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(RESEARCH ARTICLE)

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Totipotent stem cells and pluripotent stem cells are located in the reproductive organs of an adult mammal

Henry E Young [1, 2, 3, 4, *]

[1] *Dragonfly Foundation for Research and Development, Macon, GA, 31210, USA.*
[2] *Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, GA, 31207, USA.*
[3] *Department of Obstetrics and Gynecology, Mercer University School of Medicine, Macon, GA, 31207, USA.*
[4] *Department of Pediatrics, Mercer University School of Medicine, Macon, GA, 31207, USA.*

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Abstract

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. A previous study identified CEA-CAM-1 and SSEA-4 positive cells in the reproductive organs of an adult rat. This study was undertaken to test the hypothesis that the previously identified cells were totipotent stem cells (CEA-CAM-1 positive) and pluripotent stem cells (SSEA-4 positive). 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 isolation and characterization of the cells. The isolated cells were segregated using Miltenyi magnetic antibody separation columns using CEA-CAM-1, SSEA-4, and Thy-1 antibodies coupled to iron particles. The isolated and segregated cells were characterized based on immunocytochemical staining, size, Trypan blue staining, morphology, cultivation attributes, an ELICA for expressed phenotypic expression markers, and incubated with biological agents to determine their potential for differentiation. This is the first report of the existence of native populations of totipotent stem cells and pluripotent stem cells in the reproductive organs of an adult mammal.

Keywords: Stem Cells; Totipotent; Pluripotent; Testis; Ovary; Fallopian Tube

1. Introduction

Precursor cells are the resident supportive cells within the body. They consist of maintenance progenitor cells and healing stem cells. Progenitor cells provide the cellular building blocks to maintain the differentiated 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 throughout the lifespan of the individual, from conception to death. [1,2].

Four categories of precursor cells have been identified in multiple postnatal (adult) animals, including humans [3]. These four categories of precursor cells are 1. Partially differentiated cell-committed telomerase negative progenitor cells, e.g., the original tripotent progenitor mesenchymal stem cells (MSCs) that form fat, cartilage, and bone [4-7], which have been found in bone marrow [5,6], adipose tissue [9], umbilical cord [10,11], amnion [12,13], placenta [14], and other sources [15]; neural stem cells (NSCs) forming neurons and glial cells and located in the subventricular zone lining the lateral ventricles, the sub granular zone of the dentate gyrus in the hippocampus, a portion of the basal ganglia deep within the cerebral hemispheres, and the neocortex of the cerebral hemispheres [16,17]; multipotent progenitor hematopoietic stem cells (HSCs) forming all cell types of the hematopoietic lineage [18,19]; bipotent progenitor adipo-fibroblasts [20]; unipotent progenitor myoblasts [23-25], etc. [24-26]. 2. Germ layer lineage-committed telomerase

* Corresponding author: Henry E Young

positive stem cells, e.g., ectodermal stem cells (EctoSCs), mesodermal stem cells (MesoSCs), and endodermal stem cells (EndoSCs) [3,27]. 3. Telomerase positive pluripotent stem cells (PSCs) [3,28]. And 4. Telomerase positive totipotent stem cells (TSCs) [3,29]. The segregation of these postnatal (adult) precursor cells into the proposed four major categories was based on their inherent size, Trypan blue exclusion properties, unique profiles of cell surface phenotypic expression markers, genetic markers, optimal cryopreservation temperatures, presence or absence of the telomerase enzyme, log phase doubling times, growth at confluence, and capabilities for self-renewal; their unidirectional developmental lineage patterns; and their objectively measured differentiation potentials based on cell and tissue specific extracellular, cell surface, and intracellular phenotypic expression markers (Table 1, Fig. 1) [3,4].

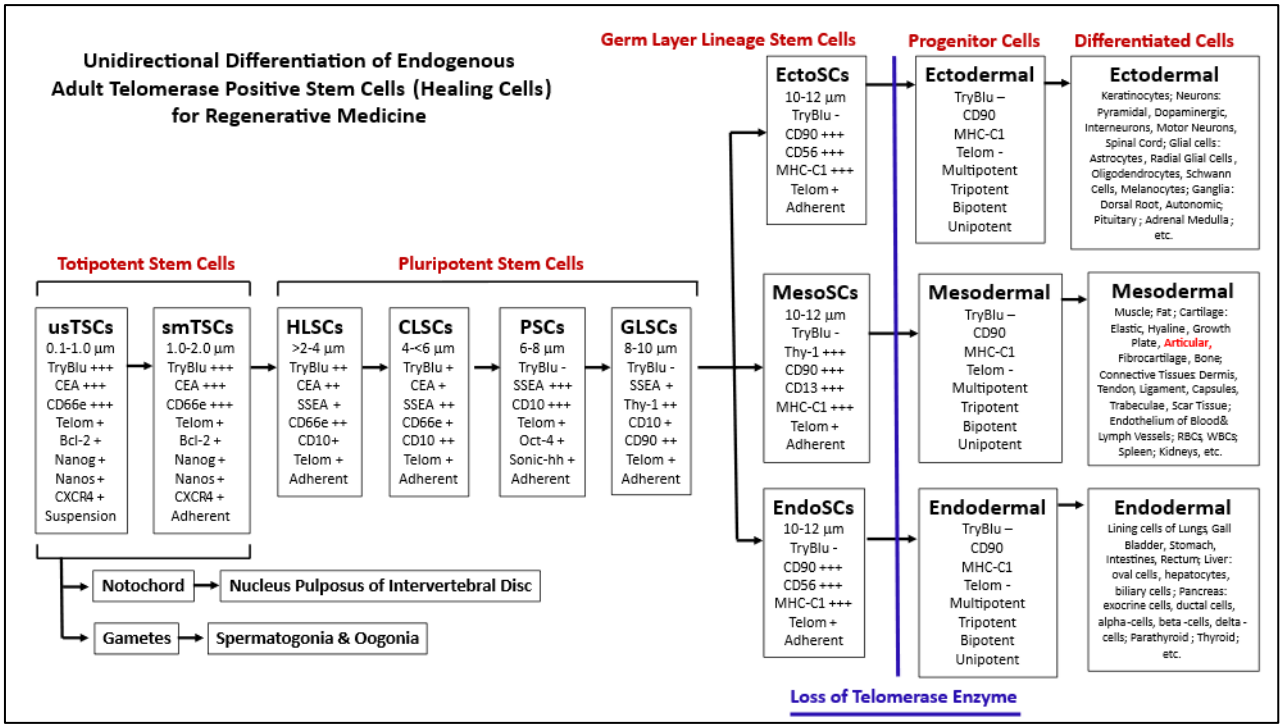
Table 1 Characteristics of Precursor Cells: Telomerase-Positive Stem Cells (TSCs, PSCs, MesoSCs) versus Telomerase-Negative Progenitor Cell (MSC)

Characteristic	**Ultra Small-Totipotent Stem Cells**	**Small-Totipotent Stem Cells**	**Pluripotent Stem cells**	**Mesodermal Stem Cells**	**Mesenchymal Progenitor Cell (MSC)**
Size, microns	0.1-1	1-2	6-8	10-12	15-30
Trypan Blue Staining	Positive	Positive	Negative	Negative	Negative
Telomerase	Present	Present	Present	Present	Absent
Numbers With Aging	Constant	Constant	Constant	Constant	Decrease
Viability Post Mortem	40+ days	30+ days	>7 days	3 days	1 day
Viability Temperature	4^{o}C	4^{o}C	4^{o}C	4^{o}C	4^{o}C
Isolated from Solid Tissues	Yes	Yes	Yes	Yes	Yes
Located in Connective Tissue Niches	Yes	Yes	Yes	Yes	Yes
Located in Bone Marrow	Yes	Yes	Yes	Yes	Yes
Located in Adipose Tissue	Yes	Yes	Yes	Yes	Yes
Located in Blood	Yes	Yes	Yes	Yes	No
Species Analyzed[1]	Av, KD, M, Rt, Rb, F, Cn, O, Cp, P, B, SpB, E, H	Av, KD, M, Rt, Rb, F, Cn, O, Cp, P, B, SpB, E, H	Av, KD, M, Rt, Rb, F, Cn, O, Cp, P, B, SpB, E, H	Av, KD, M, Rt, Rb, F, Cn, O, Cp, P, B, SpB, E, H	Rt, H
Clones Derived from Single Cells	Rat-Scl-44β,	Rat-Scl-4β, Rat-Scl-9β	Rat-Scl-40β	Rat-A_2A_2β	Rt-MSC
Contact Inhibited in Culture	No	No	No	Yes	Yes
Survive at 100% Contact Inhibition	NA	NA	NA	Yes	No
Growth in Culture	Suspension	Adherent	Adherent	Adherent	Adherent
Adherence to bare plastic	No	No	No	No	Yes
Make Their Own Substratum	NA	No	No	No	Yes
Provide Substrate	None	Type-1 Collagen	Type-1 Collagen	Type-1 Collagen	None
Default State in Serum Free Defined Medium	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence
Response to Absence of Biological Agents in SFDM	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence
Response to Inhibitors in SFDM	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence
Response to Inductive Agents in SFDM	Yes	Yes	Yes	ONLY Mesodermal Germ Layer Lineage Cells	ONLY Fat, Cartilage, Bone

