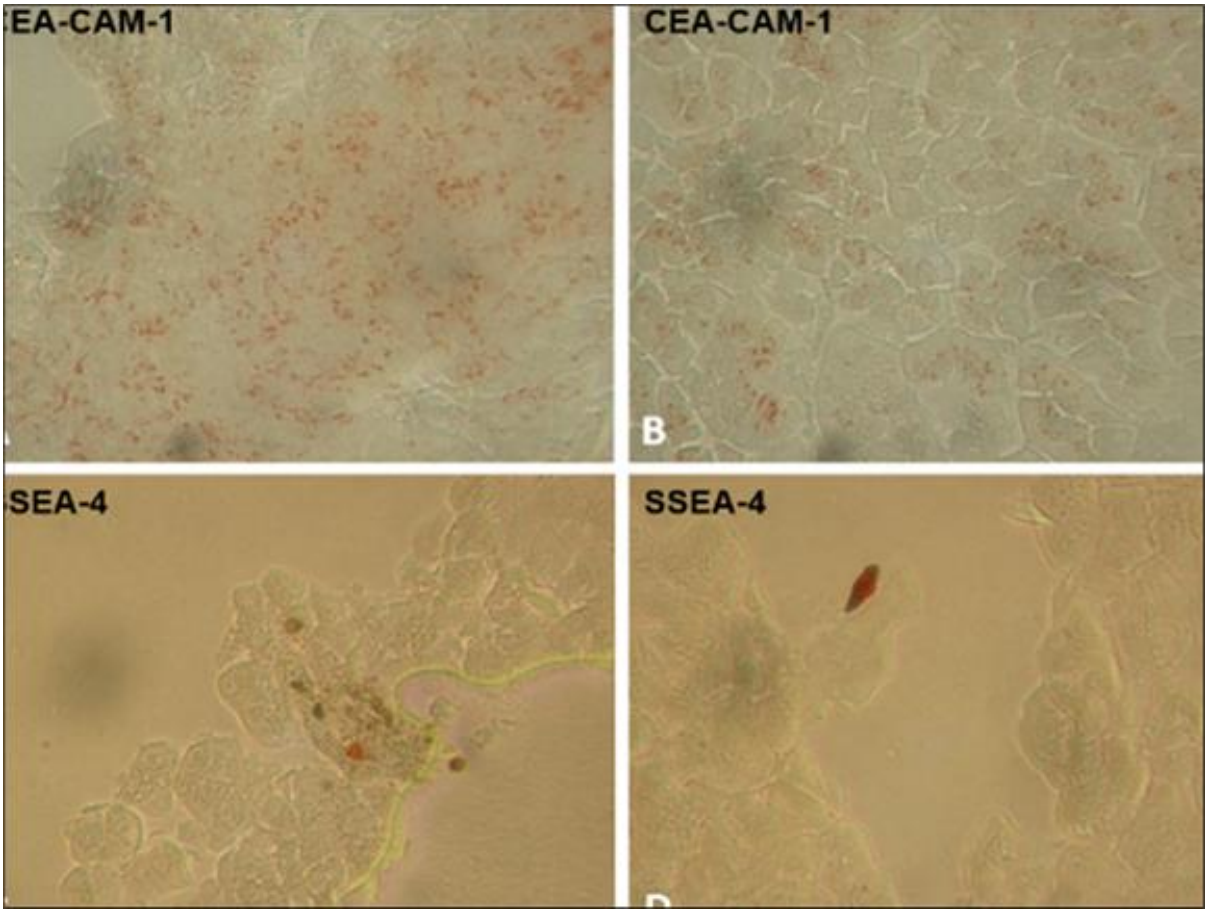

Figure 7 Pancreatic tissue from an adult rat. With IACUC-approval pancreases were harvested from euthanized rats, preserved in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. Predominantly pancreatic islet overlain with (red positive) CEA-CAM-1+ cells, 200x mag. B. Pancreatic acinar cells overlain with (red positive) CEA-CAM-1+ cells, 200x mag. C. Predominantly pancreatic islet tissue overlain with minimal (red positive) SSEA+ cells, 200x mag. D. Pancreatic acinar cells overlain with single (red positive) SSEA+ cell, 200x mag. Positive and negative procedural controls were run simultaneously, but data not shown [26]. Reprinted with permission from Young HE, Limnios JI, Lochner F, McCommon G, Cope LA, Black AC Jr. Pancreatic islet composites secrete insulin in response to a glucose challenge. J Stem Cell Res 1(1) 001: 1-12, 2017

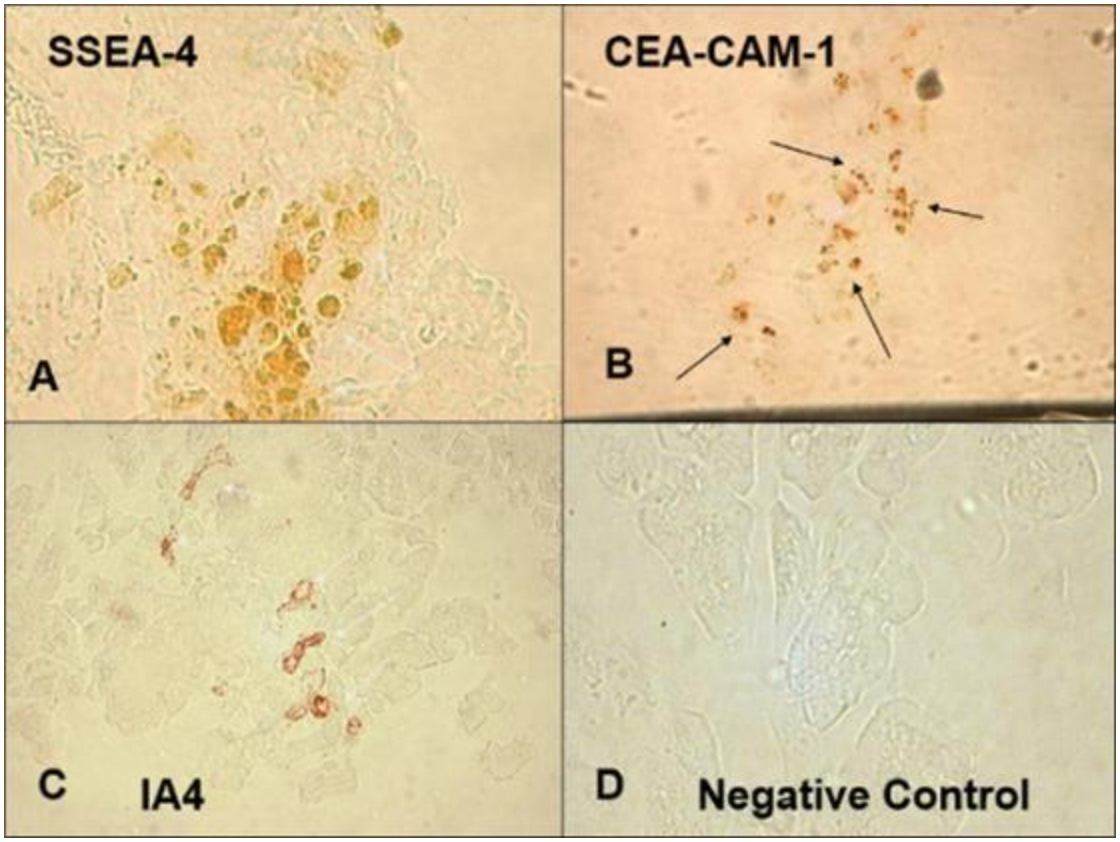

Figure 8 Adult porcine (PM0010) pancreas, brightfield microscopy, AEC HRP-substrate, Mag. x 200. A. SSEA positive stained cells overlying both islets and acinar cells. B. CEA-CAM-1 positively stained cells overlying pancreatic islets and acinar cells. C. IA4 positive staining for smooth muscle alpha-actin in small blood vessels within pancreas, positive procedural control. D. No primary antibody, negative procedural control [26]. Reprinted with permission from Young HE, Limnios JI, Lochner F, McCommon G, Cope LA, Black AC Jr. Pancreatic islet composites secrete insulin in response to a glucose challenge. J Stem Cell Res 1(1) 001: 1-12, 2017

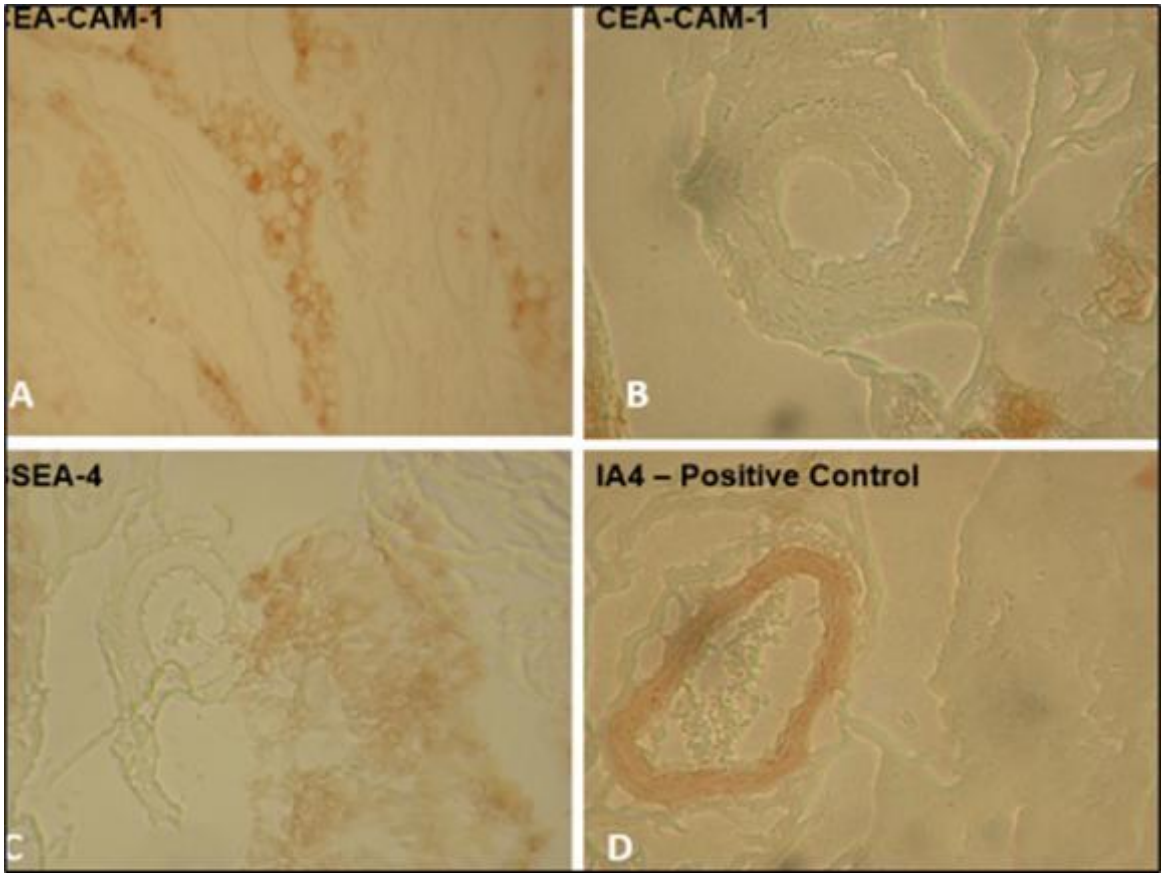

Figure 9 Antibody staining within dermis and hypodermis of adult porcine skin. With IACUC-approval the dermis and hypodermis were harvested from euthanized adult pigs, preserved in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. CEA-CAM-1+ cells within loose fibrous connective tissues surrounding unilocular adipose (fat) cells in hypodermis, 40x mag. B. CEA-CAM-1+ cells in loose fibrous connective surrounding unstained blood vessel, 200x mag. C. SSEA+ cells in loose fibrous connective tissue surrounding blood vessel in border region between reticular dermis and hypodermis, 100x mag. D: IA4+ staining of smooth muscle alpha-actin in tunica media of blood vessel (positive procedural control) in the hypodermis. Note unstained RBCs in lumen of vessel, 200x mag. Negative procedural controls were run simultaneously, but data not shown [27]. Reprinted with permission from Young HE, Limnios JI, Lochner F, McCommon G, Black GF, Coleman JA, Hawkins KC, Black Jr AC. Healing cells in the dermis and adipose tissue of the adult pig. J Stem Cell Res 2017; 1(2) 004:1-5