Response to Inductive Agents in SFDM in Presence of Inhibitors	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence
Response to Proliferation Agents in SFDM	Proliferate	Proliferate	Proliferate	Proliferate	Proliferate
Response to Proliferation Agents in SFDM in Presence of Inhibitors	Proliferate	Proliferate	Proliferate	Proliferate	Proliferate
Response to Progression Agents in SFDM	None	None	None	None	Fat, Cartilage, Bone
Response to Progression Agents in SFDM in Presence of Inhibitors	None	None	None	None	Fat, Cartilage, Bone
Differentiate Into	Small-Totipotent Stem Cells	Pluripotent Stem Cells	Mesodermal Stem Cells	Mesodermal Progenitor Cells	Fat, Cartilage, Bone
# Cell Types Objectively Identified	75	74	71	37	3
# of Lineages Objectively Identified	EctoSCs, MesoSCs, EndoSCs, Gametes, Nucleus Pulposus of Intervertebral Disc	EctoSCs, MesoSCs, EndoSCs, Gametes, Nucleus Pulposus of Intervertebral Disc	EctoSCs, MesoSCs, EndoSCs	MesoSCs	MSCs
Proliferation Rate	12-14 hours	12-14 hours	14-16 hours	18-24 hours	Days to weeks
Population Doublings	>300	>300	>400	>400	50-70
Cryopreservative Agent	Ultra-Pure DMSO	Ultra-Pure DMSO	Ultra-Pure DMSO	Ultra-Pure DMSO	Ultra-Pure DMSO
Concentration of Cryopreservative Agent	7.5%	7.5%	7.5%	7.5%	10%
# of Cells per vial Frozen	1-10 x 10^9	1-10 x 10^9	1-10 x 10^6	1-10 x 10^6	1-10 x 10^6
Optimum Freezing Temperature	-80°C	-80°C	-80°C	-70°C	-196°C
Freezing Protocol	Slow	Slow	Slow	Slow	Flash
Optimum Storage Temperature	-80°C	-80°C	-80°C	-70°C	-196°C
Flash Thaw Temperature	37°C	37°C	37°C	37°C	37°C
Recovery of Viable Cells	>98-99%	>98-99%	>98-99%	>98%	>95%
Karyotype	Normal	Normal	Normal	Normal	Normal
Expressed Genes	Telomerase, Bcl-2, Nanog, Nanos, CXCR4	Telomerase, Bcl-2, Nanog, Nanos, CXCR4	Telomerase, Oct-3/4, Sonic Hedge Hog	Telomerase	NYD
Cell Surf Markers for Animal Cells[2]	CEA-CAM-1[3]	CEA-CAM-1	SSEA-4[4]	Thy-1[5]	Thy-1
Cell Surf Markers for Human Cells	CD66e[6]	CD66e	CD10[7]	CD90[8] CD13[9] MHC-I[10]	CD105[11] CD117[12] CD123[13] CD166[14] MHC Class-I

Species utilized for studies 1, Av (chicken, Waddle Crane), KD (Komodo Dragon), M (mouse), Rt (rat), Rb (rabbit), F (feline, cat), Cn (canine, dog), O (ovine, sheep), Cp (caprine, goat), P (porcine, pig), B (bovine, cow), SpB (Spectacled

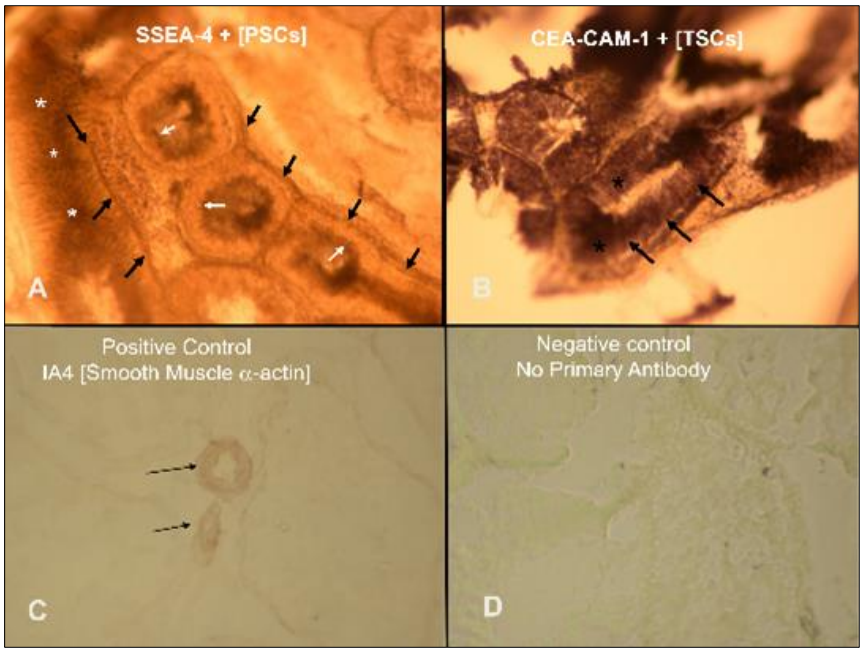
Bear), E (equine, horse), H (human); Cell Surf Markers[2], cell surface markers used for FACS (fluorescent-activated cell sorting) analysis included CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56, CD59, CD61, CD62e, CD65, CD66e, CD68, CD69, CD71, CD83, CD90, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, MHC-I, HLA-DR-II, FMC-7, Annexin-V, and Lin; CEA-CAM-1[3], carcinoembryonic antigen-cell adhesion molecule-1; SSEA[4], stage specific embryonic antigen-4; Thy-1[5], heavily N-glycosylated, glycophosphatidylinositol anchored conserved cell-surface protein with a single V-like immunoglobulin domain; CD66e[6], carcino-embryonic antigen, human variant "e"; CD10[7], cell surface enzyme with neutral metalloendopeptidase activity; CD90[8], heavily N-glycosylated, glycophosphatidylinositol anchored conserved cell-surface protein with a single V-like immunoglobulin domain; CD13[9], aminopeptidase; MHC-I[10], major histocompatibility marker-class-I, self-recognition molecule for non-hematopoietic cells; CD105[11], transmembrane glycoprotein, endoglin; CD117[12], receptor tyrosine kinase protein; CD123[13], interleukin-3 receptor alpha chain; CD166[14], a transmembrane glycoprotein that is a member of the immunoglobulin superfamily of proteins [4]. 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.



Demonstrates the unidirectional differentiation of primitive undifferentiated endogenous telomerase positive totipotent stem cells, through subsequent differentiation steps, i.e., telomerase positive pluripotent stem cells, telomerase positive ectodermal stem cells, telomerase positive mesodermal stem cells, and telomerase positive endodermal stem cells, then loss of the telomerase enzyme to become telomerase negative multipotent, tripotent, bipotent, and unipotent progenitor cells, which further differentiate into terminally differentiated adult cells. Subcategories were assessed by size, Trypan blue staining, cell surface markers, expressed genes, growth in culture, and differentiation potentials. TryBlu, 0.4% Trypan blue in sterile DPBS; CEA, antibody for carcinoembryonic antigen-cell adhesion molecule-1; CD66e, cell surface carcinoembryonic antigen; Telom, presence of telomerase enzyme; Bcl-2, Nanog, Nanos, CXCR4 are expressed genes; Suspension, growth in suspension cultures; Adherent, necessity for a substratum for cell growth; SSEA, stage-specific embryonic antigen-4 (SSEA-4); CD10, neutral endopeptidase cell surface marker; Oct-4, Oct-3/4 expressed gene; Sonic-hh, Sonic hedgehog expressed gene; CD90, equivalent to Thy-1, glycosylphosphatidylinositol (GPI)-anchored glycoprotein that is a member of the immunoglobulin superfamily; CD56, neural cell adhesion molecule; CD13, cell surface aminopeptidase; MHC Class-1, major histocompatibility complex class-1, self-recognition molecule for non-hematopoietic cells [4]. 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

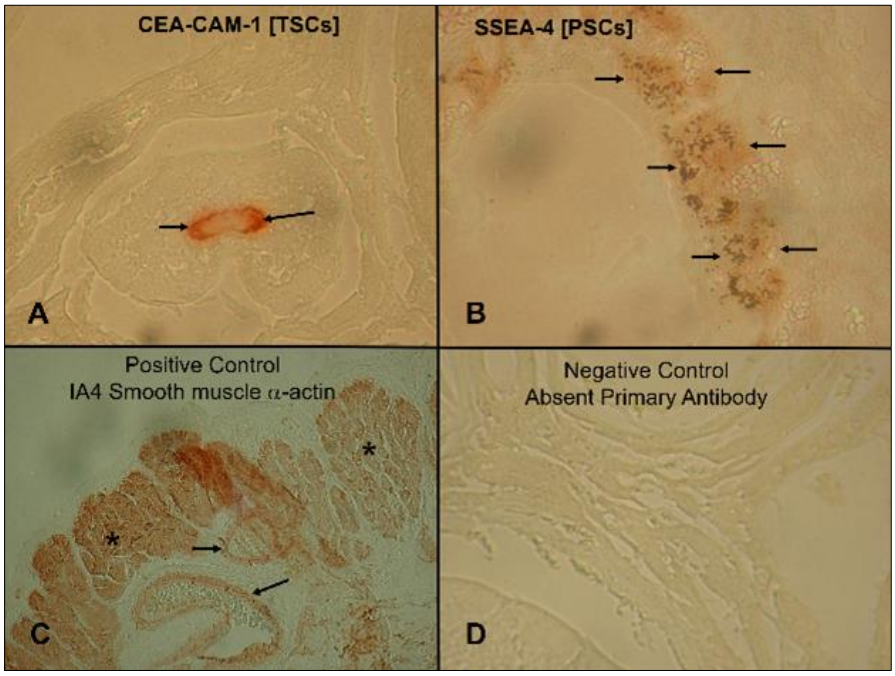
Figure 1 Unidirectional differentiation of precursor cells

Previous studies, using immunocytochemical staining of cryosectioned testis, ovary, and fallopian tube demonstrated CEA-CAM-1 and SSEA-4 positive cells in those tissues (Figs. 2-4).



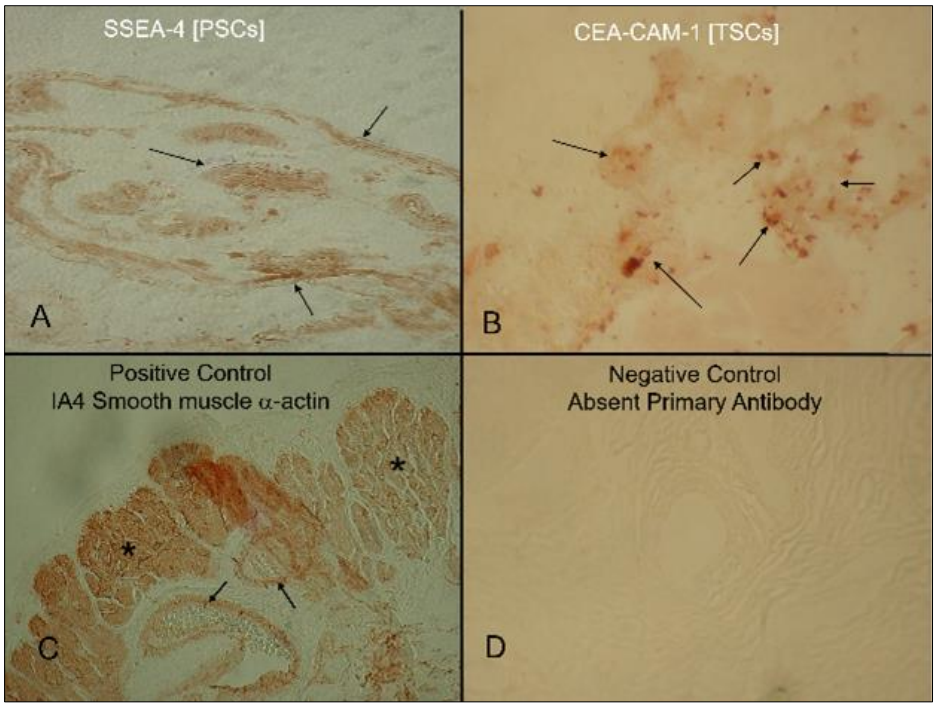
Testes were harvested, fixed in ELICA fixative, vibratome-sectioned at 25 microns, mounted on glass slides, and stained with appropriate antibodies. A. SSEA positive cells (PSCs) surrounding outside of seminiferous tubules (black arrows) as well as within the innermost layer of seminiferous tubules (white 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 [2]. Reprinted with permission from Young HE. Carcinoembryonic antigen-1 positive cells and stage-specific embryonic antigen-4 positive cells are located in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025A

Figure 2 Adult male rat



Ovary with attached fallopian tube was harvested from euthanized rats, 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 [2]. Reprinted with permission from Young HE. Carcinoembryonic antigen-1 positive cells and stage-specific embryonic antigen-4 positive cells are located in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025A

Figure 3 Adult female rat



Fallopian tube was 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 [4]. Reprinted with permission from Young HE. Carcinoembryonic antigen-1 positive cells and stage-specific embryonic antigen-4 positive cells are located in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025A

Figure 4 Adult female rat

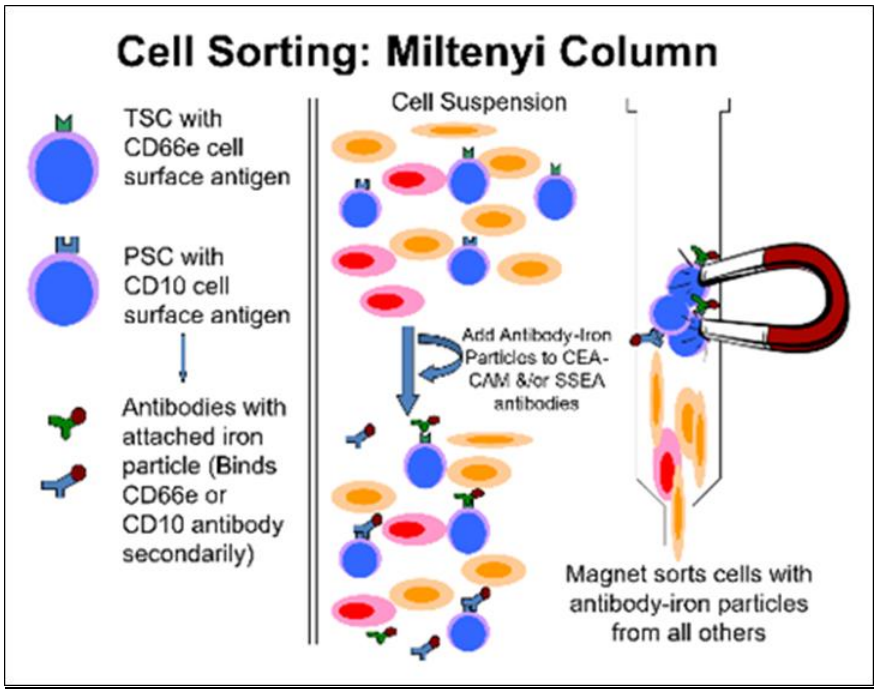
To test the hypothesis as to their specific identity, CEA-CAM-1 and SSEA-4 positive cells were isolated from the tissues, segregated with antibodies to CEA-CAM-1, SSEA-4, and Thy-1 using Miltenyi magnetic antibody separation columns, and characterized for size, Trypan blue staining, morphology, cultivation attributes, ELICA immunocytochemistry, phenotypic expression markers, potential for differentiation, and karyotypic analysis.

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 [30].
- **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 harvest and cell characterization. In females, the abdominal musculature was excised, the ovaries removed with attached fallopian tubes and placed into respective containers for harvest and cell characterization [2].

  To confirm the identity of the CEA-CAM-1 and SSEA-4 positive cells, the cells were released from the tissue by enzymatic digestion, segregated into separate populations using a Miltenyi magnetic separation column with antibodies to CEA-CAM-1 (D. Hixson), SSEA-4 (Developmental Studies Hybridoma Bank, Iowa City, IA), and Thy-1 (Sigma-Aldrige Chemical Co, St. Louis, MO) [Table 1]. Each resultant population was plated in serum-free defined medium and characterized for Trypan blue staining, size, morphology, cultivation attributes, immunocytochemistry, phenotypic expression markers, potential for differentiation, and karyotypic analysis [4, 27-29, 31, 32].

- **Enzymatic Digestion:** Adult rat testes, ovaries, and fallopian tubes were enzymatically digested individually to release the cells. The organs were minced to the consistency of orange marmalade and enzymatically digested with collagenase/dispase. This enzymatic solution consisted of 250 units per ml of type-I collagenase (CLS1, Worthington Biochemical Corp, Freehold, NJ), 33.3 units per ml of dispase (354235, R&D Systems, Minneapolis, MN), 1% antibiotic-antimycotic (153490-104, GIBCO), in calcium^{+2}-free, magnesium^{+2}-free Opti-MEM + GlutaMax, pH 7.4. The caps were tightened. Parafilm was used to seal the tubes. The 50-ml tubes were placed in an Orbital Incubator Shaker water bath (Labline) at a speed of 100 rpm for 18 hours at 37^{o}C. After 18 hours of digestion the tubes were removed from the incubator/shaker, the outside of the tubes was disinfected with 0.5% v/v Amphyl solution (VWR, Bristol, CT) in deionized water. The 50-ml tubes were centrifuged at 25 RCF (Relative Centrifugation Force) for 10 minutes. The tubes were checked for pelleted material. In some instances, undigested tissue remained in the tubes. The supernatants were removed and placed into fresh 50-ml conical tubes and centrifuged at 1800 RCF for 10 minutes. The supernatants were discarded. The pellets were re-suspended by agitation and trituration [33-35]. The disperse cell pellets were reconstituted with 2-ml of serum-free (basal) defined medium (SFDM). The basal medium consisted of Opti-MEM + GlutaMax with 2M linoleic acid (Sigma), 1M oleic acid (Sigma), 1M albumin (Sigma), 1 mg/ml fibronectin (GIBCO), 0.1 mg/ml folic acid (Sigma), 0.2 mg/ml fructose (Sigma), and 0.05 g/ml gelatin (Sigma), 1% antibiotic-antimycotic (Sigma), pH 7.4. Ten ng/ml PDGF-BB (R&D Systems) (Table 2) was added to the basal medium to form the serum-free defined propagation medium (SFDPM) [39]. Cell counts were performed as described below.
- **Cell Counts:** The final volume of each cell suspension was measured and recorded. Fifteen microliters of each cell suspension were removed and placed into separate 2.0 ml polypropylene tubes (Corning, Corning, NY). Fifteen microliters of sterile 0.4% Trypan Blue solution (filter-sterilized 0.4 g Trypan blue (Kodak Fine Chemicals, Rochester, NY) in sterile DPBS) was added to each tube. The contents were mixed by trituration 5-6 times and 15 microliters of the cell suspension/Trypan blue solution was placed on a hemocytometer for cell counting. All ultra-small Trypan blue-positive cells and small Trypan blue-negative cells within the nine large boxes of the hemocytometer were counted separately and then averaged for the number of cells of each type per each large box. Final cell number calculations were based on number of cells per gram of tissue. The isolated cells were counted on a hemocytometer by four separate individuals, the counts averaged and standardized per gram of tissue.



Miltenyi columns were used for purification of precursor cells, e.g., Thy-1 positive MSCs and MesoSCs, SSEA-4 positive PSCs, and CEA-CAM-1 positive TSCs. From a mixed population of precursor cells, the yields were routinely in the 95-98% purity range

Figure 5 Miltenyi magnetic antibody separation column

- **Miltenyi Magnetic Separation Columns:** Miltenyi magnetic separation columns (Fig. 5) allow for the isolation and purification of cells based on antibodies to their inherent cell surface markers. The animal cell surface markers used to isolate subcategories from a mixed population of precursor cells were CEA-CAM-1, SSEA-4, and Thy-1 (Table 1) [4].