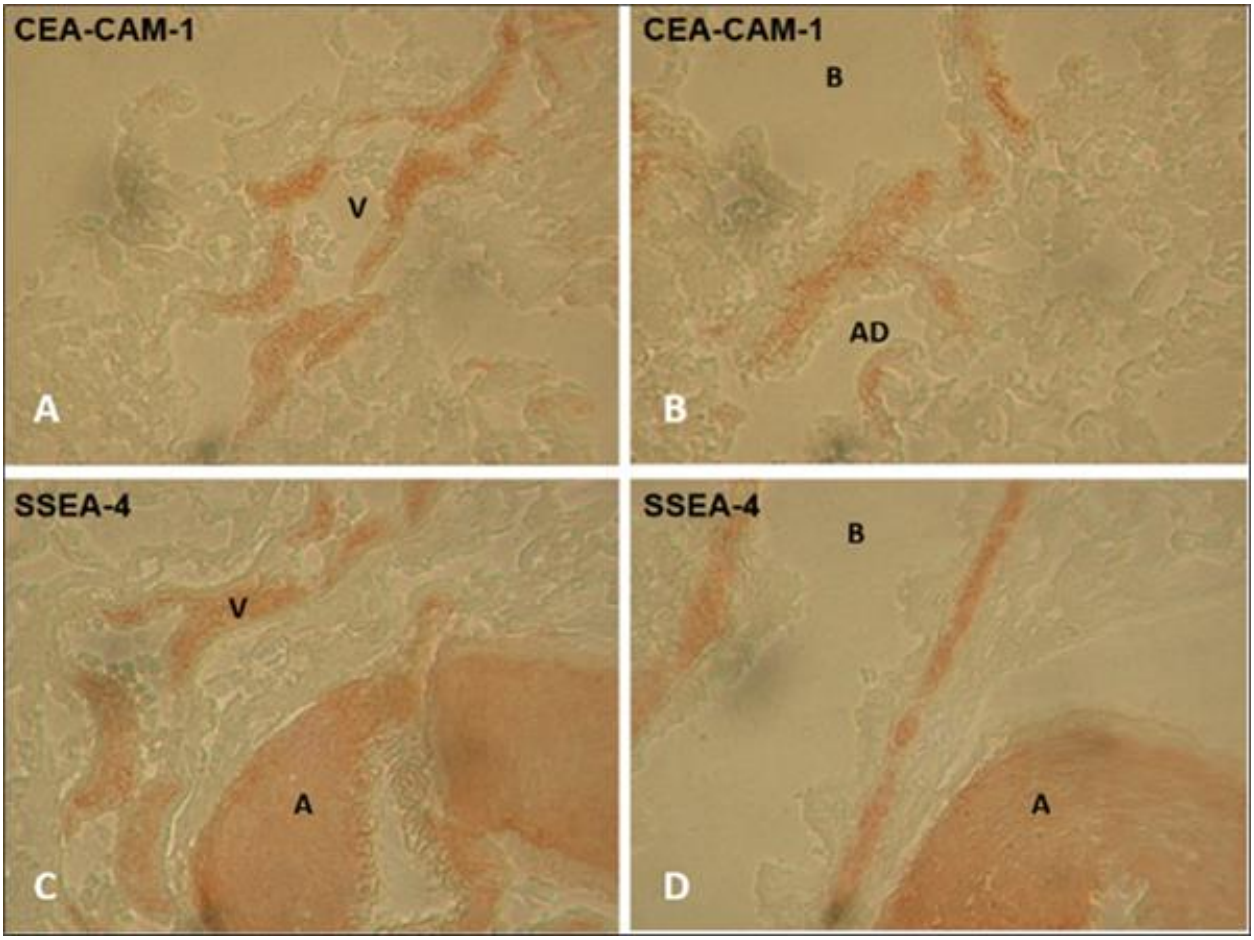

Figure 10 Normal non-regenerating lung tissue. With IACUC-approval the lungs were harvested from euthanized adult rats, preserved in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. CEA-CAM-1+ cells and SSEA+ cells are located within the smooth muscle layer of the blood vessels and structures within the bronchial tree. A. CEA-CAM-1+ cells in tunica media of a vein, 200x mag. B. CEA-CAM-1+ cells within smooth muscle layer of alveolar ducts (AD) and bronchiole (B), 200x mag. C. SSEA+ cells in tunica media (smooth muscle layer) of artery (A) and vein (V), 200x mag. D. SSEA+ cells in smooth muscle wall of bronchial (B) and artery (A), 200x mag. Positive and negative procedural controls were run simultaneously, but data not shown [28]. Reprinted with permission from Young HE, Black GF, Coleman JA, Hawkins KC, Black Jr AC. Pulmonary diseases and adult healing cells: from bench top to bedside. J Stem Cell Res 2017; 1(2) 003:1-9

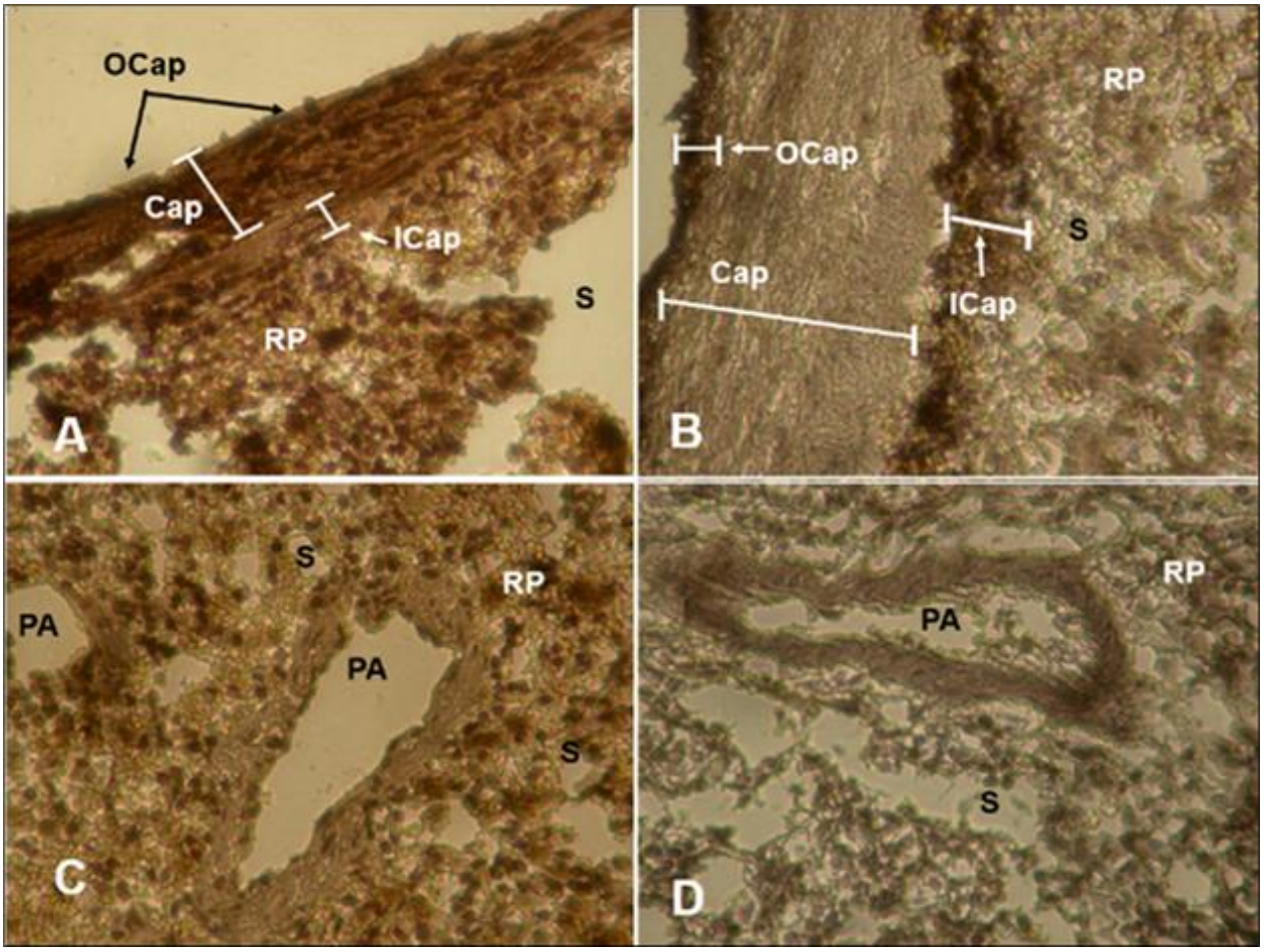

Figure 11 Antibody staining within adult spleens. With IACUC-approval the spleens were harvested from euthanized adult rats (A and C) and adult pigs (B and D). The tissues were preserved in ELICA fixative, cryosectioned at 7 microns, and stained with CEA-CAM-1 for TSCs. Legend: OCap, outer capsule; Cap, capsule; ICap, inner capsule; RP, red pulp; S, splenic sinusoid; PA, penicillar artery. A. Capsule of the adult rat spleen with underlying red pulp. Note CEA-CAM-1+ dark-stained cells amongst the connective tissues of the capsule (outer capsule, capsule, and inner capsule) as well as within the adjacent underlying parenchyma of the red pulp, mag 400x. B. Capsule of the adult pig with underlying red pulp. Note CEA-CAM- 1+ dark stained cells predominantly along the outer and inner borders of the capsule as well as fewer stained cells within the parenchyma of the red pulp, mag 400x. C. Splenic cords (cords of Billroth) of the adult rat cut in cross section. Note dark stained CEA-CAM-1+ cells within the wall of the penicillar arteriole as well as in the parenchyma of the surrounding red pulp, mag 400x. D. Splenic cords of the adult pig cut in cross section. Note a few CEA-CAM-1+ cells within the wall of the trabecular vessel and the paucity of CEA-CAM-1+ cells within the parenchyma of the red pulp surrounding the trabecular vessel, mag 400x. Positive controls (i.e., smooth muscle alpha-actin staining of smooth muscle is vessel walls) and negative controls absence of primary antibody during staining) were appropriate (data not shown) [29]. Reprinted with permission from Young HE, Limnios IJ, Lochner F, McCommon G. Telomerase-positive stem cells in adult porcine and adult rat spleens. I. Totipotent stem cells. J Regen Med Biol Res. 2020; 1(2): 1-20.