  Antibodies to the cell surface markers were bound to iron particles, and then a high intensity magnet was applied to the column. The non-antibody-bound cells separated from the antibody-bound cells in the column as the flow-through eluant from the column (Fig. 5). The best method to perform Miltenyi magnetic column separation was to perform two negative antibody sorts, followed by a positive antibody sort. If pure CEA-CAM-1 positive cells were to be obtained, the first negative sort would use the antibody Thy-1. Thy-1 antibody bound to iron particles would remove Thy-1 positive cells, e.g., MesoSCs and MSCs (Table 1) [4] from the mixed population of precursor cells and bind them to the column with the magnet, leaving CEA-CAM-1 and SSEA-4 positive cells in the flow-through eluent. A second Miltenyi column would use the antibody to SSEA-4. The SSEA-4 antibody would bind SSEA-4 positive cells, e.g., PSCs (Table 1) [4] from the CEA-CAM-1/SSEA-4 flow-through eluant and bind them to the column with the magnet, leaving CEA-CAM-1 positive cells in the flow-through eluant. A third Miltenyi column was prepared with CEA-CAM-1 bound to the iron particles. The eluant from the second column was used to purify CEA-CAM-1 positive cells bound to the column with the magnet, leaving a flow-through eluant with no cells. This was confirmed by plating the eluant from the third sort onto 1% collagen coated flask with SFDM. No cells were present (data not shown).

  If pure SSEA-4 positive cells were to be obtained, the first negative sort would use Thy-1, and the second negative sort would use CEA-CAM-1. If pure Thy-1 positive cells were to be obtained, the first negative sort would use CEA-CAM-1 and the second negative sort would use SSEA-4.

- **Plating Cells to Increase Cell Numbers:** The bound cells from each Miltenyi column were released, collected, centrifuged to pellet the cells, reconstituted in SFDPM, cell counts determined, and plated into T-75 flasks to increase cell numbers. The Thy-1 positive cells bound to the first Miltenyi column stopped proliferating at confluence, and maintained viability as long as the cells were fed with fresh SFDM. The SSEA-4 positive cells bound to the second Miltenyi column formed multiple layers of confluent cells that maintained viability as long as the cells were fed with fresh SFDM. The CEA-CAM-1 positive cells bound to the third Miltenyi column formed multiple layers of confluent cells that maintained viability as long as the cells were fed with fresh SFDM. Conditioned medium was collected from each cell population during medium changes and processed as described below.
- **Freeze/Thaw Procedures:** Excess cells were cryopreserved and stored for a minimum of 24 hours following established testing protocols [40]. Ultra-pure Dimethylsulfoxide (DMSO, Morton-Thiokol, Chicago, IL) was tested at 1, 2.5, 5, 7.5, and 10% per 1-ml of respective cell suspensions with temperatures at -20°C, -70°C, -80°C, and -196°C. Frozen vials of cells were thawed at 37°C, cell suspension withdrawn, diluted into fresh SFDM, centrifuged to pellet cells, supernatant removed, pellets reconstituted in fresh SFDM, and cells counted [36].
- **Cell Sizing Based on Flow Cytometry:** Thy-1, SSEA-4, and CEA-CAM-1 positive cells grown to their respective maximum confluence in T-75 flasks were released from the flask surface with collagenase/dispase, triturated to form a single cell suspension, added to 1% type-1 collagen solution to negate the activity of the enzymes, centrifuged to pellet the cells, and reconstituted with DPBS. Each population was stained with their respective primary antibody (e.g., CEA-CAM-1, SSEA-4, or Thy-1) bound to a fluoresceine isothiocyanate (FITC) fluorescent probe and counterstained with propidium iodide (PI) (Table 3). Sizing beads (Becton-Dickinson) of approximate size to cells in Table 1 were used to calibrate the flow cytometer. The CEA-CAM-1-FITC/PI, SSEA-4-FITC/PI, and Thy-1-FITC/PI were run on a Accuri-6 flow cytometer (Becton-Dickenson Sciences, Waltham, MA) to determine size of the cells [37].
- **Differentiated Cell Conditioned Medium:** Adult differentiated cell types, e.g., spermatogonia, chondrocytes, dispersed pancreatic exocrine and endocrine cells, skeletal muscle, and epithelium lining the gastrointestinal system, were enzymatically digested to release the cells and grown separately in 1% collagen-coated T-75-flasks (Falcon) in serum-free defined medium (SFDM). When their culture medium changed color from salmon to orange-yellow, the conditioned medium was collected, and cultures refed with fresh SFDM. The conditioned medium was processed separately for each cell type. When the separate cultures almost reached confluence (~95%), the cultures were replated separately to collect more conditioned medium. The conditioned medium from each cell type was collected at every medium change, concentrated, frozen to -20°C, and thawed at 37°C. The freeze-thaw procedure was repeated an additional three times to ensure that no live cells remained in the conditioned medium. This was followed by low-speed centrifugation to removed flocculent pelleted material, and sterile-filtered in 0.1-micron bottle-top filters. The sterile concentrated conditioned media were stored at -80°C until used.

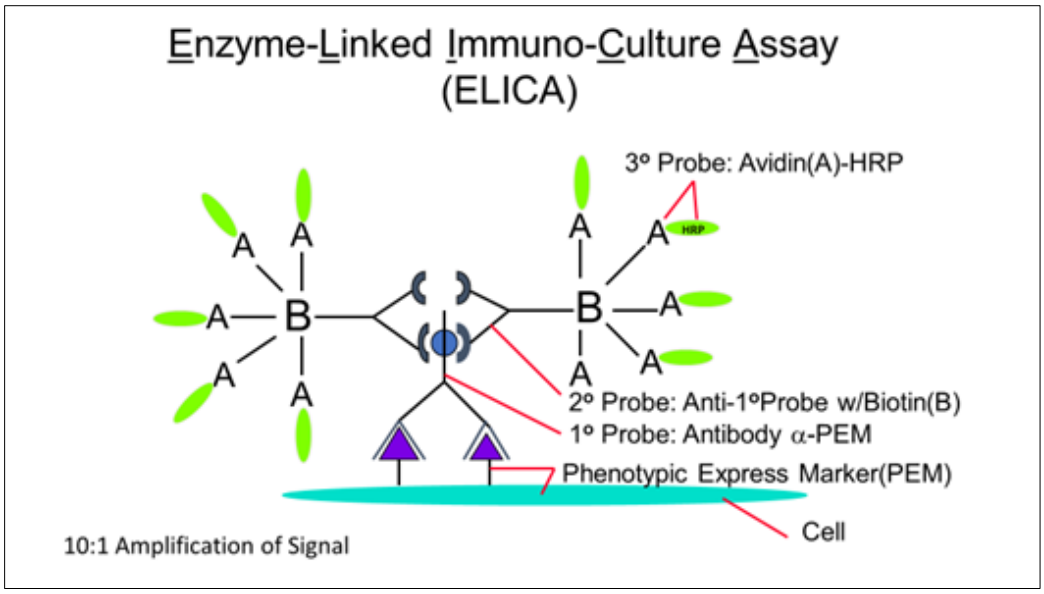
2.1. Biological Agents Incubated with Precursor Cells

Table 2 Biological Agents Incubated with Precursor Cells: TSCs, PSCs, MesoSCs, and MSCs

Name	Agent	Activity
Leukemia Inhibitory Factor[1]	LIF	Inhibits differentiation of precursor cells in presence of inductive agents
Anti-Differentiation Factor	ADF	Inhibits differentiation of precursor cells in presence of inductive agents
Platelet-Derived Growth Factor-AA[2]	PDGF-AA	Induces proliferation in precursor cells.
Platelet-Derived Growth Factor-AB[2]	PDGF-AB	Induces proliferation in precursor cells.
Platelet-Derived Growth Factor-BB[2]	PDGF-BB	Induces proliferation in precursor cells.
Insulin-Like Growth Factor-1[3]	IGF-1	Acts as a progression agent for progenitor cells to accelerate expression of phenotypic expression markers
Insulin-Like Growth Factor-2[4]	IGF-2	Acts as a progression agent for progenitor cells to accelerate expression of phenotypic expression markers
Insulin[5]	INS	Acts as a progression agent for progenitor cells to accelerate expression of phenotypic expression markers
Nerve Growth Factor[6]	NGF	Induces formation of neuronal lineage cells (neurons, oligodendrocytes, astrocytes, Schwann cells, ganglion cells) in TSCs and PSCs cells; no inductive effect on MesoSCs or MSCs.
Hepatocyte Growth Factor[7]	HGF	Induces formation of liver lineage cells (oval cells, canalicular cells, biliary cells) in TSCs and PSCs cells; no inductive effect on MesoSCs or MSCs.
Skeletal Muscle Conditioned Medium[8]	SkMCM	Induces formation of skeletal muscle cells in TSCs, PSCs, and MesoSCs; no inductive effect on MSCs.
Cartilage Conditioned Medium	CCM	Induces formation of cartilage in TSCs, PSCs, MesoSCs, and MSCs.
Pancreatic Exocrine Conditioned Medium	PanExCM	Induces formation of pancreatic stem cells in TSCs and PSCs cells; no inductive effect on MesoSCs or MSCs.
Pancreatic Islet Conditioned Medium	PanICM	Induces formation of pancreatic islet cells in TSCs and PSCs cells; no inductive effect on MesoSCs or MSCs.
Gastrointestinal Epithelial Conditioned Medium	GIECM	Induces formation of gastrointestinal epithelial cells in TSCs and PSCs cells; no inductive effect on MesoSCs or MSCs.
Spermatogonia Conditioned Medium	SpCM	Induces formation of PEM for spermatogonia in TSCs.

Table 2. Biological Agents Impacting Precursor Cells; Human recombinant proteins: 1LIF, Leukemia inhibitory factor (Sigma-Aldridge); 2PDGFs, platelet-derived growth factors-AA, -AB, -BB (R&D Systems, Minneapolis, MN); 3IGF-1 (Sigma); 4IGF-2 (Sigma); 5Insulin (Sigma); 6nerve growth factor (Sigma); 7hepatocyte growth factor (R&D Systems); CM8, Differentiated cell conditioned media

- **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. 6). 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 [4,31].



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 [4]. 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 6 Enzyme-Linked Immuno-Culture Assay (ELICA)

The ELICA procedure (Fig. 6) 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 cryosectioned 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 [4,28,32].

- **Phenotypic Expression Markers:** ~~One hundred and eleven~~ 112 immunocytochemical and histochemical staining procedures [Table 3] were used to objectively verify the identity and identifiable phenotypic expression markers for 75 separate cell types, utilizing the ELICA procedure [4,38,39].

Table 3 Antibodies, Immunocytochemistry, & Histochemistry for Phenotypic Expression Markers

Antibody	**Antigen**	**Embryological Origin**
CEA-CAM-1	Carcinoembryonic antigen-cell adhesion molecule-1	Totipotent
HCEA	Human Carcinoembryonic antigen	Totipotent
CEA	Carcinoembryonic antigen	Totipotent
CD66e	Carcinoembryonic antigen	Totipotent
DH-TuAg1	Spermatogonia	Totipotent
MC-480	SSEA-1	Pluripotent

MC-631	SSEA-3	Pluripotent
MC-813	SSEA-4	Pluripotent
CD10	Neutral endopeptidase	Pluripotent
AlkPhos	Alkaline Phosphatase	Pluripotent
CD90	Thy-1	Germ Layer Lineage
CD56	Neural cell adhesion molecule	Ectoderm
Pax-6	Neurogenic lineage	Ectoderm
FORSE-1	Neuronal precursor cells	Ectoderm
Vimentin	Cells of neurogenic lineage	Ectoderm
Nestin	Cells of neurogenic lineage	Ectoderm
R401	Nestin-neuronal lineage	Ectoderm
HNES	Nestin-neuronal lineage	Ectoderm
MAB353	Nestin-neuronal lineage	Ectoderm
RT-97	Neurofilaments = neurons	Ectoderm
NF68	Neurofilament-68 = neurons	Ectoderm
S-100	Neurofilaments-100 = neurons	Ectoderm
NF-145	Neurofilaments-145 = neurons	Ectoderm
N-200	Neurofilaments-200 = neurons	Ectoderm
8A2	Neurons	Ectoderm
NG2	Neurons	Ectoderm
TH	Tyrosine hydroxylase, precursor to neural transmitters	Ectoderm
SV2	Synaptic vesicles	Ectoderm
DOPA	Dopamine, transmitter of dopaminergic neurons	Ectoderm
T8660	Beta-tubulin-III	Ectoderm
Tuj1	Beta-tubulin	Ectoderm
GFAP	Glial-fibrillary acidic protein	Ectoderm
CNPase	Glial cells = oligodendrocytes & astrocytes	Ectoderm
Rip	Oligodendrocytes	Ectoderm
MOSP	Oligodendrocyte specific proteins	Ectoderm
MAB	Oligodendrocyte marker	Ectoderm
40E-C	Radial cells and radial glial cells	Ectoderm
VM-1	Keratinocytes	Ectoderm
CD13	Amino endopeptidase	Mesoderm
OP-137	MyoD	Mesoderm
F5D	Myogenin = skeletal muscle	Mesoderm
MF-20	Sarcomeric myosin = skeletal muscle	Mesoderm
ALD58	Myosin heavy chain	Mesoderm
A4.74	Myosin fast chain	Mesoderm

IA4	Smooth muscle alpha actin = smooth muscle	Mesoderm
Calp	Calponin	Mesoderm
MAB-3252	Cardiotin = cardiac myocytes	Mesoderm
MAB1548	Myosin heavy chain of cardiac muscle	Mesoderm
WV1D1	Bone sialoprotein II = bone	Mesoderm
MP111	Osteopontine = bone	Mesoderm
Von Kossa	Stain calcium in bone, EDTA was used as negative control prior to von Kossa stain to verify presence of calcium	Mesoderm
CIIC1	Type-II collagen	Mesoderm
II-4CII	Type-II collagen	Mesoderm
HC-II	Human type-II collagen	Mesoderm
Mallory Heidenhain One-Step	Distinguishes type-I collagen from type-II collagen	
Alcian Blue	Stains anions on carbohydrate groups	Mesoderm
AB 1.0	Alcian Blue, pH 1.0 stains sulphate groups on GAGs	Mesoderm
AB 2.5	Alcian Blue, pH 2.5 stains carboxyl groups on GAGs	Mesoderm
Alcec Blue	Stains anions on carbohydrate groups	Mesoderm
AcB 1.0	Alcec Blue, pH 1.0 stains sulphate groups on GAGs	Mesoderm
AcB 2.5	Alcec Blue, pH 2.5 stains carboxyl groups on GAGs	Mesoderm
Safranin-O	Stains anions on carbohydrate groups	Mesoderm
SO 1.0	Safranin-O, pH 1.0 stains sulphate groups on GAGs	Mesoderm
SO 2.5	Safranin-O, pH 2.5 stains carboxyl groups on GAGs	Mesoderm
Glycosidase enzymes	hyaluronidase, chondroitinase-AC, chondroitinase-ABC, keratanase, were included with above histochemistry to serve as negative controls to verify identity of stained GAGs	
D1-9	Type-IX collagen = cartilage	Mesoderm
9/30	Cartilage link protein	Mesoderm
12/21	Cartilage proteoglycan hyaluronate binding region	Mesoderm
12C5	Versican hyaluronate binding region	Mesoderm
H-DC34	Sialomucin-containing hematopoietic/endothelial cells	Mesoderm
CD31	PECAM, Peripheral endothelial cell adhesion molecule	Mesoderm
P1H12	Human endothelial cell surface marker	Mesoderm
P2B1	Peripheral endothelial adhesion molecule	Mesoderm
P8B1	VCAM, vascular cell adhesion molecule	Mesoderm
P2H3	CD62e, E-selectin (vasculature)	Mesoderm
H-endo	CD146, Endothelial cells	Mesoderm
H5A4	CD11b, granulocytes, monocytes, NK-cells	Mesoderm
H4C4	CD44, hyaluronate receptor	Mesoderm
Hermes-1	CD44, hyaluronate receptor	Mesoderm

H5A5	CD45, all hematopoietic cells except RBCs	Mesoderm
H5C6	CD63, macrophages, monocytes, platelets	Mesoderm
HFSP	Human fibroblast specific protein	Mesoderm
1B10	Fibroblast-specific protein	Mesoderm
Sudan Black-B	Stains fat (adipocytes)	Mesoderm
Oil Red-O	Stains fat (adipocytes)	Mesoderm
Hydrocarbon	Toluene was used as negative control to confirm identity of unilocular and/or multilocular adipocytes	
CD95	Used to distinguish apoptotic cells from multilocular fat cells	
H-AFP	Human alpha-fetoprotein = fetal liver	Endoderm
R-AFP	Rat alpha-fetoprotein = fetal liver	Endoderm
DESMO	Endodermal epithelial marker of liver	Endoderm
LAP	Canalicular cell surface protein of liver	Endoderm
151-Ig	Liver epithelial growth factor	Endoderm
HA4c19	Bile canalicular cells of liver	Endoderm
OC2	Progenitor cells, oval cell, & biliary cells of liver	Endoderm
OC3	Progenitor cells & biliary cells of liver	Endoderm
OC4	Progenitor cells & biliary cells of liver	Endoderm
OC5	Progenitor cells & biliary cells of liver	Endoderm
OC10	Progenitor cells & biliary cells of liver	Endoderm
H.4	Intracellular staining of liver hepatocytes	Endoderm
H.1	Liver hepatocytes cell surface marker	Endoderm
DPPIV	Progenitor, canalicular, and biliary cells of the liver	Endoderm
OV6	Biliary and oval cells of liver; biliary cells of liver	Endoderm
HESA	Human GI (Gastrointestinal) Epithelium	Endoderm
YM-PS087	Glucagon-secreting cells of endocrine pancreas	Endoderm
INS	Insulin-secreting cells of endocrine pancreas	Endoderm
YM-PS5088	Insulin-secreting cells of endocrine pancreas	Endoderm
SOMA	Somatostatin-secreting cells of the endocrine pancreas	Endoderm
11180	Somatostatin-secreting cells of the endocrine pancreas	Endoderm
CK-19	Ductal cells of the exocrine pancreas	Endoderm
Telom	Telomerase positive cells	aTPSCs
Thy-1	Equivalent to CD90 for animal cells expressing self-recognition molecules	Germ layer lineage stem cells and progenitor cells
PI	Propidium Iodide, measure of live cells with flow cytometry	Live Cells
DAPI	Fluorescent marker to visualize living and fixed DNA	Live & Dead Cells
Gal-19	Insect beta-galactosidase genomic marker	Cell tracking marker

Table 3. Immunocytochemistry and Histochemistry; Antibodies, histochemical procedures, and negative controls (left column) used to identify specific phenotypic expression markers, PEMs (center column) for particular cell types (right column) [38]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014.