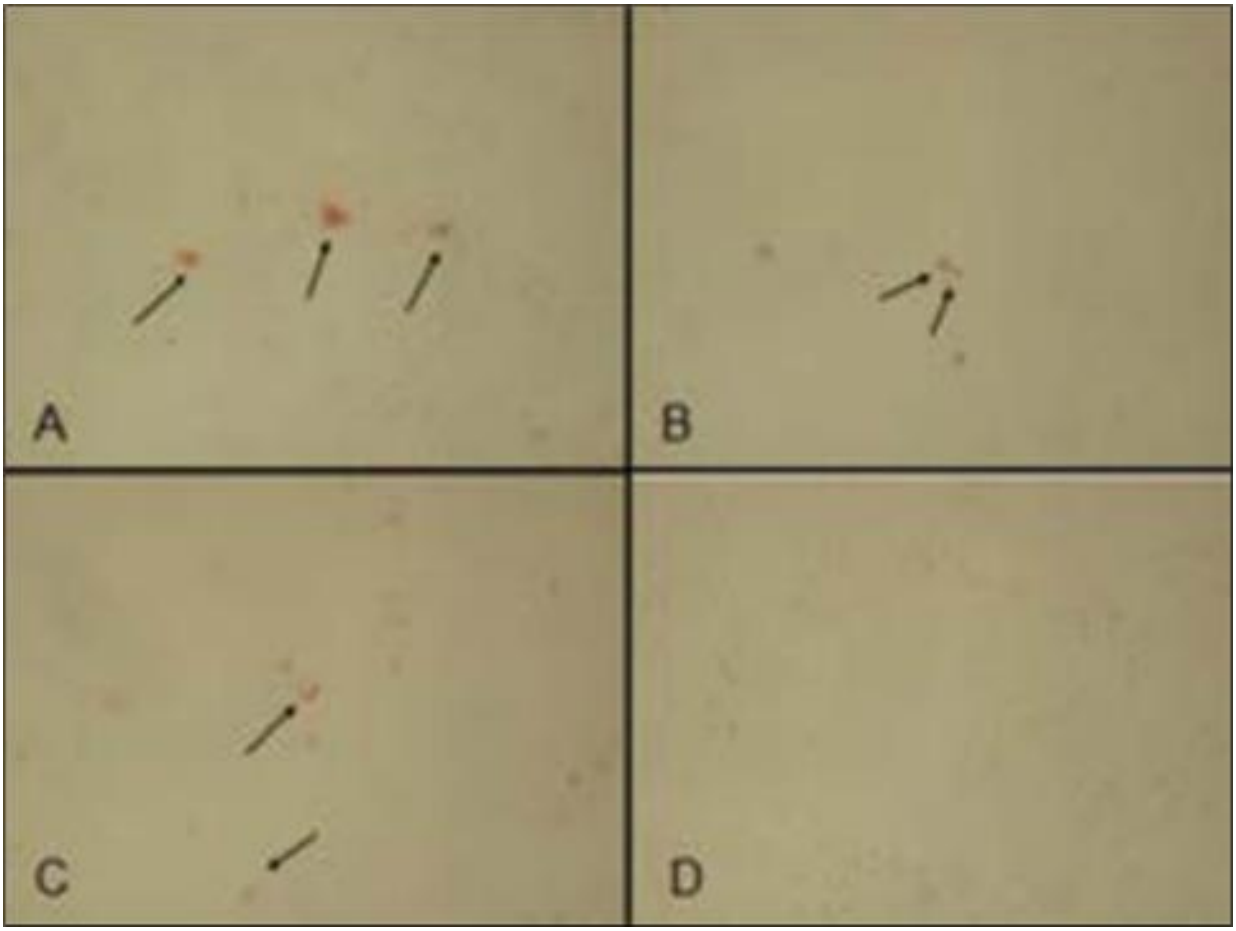

Figure 12 Staining of non-hematopoietic marrow cells from adult rats. With IACUC-approval bone marrow was harvested from adult rats, preserved in ELICA fixative, smeared onto a glass slide, and stained immunocytochemically. A. A cluster of CEA-CAM-1+ cells (red, arrows), mag. 400x. B. A small cluster of SSEA-4+ cells (arrows), mag, 200x. C. IA4+ arterioles (arrows) present in the bone marrow preparation as the positive procedural control, mag 200x. D. Negative procedural control, immunocytochemical staining of bone marrow smears without primary antibody, mag 200x [30]. Reprinted with permission from Young HE, Henson NL, Black GF, Hawkins KC, Coleman JA, Black Jr AC. Stage-Specific Embryonic Antigen-4-Positive Cells and Carcinoembryonic Antigen Cell Adhesion Molecule-1-Positive Cells are Located in the Bone Marrow of the Adult Rat. J Stem Cell Res. 1(2) 001: 1-3, 2017

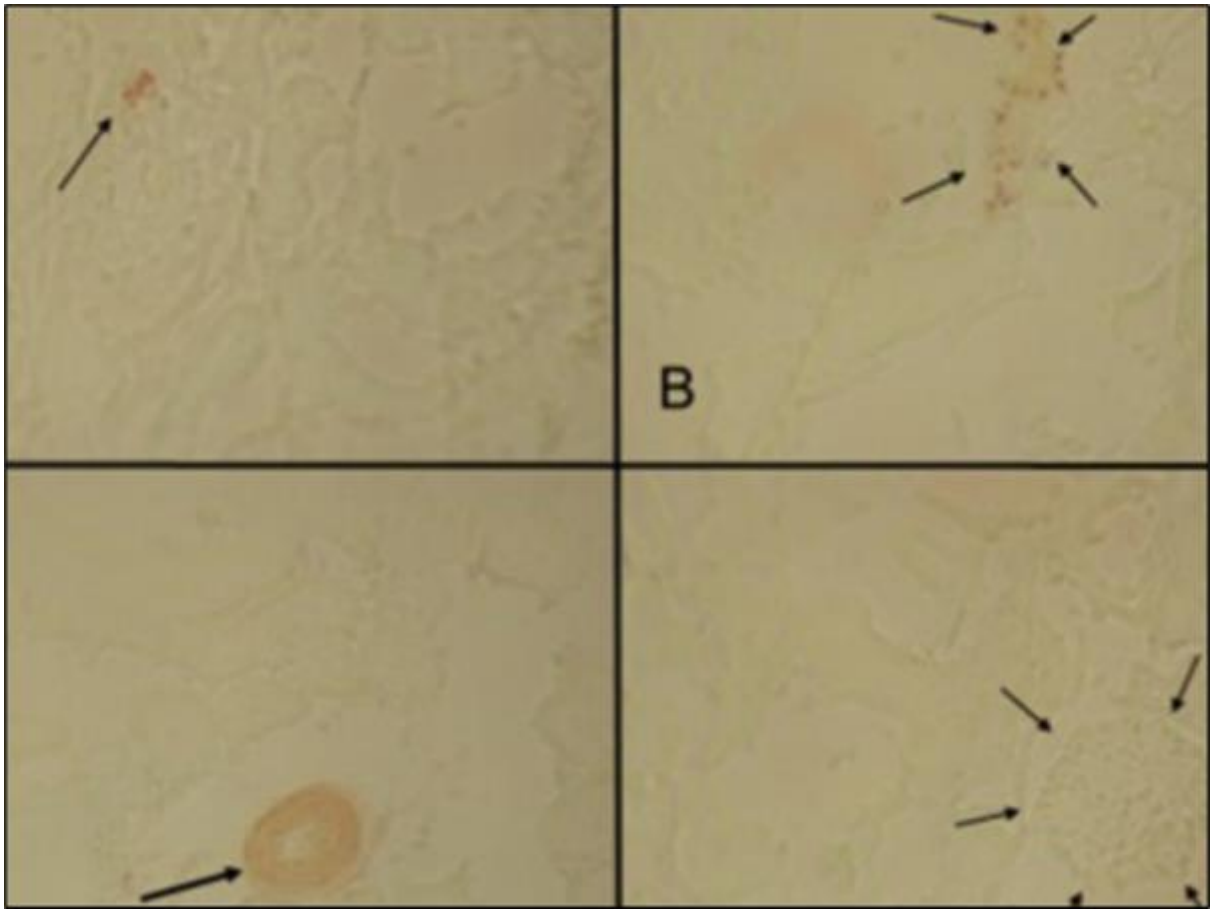

Figure 13 Kidney of the adult rat. With IACUC-approval, adult rats were euthanized, kidneys harvested, preserved with ELICA fixative, frozen, and cryosectioned at 7 microns. The resultant sections were stained immunocytochemically using the antibodies: CEA-CAM-1, SSEA-4, and IA4. A. A small cluster of six cells staining for SSEA-4 were noted located among the tubules in the medulla of the kidney, 100x mag. B. A larger cluster of 26 cells staining for CEA-CAM-1 were located among the tubules at the border between the cortex and medulla of the kidney, 200x mag. C. Cells staining for IA4 (denoting presence of smooth muscle alpha-actin) were noted in the tunica media of blood vessels within the kidney, 40x mag. D. All negative procedural controls demonstrated complete absence of staining of any kind throughout the kidney, 100x mag [31]. Reprinted with permission from Young HE, Black GF, Coleman JA, Hawkins KC, Williams S, Black Jr AC. Healing cells in the kidney of the adult rat. J Stem Cell Res 2017; 1(3) 001:1-4.

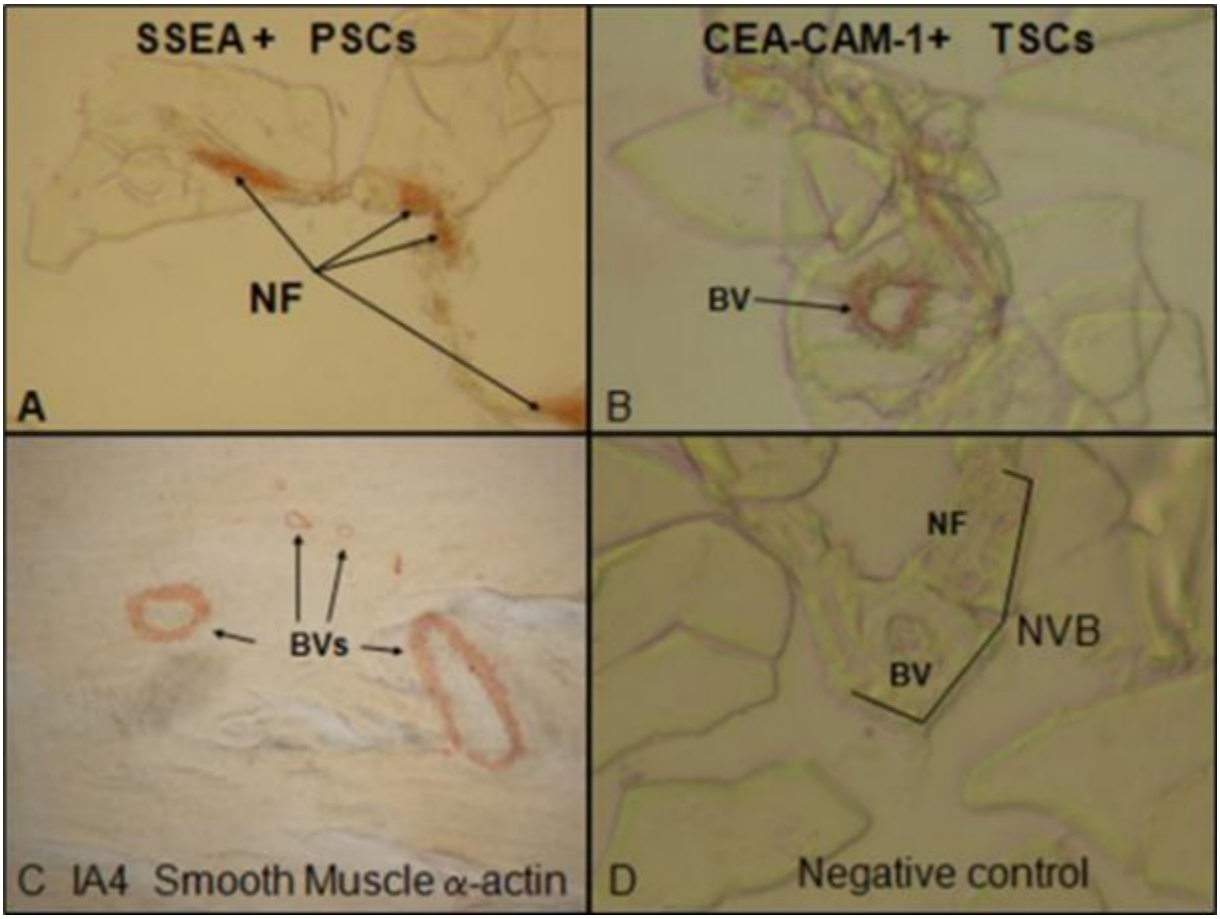

Figure 14 Adult rat skeletal muscle. Post-traumatic adult male rat skeletal muscle (RM1025), euthanized, skeletal muscle harvested, preserved with ELICA fixative, cryosectioned at 7 microns, mounted on positively charged glass slides, and incubated with designated antibodies. A. SSEA positive (PSCs) within nerve fiber connective tissues. B. CEA-CAM-1 positive (TSCs) staining within tunica intima of blood vessels. C. IA4 positive staining for smooth muscle alpha-actin in the tunica muscularis of medium and small blood vessels within the perimysium of skeletal muscle, positive procedural control. D. No primary antibody, as negative procedural control [32]. Reprinted with permission from Young HE, Henson NL, Black GF, Hawkins KC, Coleman JA, Black Jr AC. Location and characterization of totipotent stem cells and pluripotent stem cells in the skeletal muscle of the adult rat. J Stem Cell Res 1(1) 002: 1-17, 2017

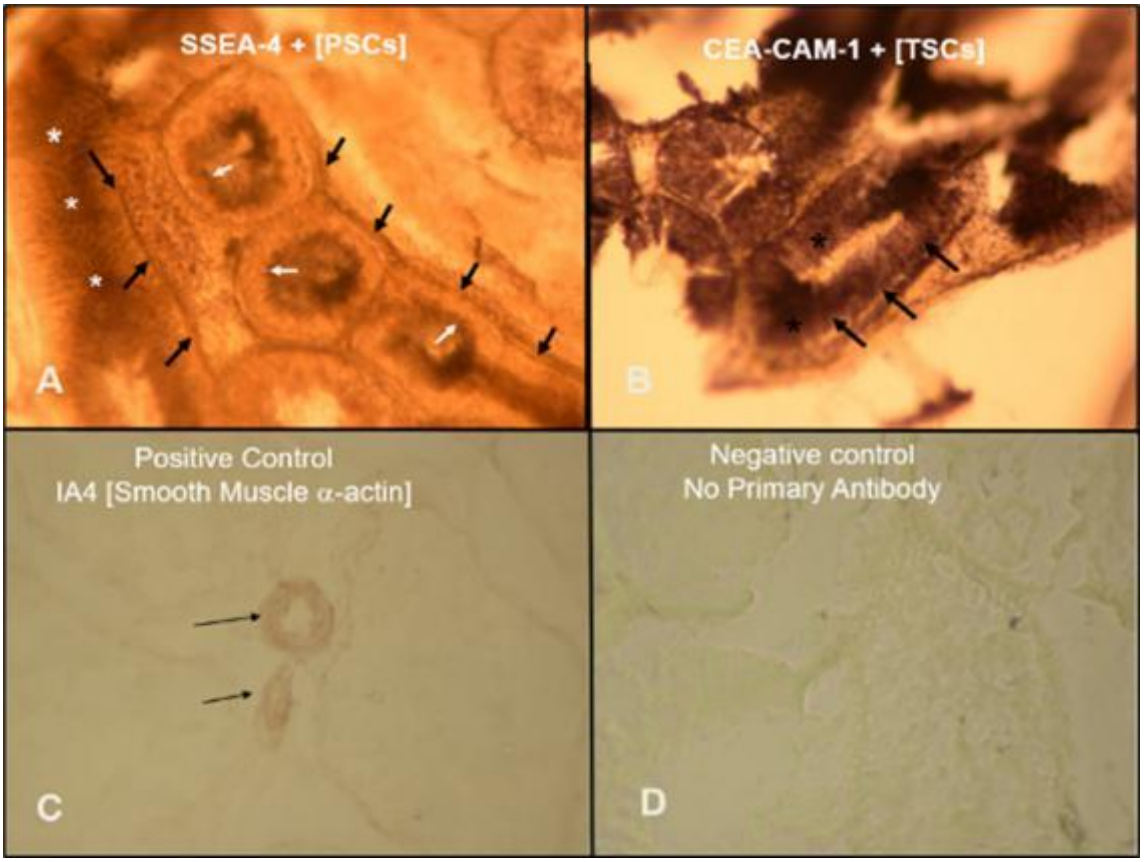

Figure 15 Adult rat testis (RM1021), tissue harvested, preserved in ELICA fixative, vibratome-sectioned at 25 microns, mounted on glass slides, and stained with appropriate antibodies. A. SSEA positive cells (PSCs) surrounding outside of seminiferous tubules (black arrows) as well as within the innermost 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 [18]. Reprinted with permission from Young HE. Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 are present in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025; 23(03): 149-157

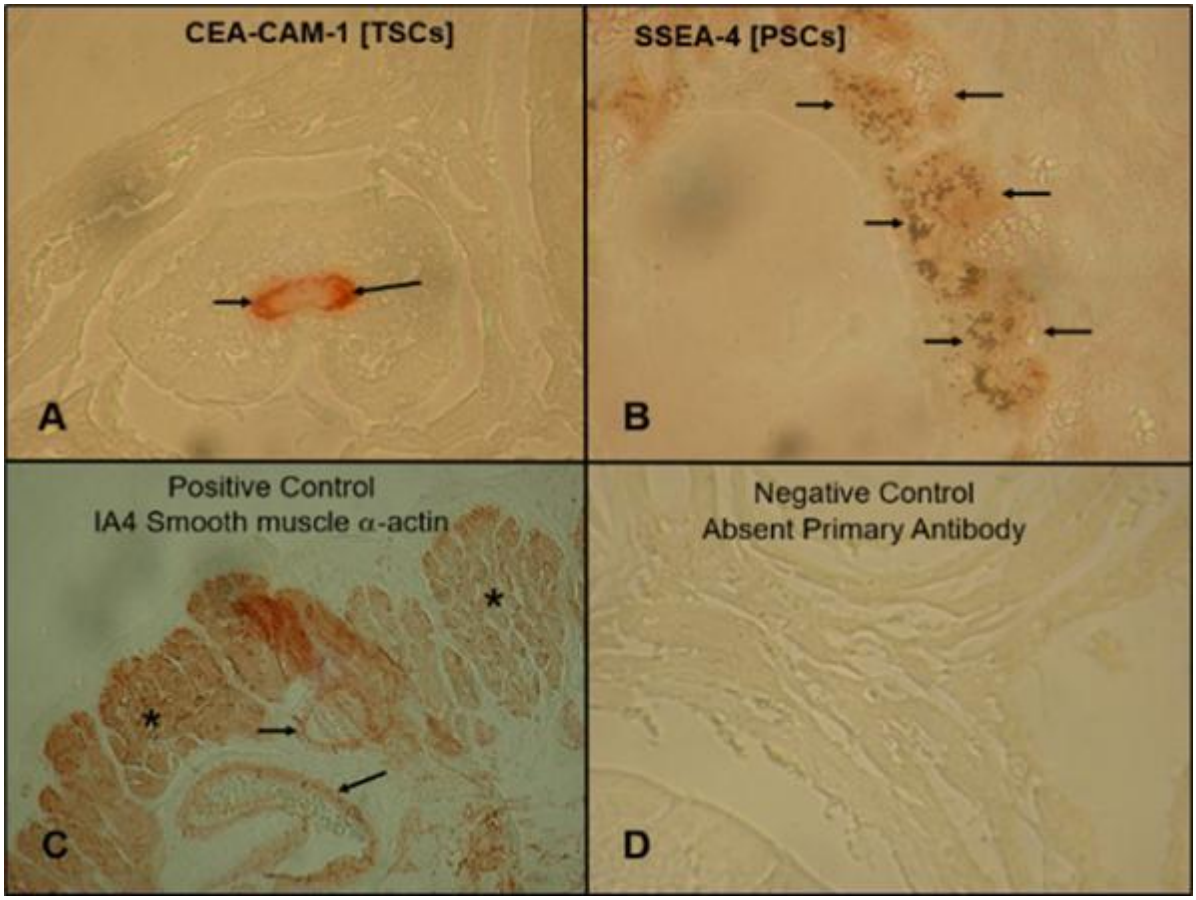

Figure 16 Adult female rat (RF1022) ovary with attached fallopian tube harvested from euthanized rat, fixed in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. CEA-CAM-1 positive staining in area of zona pellucida of ovum inside tertiary follicle (arrows), 400x. B. SSEA positive cells interspersed among granulosa cells of Graafian follicle (arrows), 400x. 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. This particular slide was used in this staining series as the positive procedural control because of the amount of smooth muscle present in the wall musculature and the vascular supply, 200x. D. No primary antibody, no staining present within ovary, used as the negative procedural control, 400x [18]. Reprinted with permission from Young HE. Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 are present in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025; 23(03): 149-157