- ***In Vitro* Characterization of Differentiation Potentials:** CEA-CAM-1 and SSEA-4 positive cells with an optimum freezing temperature of -80°C, Thy-1 positive cells with an optimum freezing temperature of -70°C, and Thy-1 positive cells with an optimum freezing temperature of -196°C were plated at 1.0 x 10^3 cells per well in stem cell serum-free basal medium (SFBM), pH 7.4, using multiple 96-well tissue culture plates coated with 1% type-1 collagen. The cells were incubated in a humidified incubator (HERA, Kendro, Thermo-Fisher Scientific) containing 95% air/5% carbon dioxide at 37° C for 24 hours, washed with DPBS, refed with SFDM containing 10-ng/ml PDGF-BB and allowed to propagate to 50% confluence. The cells had their propagation medium removed, washed with DPBS and processed for cell characterization in SFD with biological agents (Table 2) and assayed with 111 immunocytochemical and histochemical procedures (Table 3) [4,38,39]. The CEA-CAM-1, SSEA-4, and Thy-1 positive cells were assayed individually for unique cell surface markers, cultivation with SFDM, activity in SFDM with differentiation inhibitors, proliferation agents, log phase doubling time, progression agents, growth at confluence, replicative potential, human recombinant proteins and conditioned medium-induced differentiation potentials (Table 2). Positive and negative controls were included to assure validity of the immunocytochemical staining. Stained cells were visualized using a Nikon TMS inverted phase/bright field microscope with bright field microscopy at 400x and photographed as above.
- **Karyotypic Analysis:** Karyotypic analysis of isolated CEA-CAM-1 positive cells and SSEA-4 positive cells was performed as described [31]. Isolated cells from the SSEA-4 and CEA-CAM-1 Miltenyi sorts were plated onto 1% type-1 collagen coated T-25 flasks (Falcon, Thermo-Fisher Scientific) at a plating density of 0.5x10^6 cells/ml of serum-free propagation medium (SFPM). After 24-hours, the medium was removed and replaced with serum-free propagation medium (SFDM with 10-ng/ml PDGF-BB) containing 0.1-g/ml colcemid (10-ml/ml KaryoMAX™ Colcemid, GIBCO). Cells were incubated in this propagation medium for 4-hours. Approximately 50% of the cells displayed mitotic profiles, characterized by loss of bipolar (CEA-CAM-1 positive cells) or stellate (SSEA-4 positive cells) morphology, spherical shape, and appearance of metaphase plates within the area of the nucleus. After colcemid incubation, the CEA-CAM-1 positive cells and SSEA-4 positive cells were released from their respective flasks, centrifuged, and processed separately. Pelleted cells were resuspended in 200-microliters of SFPM, resuspended in 10-ml of 0.075M potassium chloride (KCl, Sigma) and incubated for 15-min in a 37°C water bath. [Note that the KCl solution was added very slowly for the first few milliliters of solution.] Three to five drops of freshly prepared fixative (3:1 methanol: glacial acetic acid) (methanol and glacial acetic acid, Sigma) were added to stop the reaction. Cells were centrifuged at 500 RCF for 5-min at ambient temperature. Following centrifugation the supernatant was discarded, the cells resuspended, and 10-ml of freshly prepared fixative was added to the cells. This fixation regimen was performed three times in succession to ensure proper fixation of the cells. Following the final centrifugation, the cells were resuspended in sufficient fixative to yield a slightly turbid mixture [31].

  Chromosomes spreads were generated by applying one to two drops of turbid cell suspension from a glass Pasteur pipette (Thermo-Fisher Scientific) to a clean cold (~4°C) wet glass slide. The pipette was maintained approximately 6 inches above the surface of the slide at a 30-degree angle to facilitate even distribution of the chromosomes [31].

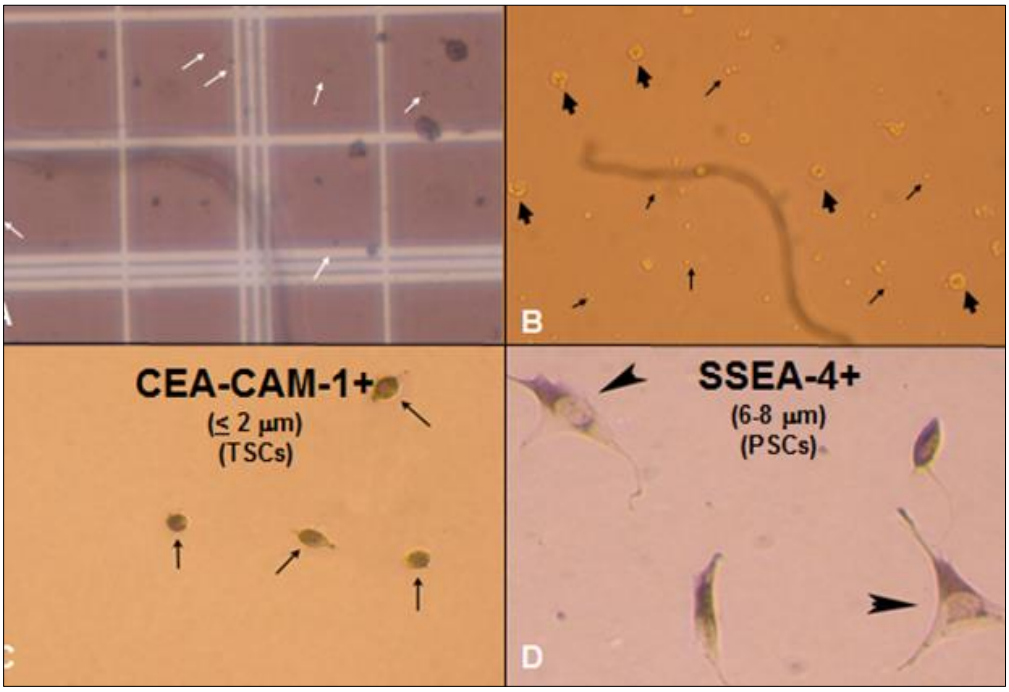
  Slides were stained utilizing "GTG" Banding (Giemsa Banding) protocol as per manufacturer's directions (GIBCO). In brief, slides were dried/aged at 60°C for a minimum of 18 hours. Slides were incubated in the trypsin solution for 30-60 seconds and then stained with the KaryoMAX™ Giemsa stain (GIBCO) in Gurr's buffer, Ph 6.8, for five minutes. After staining, slides were allowed to dry to ambient temperature and cover slipped with Cytoseal-60. Slides were allowed to dry under a fume hood for 24 hours prior to analysis [31].

- **Visual Analysis:** Chromosome spreads were visualized with a Nikon Fluor Phot compound microscope using brightfield microscopy at 1000x. Photographs were taken with a Nikon CoolPix 995 digital camera. Digital photographs were cropped and color adjusted to optimize chromosome banding using Adobe Photoshop 7.0.
- **Chromosomal Analysis:** Chromosomes were paired based on banding patters and positions of the centromere. The chromosomes were arranged based on the nomenclature rules for rat-chromosome G-bands, as described by Levan [44].

3. Results

Cell counting of CEA-CAM-1 positive sort from Miltenyi column demonstrated ultra-small and small Trypan blue positive cells and some positive cellular debris (Fig. 7A). Phase contrast microscopy of just plated cells from Thy-1 negative sort demonstrated small (arrows) and medium-sized cells (larger arrowheads) (Fig. 7B). Miltenyi sorted CEA-

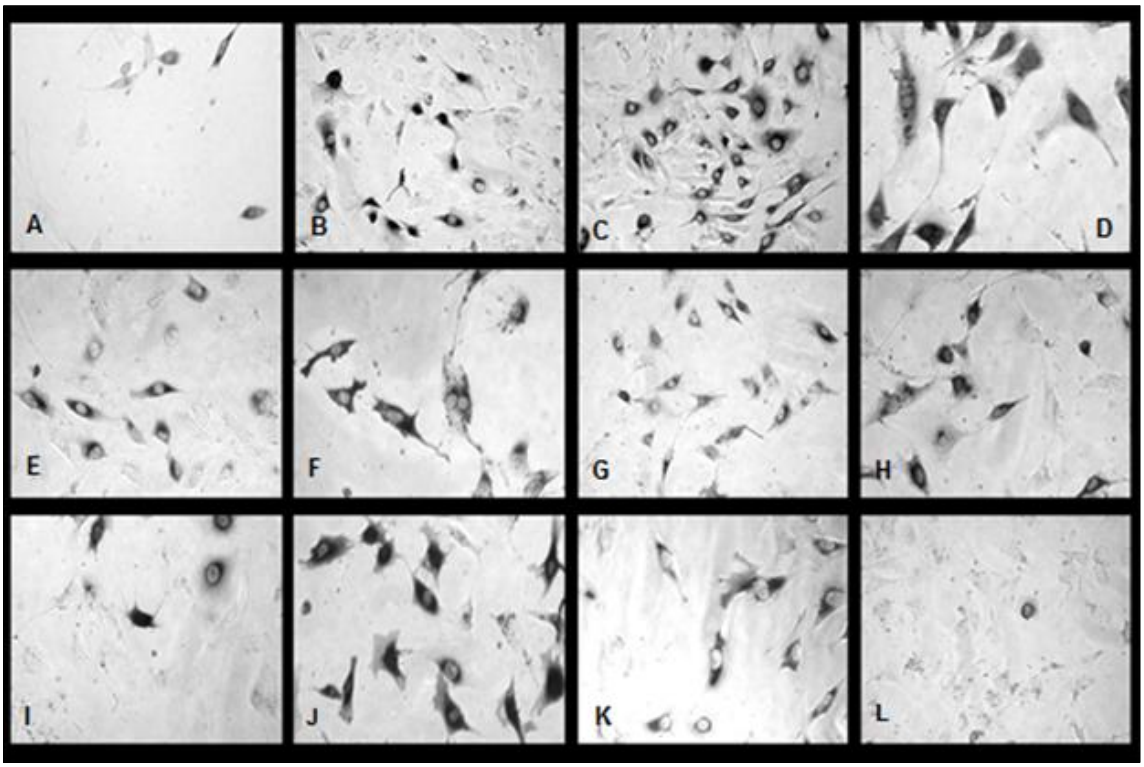
CAM-1 positive ultra-small and small cells are ≤ 2 microns in size based on flow cytometry (Fig. 7C). Miltenyi sorted SSEA-4 positive medium-sized cells are 6-8 microns in size based on flow cytometry (Fig. 7D).