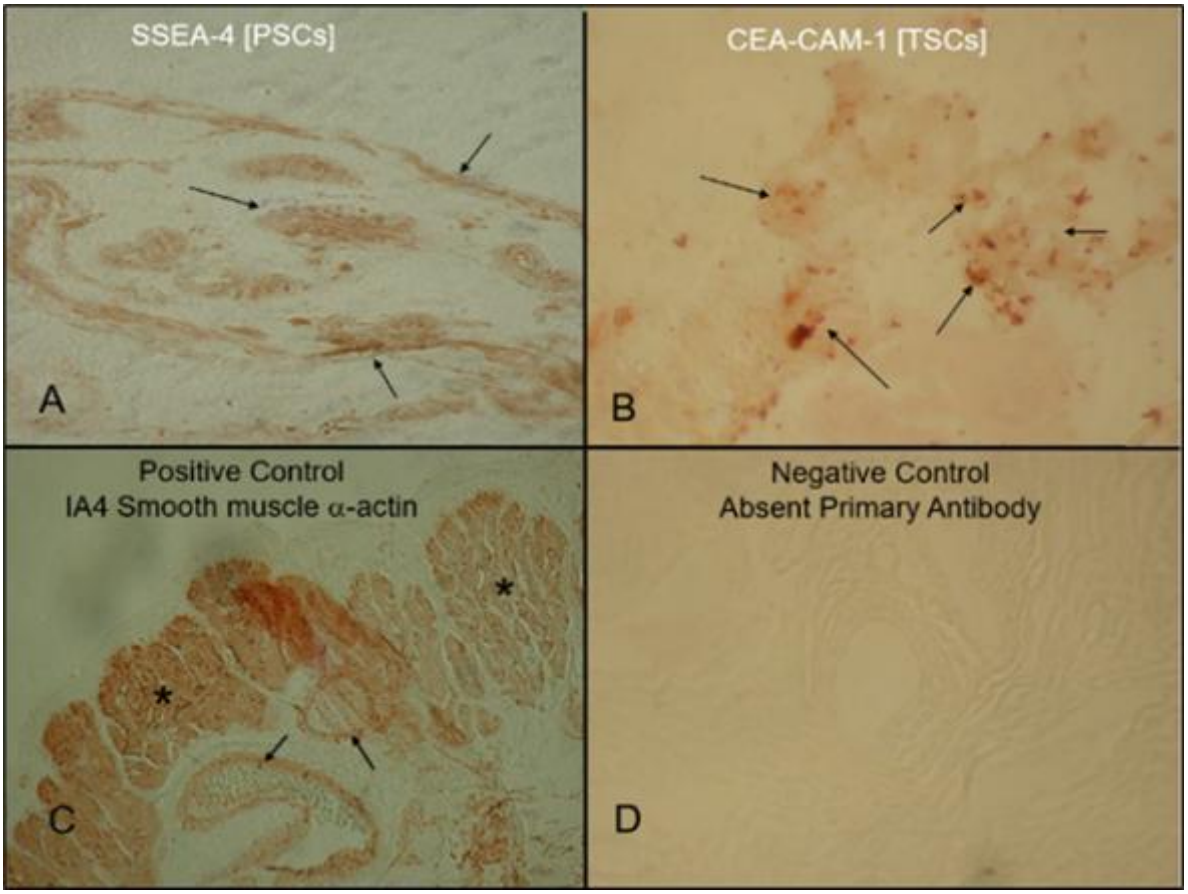

Figure 17 Adult female rat (RF1022) fallopian tube harvested from euthanized rat, preserved in ELICA fixative, cryosectioned at 7 microns, and stained with appropriate antibodies. A. SSEA-4 positive PSCs within area of ampulla of fallopian tube (arrows), 200x. B. CEA-CAM-1 positive TSCs in the connective 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 [18]. Reprinted with permission from Young HE. Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 are present in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025; 23(03): 149-157

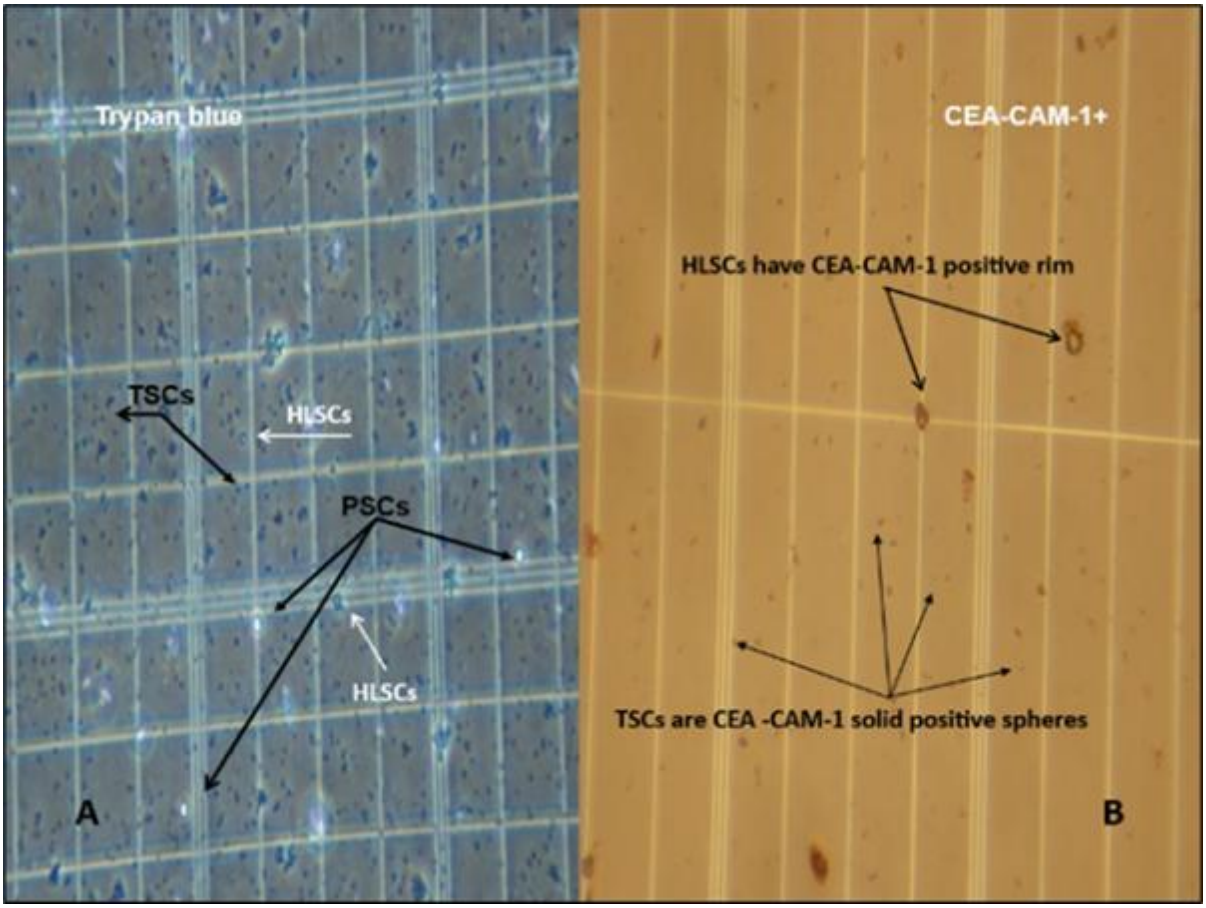

Figure 18 Human stained plasma fraction. With IRB-approval, adult human blood from a chronically-ill individual was harvested by venipuncture. A haematocrit was formed and the plasma supernatant removed and processed as described. A. Human cells stained 1:1 with 0.4% Trypan blue solution, diluted 1:1000 with sterile saline and mounted onto a hemocytometer, magnification 200X. Note cells that are spherical and solid blue are large and small TSCs. Spherical cells that have a rim of Trypan blue positive staining with centers void of Trypan blue positive staining are HLSCs. Trypan blue negative cells which are white "glowing" spheres are PSCs. B. Human cells stained with CEA-CAM-1 antibody, diluted 1:1000 with sterile saline and mounted onto a hemocytometer, magnification 200X. Note very small cells that display CEA-CAM-1 throughout are small TSCs. Note larger cells with rim of CEA-CAM-1 positive material with relatively "clear" centers are HLSCs [20]. Reprinted with permission from Young HE, Lochner F, Lochner D, Lochner D, Black GF, Coleman JA, Young VE, McCommon G, Black Jr AC. Primitive stem cells in adult human peripheral blood. J Stem Cell Res. 1(2) 001:1-8, 2017

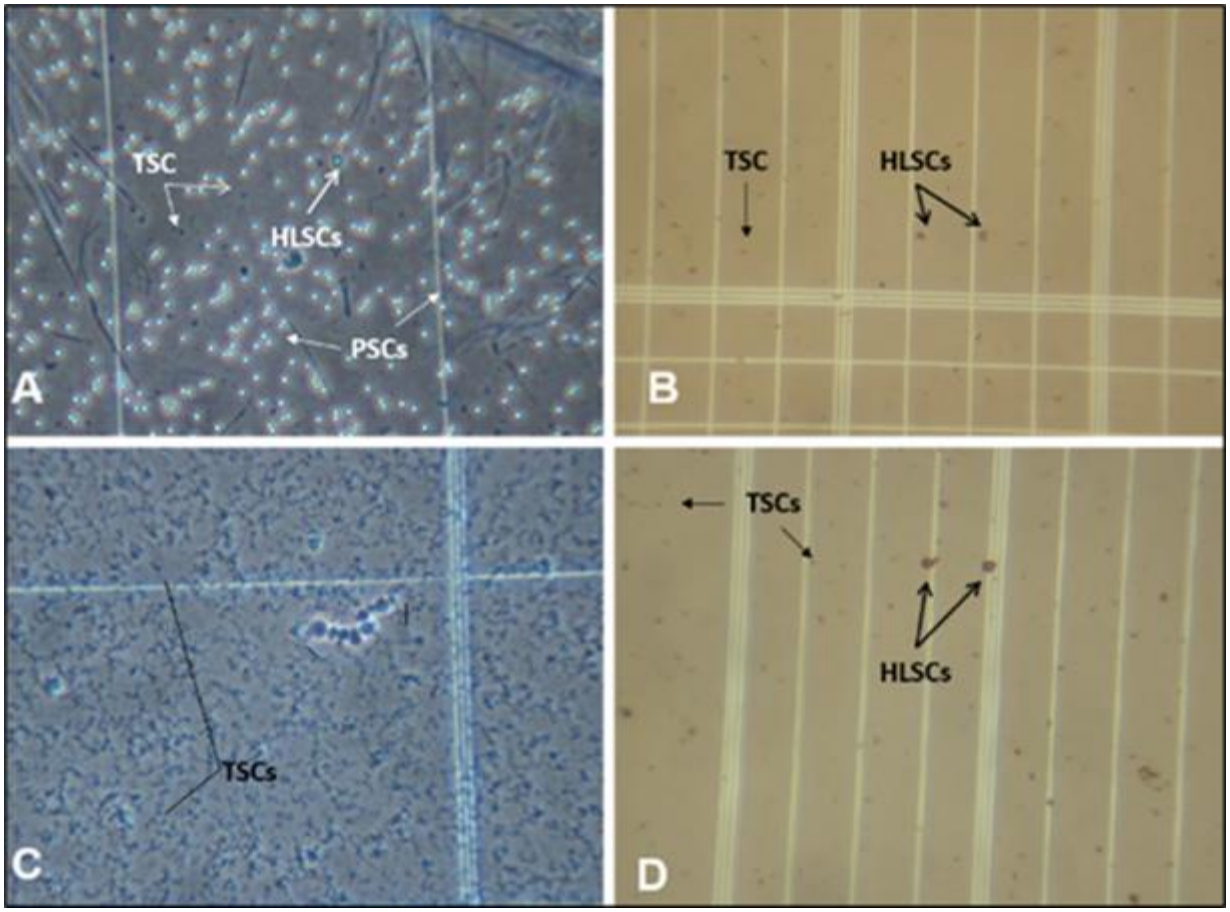

Figure 19 Feline (Cat) and Canine (Dog) stained plasma fractions. With IACUC-approval, adult blood was harvested by venipuncture. A haematocrit was formed and the plasma supernatant removed and processed as described. A. Feline plasma fraction stained with 0.4% Trypan blue. TSCs are small solid dark spheres. HLSCs are spheres with a dark rim of blue-staining and relatively clear centers. PSCs are unstained spheres with glowing white centers, 100x mag. B. Feline plasma fraction stained with CEA-CAM-1. TSCs are small solid dark-red round spheres. HLSCs are spherical with a dark red-brown rim of staining and a relatively clear center, 100x mag. C. Canine plasma fraction stained with 0.4%Trypan blue. TSCs are small solid dark round spheres, 100x mag. D. Canine plasma fraction stained with CEA-CAM-1. TSCs are small solid dark-red spheres and HLSCs are spheres with a dark-red rim and a clear center, 100x mag [21]. Reprinted with permission from Young HE, Lochner F, Lochner D, Lochner D, McCommon G, Black AC Jr. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine, and Equine Peripheral Blood. J Stem Cell Res. 2017; 1(1) 004: 1-6

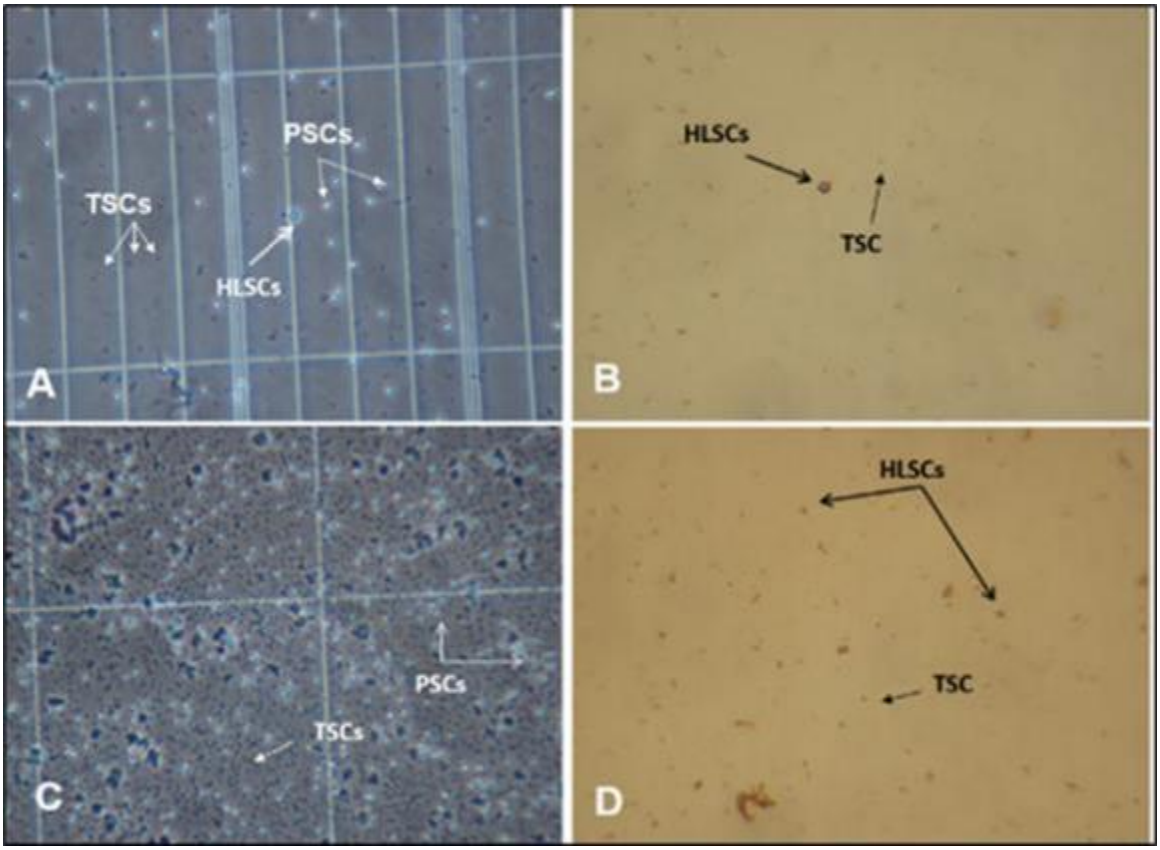

Figure 20 Ovine (Sheep) and Caprine (Goat) stained plasma fractions. With IACUC-approval, adult blood was harvested by venipuncture. A haematocrit was formed and the plasma supernatant removed and processed as described. A. Ovine plasma fraction stained with 0.4% Trypan blue. TSCs are small dark blue-stained spherical cells. HLSCs are a spherical cell with a rim of dark blue staining and a relatively clear center. PSCs are spherical unstained cells with white centers, 100x mag. B. Ovine plasma fraction stained with CEA-CAM-1. TSCs are small dark red-brown spherical cells. HLSCs are a spherical cell with a rim of dark red-brown staining and a relatively clear center. C. Caprine plasma fraction stained with 0.4%Trypan blue. TSCs are small solid dark spheres, 100x mag. D. Caprine plasma fraction stained with CEA-CAM-1. TSCs are small dark-red round circles. HLSCs are a spherical cell with a rim of dark red-brown staining and a relatively clear center. 100X Mag [21]. Reprinted with permission from Young HE, Lochner F, Lochner D, Lochner D, McCommon G, Black AC Jr. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine, and Equine Peripheral Blood. J Stem Cell Res. 2017; 1(1) 004: 1-6

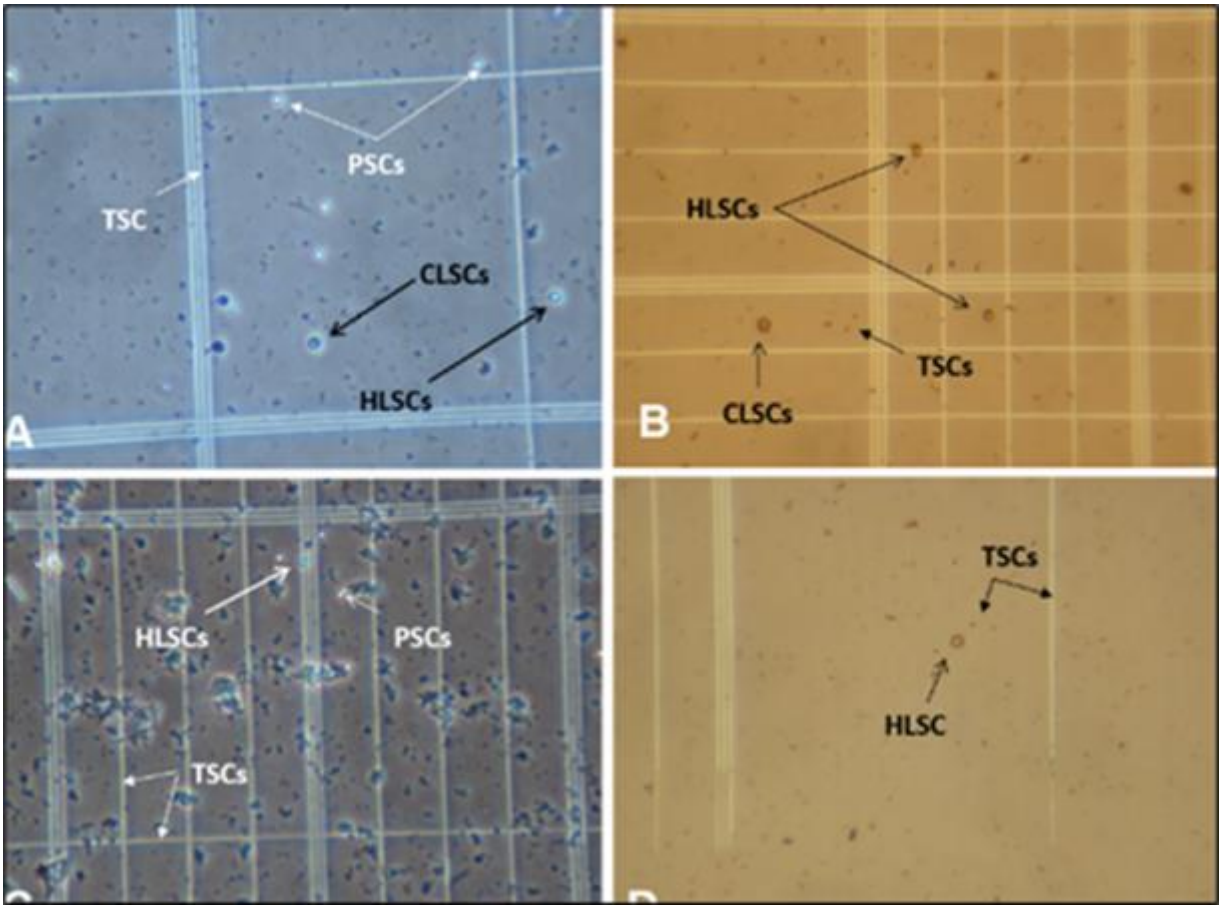

Figure 21 Bovine (Cow) and Equine (Horse) stained plasma fractions. With IACUC-approval-approval, adult blood was harvested by venipuncture. A haematocrit was formed and the plasma supernatant removed and processed as described. A. Bovine plasma fraction stained with 0.4% Trypan blue. TSCs are small solid dark spheres; HLSCs are spheres with a peripheral rim of staining and relatively clear centers; CLSCs are spheres with a "crown" of dark blue staining along one side of cell and relatively clear centers; PSCs are unstained spheres with glowing white centers, 100x mag. B. Bovine plasma fraction stained with CEA-CAM-1. TSCs are small solid dark-red spheres. HLSCs are spheres with a peripheral rim of red-brown staining and relatively clear centers; CLSCs are spheres with a "crown" of red-brown staining along one side of cell and relatively clear centers, 100x mag. C. Equine plasma fraction stained with 0.4%Trypan blue. TSCs are small solid dark spheres. HLSCs are spheres with a peripheral rim of staining and relatively clear centers. PSCs are unstained spheres with glowing white centers, 100x mag. D. Equine plasma fraction stained with CEA-CAM-1. TSCs are small solid dark-red spheres. HLSCs are spheres with a peripheral rim of red-brown staining and relatively clear centers, 100x mag [21]. Reprinted with permission from Young HE, Lochner F, Lochner D, Lochner D, McCommon G, Black AC Jr. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine, and Equine Peripheral Blood. J Stem Cell Res. 2017; 1(1) 004: 1-6

Table 1 Species, Age, and Location of TSCs and PSCs

Loca[1]	**Sal[2]**	**R[3]**	**Av[4]**	**M[5]**	**Rt[6]**	**R[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]					Is Im						Im				
Ub[58]					Is Im						Im				
Thy[59]					Is Im						Im				
Tng[60]					Is Im						Im				
Testis					Is Im										
Ovary					Im										
FT[61]					Im										
Kar[62]				Dip 40[63]	Dip 42						Dip38			Dip 64	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 mater); Men[52], meninges (dura mater, arachnoid mater, pia mater); SpC[53], spinal cord (white mater, gray mater); 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; FT[61], Fallopian Tube; Kar[62], karyotypic analysis; Dip[63], diploid number of chromosomes correct for respective species [18,33]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014; Young HE. Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 are present in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025; 23(03): 149-147.