Segregated precursor cells isolated from the adult rat reproductive organs (testis, RM 1021). A, Trypan blue positive TSCs (white arrows) as seen on a hemocytometer during cell counting, 400x; B, TSCs (small arrows) and PSCs (larger arrowheads) just plated in SFDM-TSC culture medium, 400x; C, Miltenyi Sorted CEA-CAM-1 positive cells, ≤ 2 microns in size, TSCs, 1600x; D, Miltenyi Sorted SSEA-4 positive cells, 6-8 microns in size, PSCs, 800x. Figures 7C & 7D [41] were Reprinted with permission from Young HE and Black Jr AC. Naturally occurring adult pluripotent stem cells. In: Stem Cells: From Biology to Therapy, Advances in Molecular Biology and Medicine. 1st Ed, R.A. Meyers, Ed, WILEY-BLACKWELL-VCH Verlag GmbH & Co. KGaA. Chap 3, pp. 63-93, 2013

Figure 7 Adult rat testis

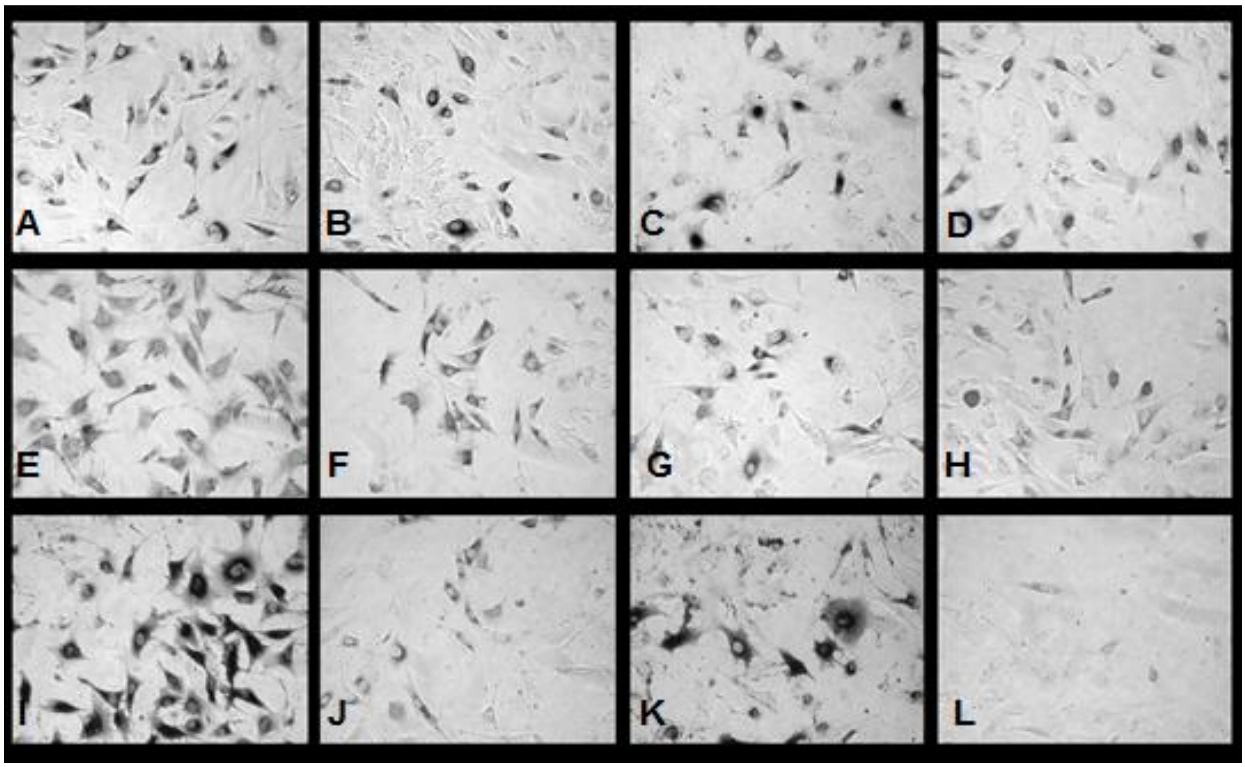
- **Phenotypic Expression Markers:** Human recombinant proteins, e.g., nerve growth factor (NGF and hepatocyte growth factor (HGF); conditioned medium from differentiated cells, e.g., skeletal muscle conditioned medium (SkMCM), cartilage conditioned medium (CCM), pancreatic exocrine conditioned medium (PanExCM), pancreatic islet conditioned medium (PanICM), gastrointestinal epithelial conditioned medium (GIECM), and spermatogonia conditioned medium (SpCM); pluripotent stem cell conditioned medium from stem cells (Table 2), were used to induce cell-specific phenotypic expression markers in CEA-CAM-1 and SSEA-4 positive cells (Figs. 8, 9).



Starting population consisted of Miltenyi sorted CEA-CAM-1 positive cells from adult rat testis (RM 1021). Representative examples of phenotypic expression markers identifying 75 separate cell types induced from CEA-CAM-1 positive cells, forming cells with phenotypic expression markers indicative of ectodermal, mesodermal, and endodermal germ layer lineage cells and spermatogonia. All pictures (A-L) were taken with bright-field microscopy to demonstrate stained cells, magnification 400x. A, SSEA-4, stage specific embryonic antigen (PSC-CM-induced PSCs in TSCs); B, RT-97, neuron (NGF-induced ectodermal lineage); C, DOPA, dopaminergic neurons (NGF-induced ectodermal lineage); D, MF-20, skeletal muscle (SkMCM-induced mesodermal lineage); E, CIIC1, type-II collagen (CCM-induced mesodermal lineage); F, RAFP, rat alpha fetoprotein (HGF-induced endodermal lineage); G, Pax6, pancreatic stem cell (PanExCM-induced endodermal lineage); H, INS, insulin, pancreatic beta-cells (PanICM-induced endodermal lineage); I, SOMA, somatostatin, pancreatic delta-cells (PanICM-induced endodermal lineage); J, H1, hepatocytes (HGF-induced endodermal lineage); K, OC5, liver cells (HGF-induced endodermal lineage); and L, DHTuAG1, spermatogonia, male germ cell (SpCM-induced spermatogonia) [42]. Reprinted with permission from Young HE, et al. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys, 40(1):1-80, 2004

Figure 8 Rat testis CEA-CAM-1 positive cells

Figure 8 demonstrates induction of multiple cell types across all three germ layer lineages (e.g., ectoderm, mesoderm, and endoderm) and the totipotent lineage (Fig. 1) in CEA-CAM-1 positive cells. Pluripotent stem cell-conditioned medium (PSC-CM) induced expression of SSEA-4 PEM for pluripotent stem cells (Fig. 8A). Nerve Growth Factor (NGF) induced expression of RT-97 PEM for neurons (Fig. 8B) and DOPA PEM for dopaminergic neurons (ectodermal lineage cells) (Fig.8C). Skeletal muscle conditioned medium (SkMCM) induced MF-20 PEM for skeletal muscle (mesodermal lineage cell) (Fig. 8D). Cartilage conditioned medium (CCM) induced CIIC1 PEM for cartilage (mesodermal lineage cell) (Fig. 8E). Hepatocyte Growth Factor (HGF) induced expression of RAFP PEM for rat alpha fetoprotein (endodermal lineage cell) (Fig. 8F). Pancreatic Exocrine Conditioned Medium (PanExCM) induced expression of Pax6 PEM for pancreatic stem cells (endodermal lineage cell) (Fig. 8G). Pancreatic Islet Conditioned Medium (PanICM) induced INS PEM for insulin secreting beta-cells (Fig. 8H) and SOMA PEM for somatostatin secreting delta cells (Fig. 8I) in pancreatic islets (endodermal lineage cell). HGF induced expression of HI PEM for hepatocytes (Fig. 8J) and OC5 PEM for liver cells (Fig. 8K) (endodermal lineage cells). And Spermatogonia Conditioned Medium (SpCM) induced expression of DHTuAG1 PEM for spermatogonia (totipotent germ layer lineage) (Fig. 8L).



Starting population consisted of Miltenyi sorted SSEA-4 positive cells from adult rat testis (RM 1021). Representative examples of the phenotypic expression markers of 71 potential cell types (Table 2) induced from SSEA-4 positive cells (B-K). Pictures A-K were taken with bright-field microscopy. Picture L was taken with phase contrast microscopy to demonstrate absence of any stained cells, 400x. A, Starting population of naïve stage specific embryonic antigen-4 (SSEA-4) positive cells; B, S-100, neuron (NGF-induced ectodermal lineage); C, RT-97, neuron (NGF-induced ectodermal lineage); D, Rip, glial cells (NGF-induced ectodermal lineage); E, ALD-58, Skeletal Muscle (SkMCM-induced mesodermal lineage); F, CIIC1, Type-II collagen (CCM-induced mesodermal lineage); G, D1-9, Type-IX collagen (CCM-induced mesodermal lineage); H, 151-Ig, Gastrointestinal epithelium (GIECM-induced endodermal lineage); I, SOMA, somatostatin (PanICM-induced endodermal lineage); J, OC2, liver cells (HGF-induced endodermal lineage); K, DPPIV, liver cells (HGF-induced endodermal lineage); L, DHTuAG1, SpCM-induced PSCs to potentially form spermatogonia, no staining, no spermatogonia present [42]. Reprinted with permission from Young HE, et al. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys, 40(1):1-80, 2004

Figure 9 Adult rat testis SSEA-4 positive cells

Figure 9 demonstrates induction of multiple cell types across all three germ layer lineages (e.g., ectoderm, mesoderm, and endoderm), but not in the germ cell totipotent lineage in SSEA-4 positive cells. NGF induced expression of S-100 and RT-97 PEMs for neurons and Rip for glial cells (ectodermal lineage cells) (Fig. 9B, 9C, 9D). SkMCM induced expression of ALD-58 PEM for skeletal muscle (mesodermal lineage cell) (Fig. 9E). CCM induced expression of CIIC1 (type-2 collagen) and D1-9 (type-9 collagen) PEMs for cartilage (mesodermal lineage cell) (Figs. 9F, 9G). GIECM induced expression of 151-Ig PEM for gastrointestinal epithelium (endodermal lineage cell) (Fig. 9H). PanICM induced expression of SOMA for somatostatin secreting delta cells in pancreatic islets (endodermal lineage cell) (Fig. 9I). And HGF induced expression of OC2 and DPPIV PEMs for liver cells (endodermal lineage cells) (Figs. 9J, 9K). Spermatogonia conditioned medium did NOT induce expression of DHTuAG1 PEM for spermatogonia (totipotent germ layer lineage) (Fig. 9L).