Table 2 Attributes of Endogenous Adult Telomerase Positive Stem Cells

Attributes	**TSCs[1]**	**HLSCs[2]**	**CLSCs[3]**	**PSCs[4]**	**GLSCs[5]**	**EctoSCs[6]**	**MesoSCs[7]**	**EndoSCs[8]**
Size, microns	0.1-2.0	>2-4	>4-<6	6-8	>8-<10	10-12	10-12	10-12
0.4% Trypan blue	Entire Cell Positive[9]	Halo[10] Positive, Negative	Corona[11], Positive Negative	Entire Cell Negative[12]	Entire Cell Negative	Entire Cell Negative	Entire Cell Negative	Entire Cell Negative
Animal Cell Surf Markers	CEA-CAM-1[13]	CEA-CAM-1^{high} SSEA-4^{low}	CEA-CAM-1^{low} SSEA-4^{high}	SSEA-4[14]	SSEA-4, Thy-1[15]	Thy-1	Thy-1	Thy-1
Human Cell Surf Markers	CD66e[16]	CD66e^{high} CD10^{low}	CD66e^{low} CD10^{high}	CD10[17]	CD10 CD90[18]	CD56, CD90 MHC-1[19]	CD13, CD90 MHC-1	??? CD90 MHC-1
Culture Conditions	Substrate Adhesion[20]	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion
Maximum Population Doublings To Date	>300 Rat	>300 Rat	>300 Rat	>400 Rat	>400 Rat	>400 Rat	>690 Human	>400 Rat
Differentiation Capabilities[21]	All Cells[22]	Somatic Cells only[23]	Somatic Cells only	Somatic Cells only	Somatic Cells only	Ectoderm Lineage Only[24]	Mesoderm Lineage Only[25]	Endoderm Lineage Only[26]

Table 1. TSCs[1], totipotent stem cells; HLSCs[2], halo-like stem cells; CLSCs[3], corona-like stem cells; PSCs[4], GLSCs[5], germ layer lineage stem cells; EctoSCs[6], ectodermal stem cells; MesoSCs[7], mesodermal stem cells; EndoSCs[8], endodermal stem cells; entire cell demonstrating Positive[9] staining for 0.4% Trypan blue; Halo[10], complete peripheral rim of positive trypan blue staining with central area of cell negative for Trypan blue staining; Corona[11], crown of positive Trypan blue staining, remainder of cell is negative for Trypan blue staining; entire cell demonstrating Negative[13]staining for 0.4% Trypan Blue; CEA-CAM-1[13], carcinoembryonic antigen-cell adhesion molecule-1; SSEA-4[14], stage-specific embryonic antigen-4; Thy-1[15], N-glycosylated glycophosphatidylinositol (=CD90); CD66e[16], carcinoembryonic antigen; CD10[17], common acute lymphoblastic leukemia antigen (CALLA); CD90[18], N-glycosylated glycophosphatidylinositol (=Thy-1); MHC-1[19], self-recognition molecule Major Histocompatibility Complex-Class-1; Substrate Adhesion[20], only grows attached to a type-1 collagen-based substratum; Differentiation Capabilities[21], differentiation capabilities; All Cells[22], will form all somatic cells of the body, the gametes (spermatogonia and oogonia), and the nucleus pulposus of the intervertebral disc (the only tissue derived from the notochord in adults); Somatic Cells Only[23], will only form somatic cells of the body, will not form gametes, will not form nucleus pulposus of the intervertebral disc; Ecto Lineage Only[24], will only form cells of the ectodermal germ layer lineage, will NOT form cells of either the mesodermal or the endodermal germ layer linages; Meso Lineage Only[25], will only form cells of the mesodermal germ layer lineage, will NOT form cells of either the ectodermal or the endodermal germ cell lineages; Endo Lineage Only[26], will only form cells of the endodermal germ layer lineage, will NOT form cells of either to ectodermal or mesodermal germ layer lineages; NYD[28] , not yet determined (22). 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.

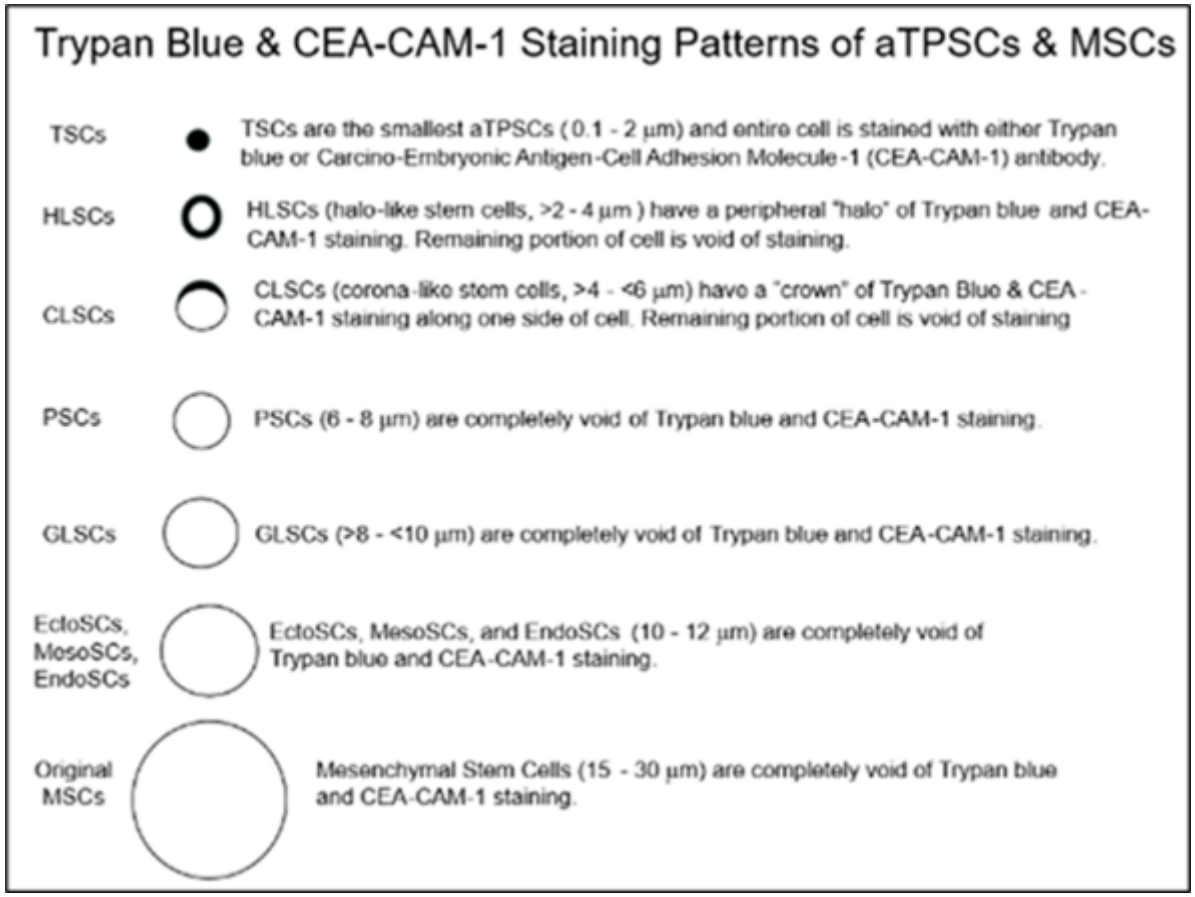

Figure 22 Diagrammatic representation of size comparison and visual staining patterns viewed by brightfield microscopy of aTPSCs (e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, and EndoSCs) and Mesenchymal Stem Cells (MSCs) when stained with either 0.4% Trypan Blue or Carcino-Embryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1)

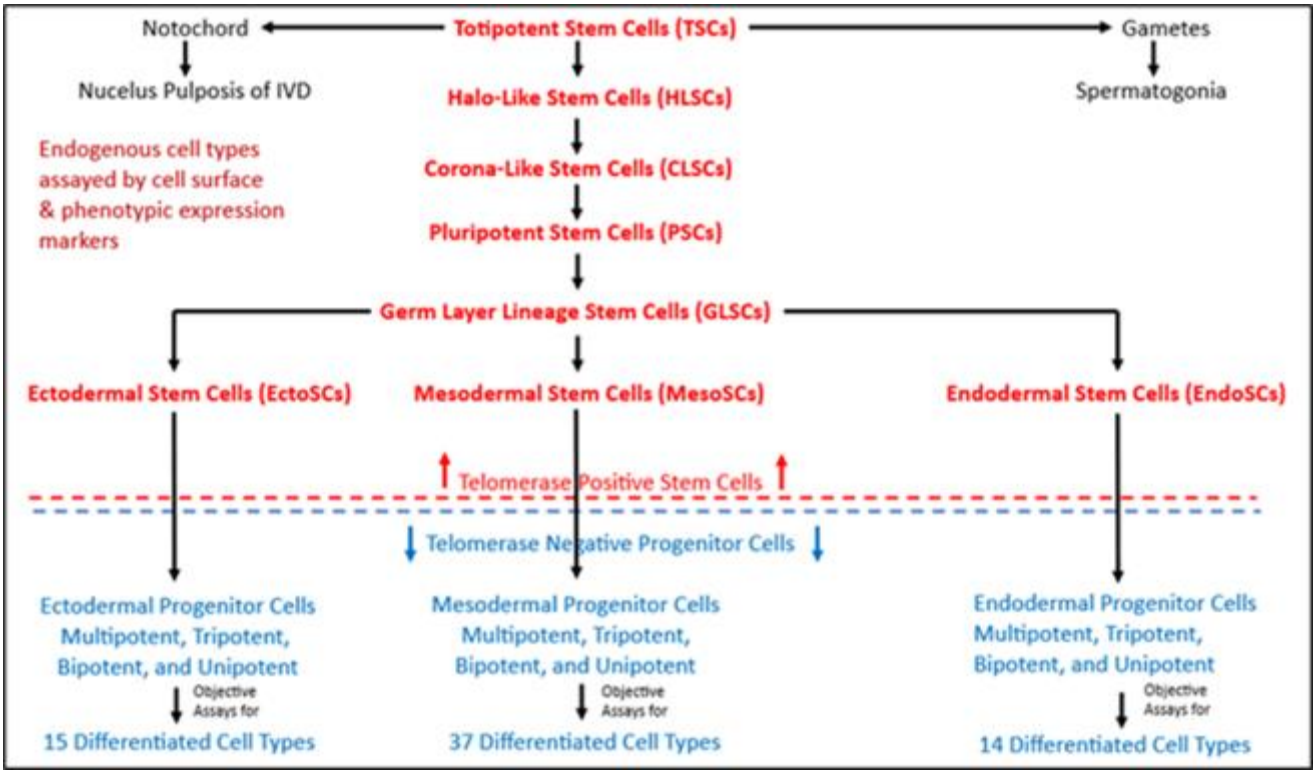

Figure 23 Identification of eight categories of aTPSCs (red) and their unidirectional differentiation potentials into telomerase negative progenitor cells and telomerase negative differentiated cells (blue) using objective assays for cell surface and 130 phenotypic expression markers to identify 76 distinct cell types [22]. 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

3.1. Current Knowledge of aTPSCs

I discovered naturally-occurring endogenous adult telomerase positive stem cells (aTPSCs) in 1975 and have been working with them ever since. These cells are located in the connective tissue matrices throughout the body as dormant quiescent cells. We have located (via ELICA), isolated, propagated, differentially cryopreserved, sorted, cloned from single cells, sized by flow cytometry, and applied various recombinant growth factors, morphogenetic proteins, and cell-specific exosomes to determine differentiation potential. There are five basic categories of aTPSCs, e.g., TSCs, PSCs, EctoSCs, MesoSCs, and EndoSCs.

TSCs are totipotent stem cells that are 0.1 to 2.0-microns in size. They can be seen as early

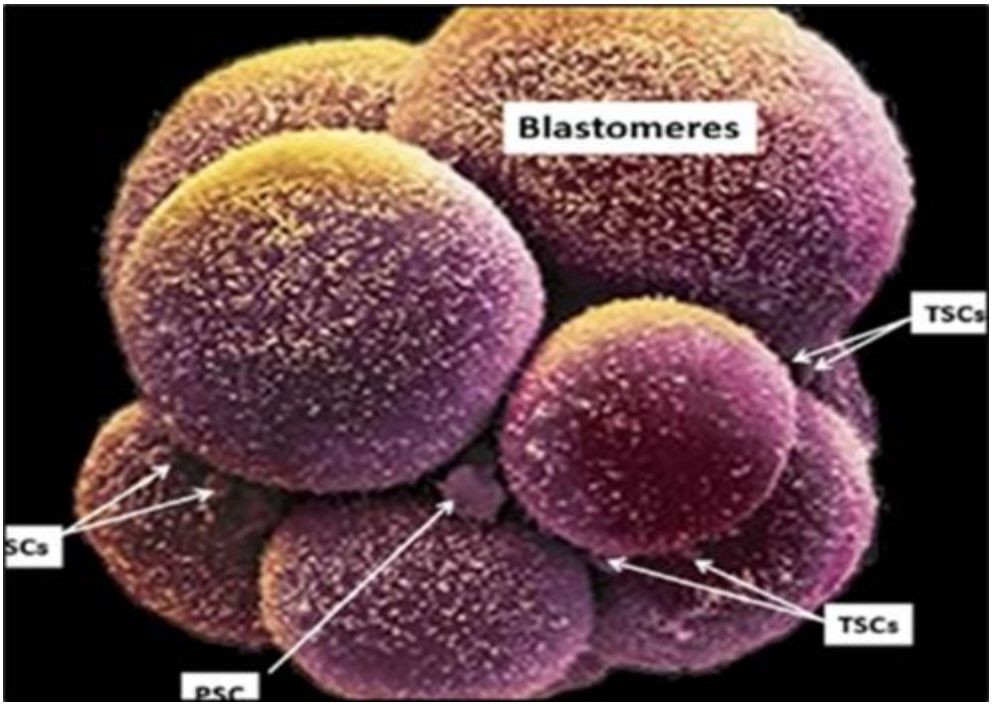

Figure 24 Scanning electron micrograph of a male embryo, somewhere between 8-cell stage and morula. Note presence of three pairs of totipotent stem cells (TSCs) and one pluripotent stem cell (PSC). Size approximation same as seen with flow cytometry (TSCs are 0.1 to 2 microns and PSC are 6 to 8 microns in size) [36]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014

as the eight-cell stage in a developing male embryo (via scanning electron microscopy) (Fig. 24), well before differentiation of the blastocyst into inner cell mass (embryo) and trophectoderm (placenta). They can be induced to form all somatic cells of the body, the nucleus pulposis of the intervertebral disc (IVD) (the only functional adult derivative of the notochord), gender-specific gametes (male TSCs only form spermatogonia and female TSCs only form oogonia), and the placenta (syncytiotrophoblast secreting chorionic gonadotropin) (Fig. 23). They retain the telomerase enzyme thus giving them essentially, an unlimited lifespan until they are induced to differentiate, at which point they assume the replicative ability of progenitor cells. In humans, the replicative ability is 70 population doublings (i.e., Hayflick's Limit) before their pre-programing is activated for senescence and cell death [37].

PSCs are pluripotent stem cells that are >2 to 8-microns in size. There are four populations of pluripotent stem cells, based on size and cell surface staining (and I apologize for the naming system), e.g., halo-like stem cells (>2.0 to 4-microns) and corona-like stem cells (>4 to <6 -microns) were named for the Trypan blue staining patterns (Fig. 22), pluripotent stem cell (6 to 8-microns), and germ layer lineage stem cells (>8 to <10-microns). Pluripotent stem cells can be seen in the inner cell mass of the developing embryo (via ELICA). They can be induced to form all somatic cells of the body, across all three germ layer lineages, but will NOT form nucleus pulposis of IVD, gametes, or placenta. They retain the telomerase enzyme until induced to differentiate. This gives them, essentially, an unlimited lifespan until they are induced to differentiate. With differentiation, they assume the replicative ability of progenitor cells. In humans, that replicative ability is 70 population doublings (i.e., Hayflick's Limit) before their pre-programing is activated for senescence and cell death [37].

EctoSCs are ectodermal stem cells and are 10 to 12 microns in size. They can be seen in the ectodermal germ layer lineages, e.g., surface ectoderm, neural ectoderm, and neural crest (via ELICA). They can be induced to form all somatic cells of the ectodermal germ layer lineage [Fig. 23]. We have not been able to force them into transdifferentiation into either of the other two germ layer lineages using inductive agents, e.g., recombinant proteins, morphogenetic proteins, or cell-specific exosomes. EctoSCs retain the telomerase enzyme thus giving them essentially, an unlimited lifespan until they are induced to differentiate, at which point they assume the replicative ability of progenitor cells. In humans, the replicative ability is 70 population doublings (i.e., Hayflick's Limit) before their pre-programing is activated for senescence and cell death [37].

MesoSCs are mesodermal stem cells, originally called pluripotent mesenchymal stem cells, and are 10-12 microns in size. They can be seen in the mesodermal germ layer lineages, e.g., somitic mesoderm, urogenital mesoderm, and lateral plate mesoderm (via ELICA). They can be induced to form all somatic cells of the mesodermal germ layer lineage (Fig. 23). They retain the telomerase enzyme thus giving them essentially, an unlimited lifespan until they are induced to differentiate, at which point they assume the replicative ability of progenitor cells. In humans, the replicative ability is 70 population doublings (i.e., Hayflick's Limit) before their pre-programing is activated for senescence and cell death [37]. For a (20-year) longevity experiment, MesoSCs were propagated through 2^690 populations doublings. MesoSCs double every 14-16 hours. Therefore, by titrating a proliferation agent to their doubling rate + 2 hours, no alterations in the genome, assessed by karyotypic analysis, were seen [38].

EndoSCs are endodermal stem cells. They can be seen in the endodermal germ layer lineage (via ELICA). They can be induced to form all somatic cells of the endodermal germ layer lineage. They retain the telomerase enzyme, thus giving them essentially, an unlimited lifespan.

Adult TPSCs exist in the body as "newborn" cells no matter the chronological age of the individual. Once aTPSCs are induced to form differentiated cell types, they lose their telomerase enzyme and recapitulate maturation from newborns to geriatric-aged individuals, assuming all the characteristics of progenitor cells, and pre-programmed for a definitive lifespan before senescence and/or cell death. aTPSCs do not turn back any clock. Rather, they start at the newborn state and move forward. But as they are recapitulating normal maturation from time of transplant forward, it may seem that they are "turning back the clock" for the entire organism.

Our original game plan for characterizing aTPSCs from multiple animals entailed localization (this study), isolation, plating, propagation, differential cryopreservation, identification of cell surface markers, cell sorting, generation of cell-specific exosomes, repetitive single cell clonogenic analysis, and testing with recombinant proteins, morphogenetic proteins, cell-specific exosomes, hormones, and chemicals to ascertain their differentiation potentials with respect to various biological agents. Unfortunately, or fortunately as the case may be, aTPSCs are uniquely different from progenitor cells and differentiative cells with respect to reagents and particular protocols used for isolation, plating, propagation, differential cryopreservation, identification of cell surface markers, cell sorting, response to cell-specific exosomes, and repetitive single cell clonogenic analysis. Following established reagents and protocols, we hit a road block at every single step in the game plan. Therefore, we had to develop technologies (reagents and methodologies) that matched the aTPSCs, rather than trying to make the aTPSCs conform to established reagents and procedures. As Dr. PM Johnston drummed into me when I was studying limb regeneration in adult terrestrial salamanders, "Know your model system, and tissue never lies. You just need to be smart enough to understand what it is trying to tell you". And another saying of his was "Just because something hasn't been reported, doesn't mean it does not exist, it only means that it hasn't been discovered yet".

Our next approach was to isolate, plate, propagate, release intact from culture vessels, cryopreservation, differential centrifugation, identify cell surface markers, cell sort using those same markers, generate cell-specific exosomes, isolate single cell-derived clones by repetitive single cell clonogenic analysis, insert a genomic label into the clones to be able to track them in vitro and in vivo, and begin characterization and experimentation with the adult telomerase positive stem cells. Unfortunately, besides differences in size compared to progenitor cells or differentiated cells, the aTPSCs also expressed multiple unique qualities that made it difficult to work with the cells. In other words, they did not conform to ANY established procedures for isolation, plating, propagation, release from culture vessels, cryopreservation, thawing, cell surface markers, or response to biological agents. To study the aTPSCs we needed to establish procedures for these cells, rather than relying on procedures established for progenitor cells or differentiated cells.

This is the first in a series of papers describing in extensive detail the reagents, manufacturers, and methodologies we identified that were necessary to analyze aTPSCs.

4. Conclusion

Adult telomerase positive stem cells, specifically TSCs and PSCs, are a conserved cell population that is located in multiple species of animals, thus proving the Hypothesis that "adult telomerase positive stem cells are a conserved population of cells located within the connective tissues of many organs in multiple animals".

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest was disclosed.

Statement of ethical approval

Animal Use The use of animals in this study complied with the guidelines of Mercer University's Institutional Animal Care and Use Committee (ACUC). 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

Statement of informed consent

Human Use The use of human biopsy specimens in this study complied with the guidelines of Mercer University's Institutional Review Board (IRB). These guidelines reflect the Federal Regulations for Protection of Human Research Subjects, HHS Office for Human Research Protections – 45 and 46 CFR: 46.102(I)

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