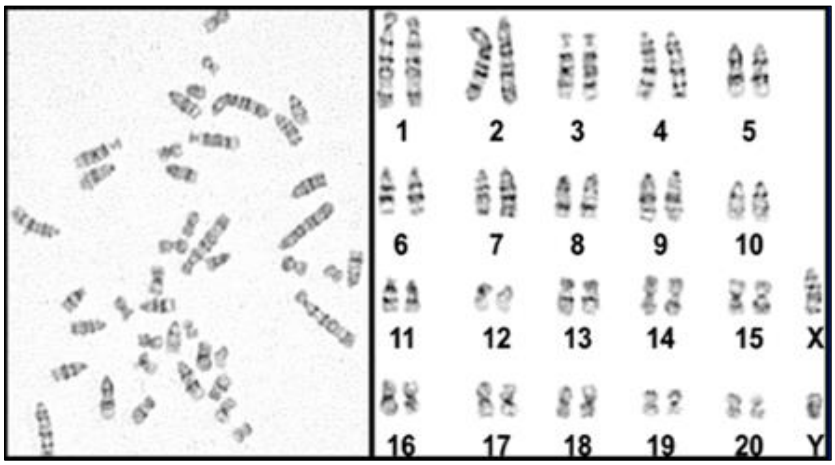
Table 4 Characteristics of Precursor Cells Isolated from Adult Rat Testes, Ovaries, and Fallopian Tubes

Characteristic	**Progenitor Cells (MSCs)**	**Mesodermal Lineage Stem Cells (MesoSCs)**	**Pluripotent Stem Cells (PSCs)**	**Small-Totipotent Stem Cells (sm-TSCs)**	**Ultra-Small Totipotent Stem Cells (us TSCs)**
Cell Surface Markers	Thy-1	Thy-1	SSEA-4	CEA-CAM-1	CEA-CAM-1
Size in microns	15-30	10-12	6-8	1-2	0.1-1
Trypan blue staining	Negative	Negative	Negative	Positive	Positive
Chromosome Number	42	42	42	42	42

Optimum Cryopreservative	Ultra-pure DMSO	Ultra-pure DMSO	Ultra-pure DMSO	Ultra-pure DMSO	Ultra-pure DMSO
Percent DMSO	10	7.5	7.5	7.5	7.5
Freezing & Storage Temperature	-196°C	-70°C	-80°C	-80°C	-80°C
Freezing Procedure	Flash	Slow 1° Per Minute	Slow 1° Per Minute	Slow 1° Per Minute	Slow 1° Per Minute
Thaw Procedure	37°C	37°C	37°C	37°C	37°C
Recovery Percentage	~95%	>95	98-99	98-99	98-99
Make Own Culture Substrates	Yes	No	No	No	No
Adherent Cultures	Yes	Yes	Yes	Yes	No
Suspension Cultures	No	No	No	No	Yes
Ca^{+2}-dependent binding sites[1]	Yes	Yes	Yes	Yes	No
RGD-dependent binding sites[2]	Yes	Yes	Yes	Yes	No
Cultivation in SFDM with GLSC Reagents	Yes	Yes	No	No	No
Cultivation in SFDM with PSC Reagents	Yes	Yes	Yes	No	No
Cultivation in SFDM with TSC Reagents	Yes	Yes	Yes	Yes	Yes
Activity in SFDM without Inhibitor[3]	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
Activity in SFDM with Inhibitor & Inductive Agents	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
SFDM with Progression Agents[4]	Yes	No	No	No	No
SFDM with Inductive Agent[5]	Yes: But Only Fat, Cartilage, and Bone	Yes: But Only Mesodermal Lineage Cells	Yes: All Somatic Cells	Yes: ALL Somatic Cells, Gametes, Nucleus Pulposis of IVD	Yes: ALL Somatic Cells, Gametes, Nucleus Pulposis of IVD
SFDM with Proliferative Agent[6]	Yes	Yes	Yes	Yes	Yes
Log Phase Doubling Times	Days to Weeks	18-24 Hours	14-16 Hours	12-14 Hours	12-14 Hours
Growth at Confluence	Contact Inhibited	Contact Inhibited	Multiple Layers	Multiple Layers	High Cell Density
Replicative Potential	6-8 Cell Doublings	>400 Cell Doublings	>400 Cell Doublings	>300 Cell Doublings	>300 Cell Doublings
Expressed Genes	MHC Class-1	Telomerase, MHC Class-1	Telomerase, Oct ¾, Sonic Hedge Hog	Telomerase, BCL-2, Nanog, Nanos, CXCR4	Telomerase, BCL-2, Nanog, Nanos, CXCR4
Differentiation Potential	Fat, Cartilage, Bone	Ectodermal, Mesodermal, Endodermal Lineages	All Somatic Cells	All Somatic Cells, Spermatogonia	All Somatic Cells, Spermatogonia

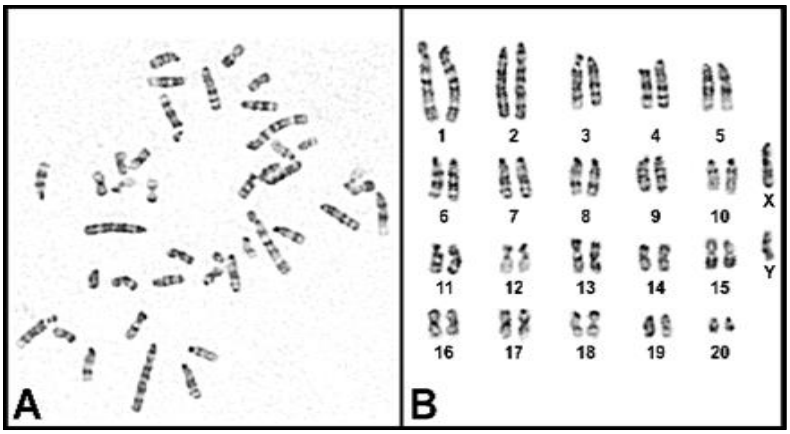
Number of Cell Types Formed[7]	3	EctoSCs: 15; MesoSCs: 37; EndoSCs: 14	71	75	75

Ca+2-dependent sites1, calcium-dependent binding sites; 2RGD-dependent, fibronectin-dependent binding sites; Replicative Potential3, absolute number of cell doublings before pre-programmed senescence and cell death [rodent progenitor cells = 6-8, GLSCs >690; PSCs > 400+; TSCs > 300+]; Activity in SFDM without Inhibitor3, Inhibitory Agents: Leukemia Inhibitory Factor (LIF) and Anti-Differentiation Factor (ADF); SFDM with Progression Agents4, Progression agents: Insulin, insulin-like growth factor-1 (IGF-1), and insulin-like growth factor-2 (IGF-2); SFDM with Inductive Agent5, Inductive Agents: Bone morphogenetic protein-2 (BMP-2), Cartilage conditioned medium (CCM), Adipocyte conditioned medium (AdipCM), Endothelial cell growth factor (ECGF), Fibroblast morphogenetic protein (FMP), Erythropoietin (EPO), Nerve growth factor (NGF), Hepatocyte growth factor (HGF), Lung conditioned medium (LngCM), Pancreatic exocrine conditioned medium (PanExCM), Pancreatic islet conditioned medium (PanICM), Gastrointestinal epithelial conditioned medium (GIECM), Keratinocyte conditioned medium (KerCM), Skeletal Muscle conditioned medium (SkMCM), Smooth muscle conditioned medium (SmMCM), Cardiac muscle conditioned medium (CdMCM), Tendon conditioned medium (TenCM), Ligament conditioned medium (LigCM); SFDM with Proliferative Agent6, Platelet-derived growth factor-AA, Platelet-derived growth factor-AB, Platelet-derived growth factor-BB; Number of cell types formed7, Number of 220+ differentiated cell types present in the body (limited numbers of verifiable differentiated cells due to limited numbers of objective assays present in laboratory). [Progenitor cells = multipotent (e.g., hematopoietic stem cells), tripotent (e.g., mesenchymal stem cells: fat, cartilage, and bone), bipotent (e.g., adipo-fibroblasts), unipotent (e.g., myoblasts, chondroblasts, osteoblasts)]; Germ layer lineage stem cells = EctoSCs (15), MesoSCs (37), EndoSCs (14); Pluripotent Stem Cells formed = 71; Totipotent Stem Cells formed = 75.



Representation karyotypic analysis of CEA-CAM-1 positive cells isolated from the adult rat testis (RM 1021). Note, chromosome spread on left, karyotype on right demonstrating the normal number of 42 chromosomes for the rat [31]. Reprinted with permission from Henson NL, et al. Karyotypic analysis of adult pluripotent stem cells. Histology and Histopathology, 20: 769-784, 2005

Figure 10 Adult rat testis CEA-CAM-1 karyotype



Representation karyotypic analysis of SSEA-4 positive cells isolated from the adult rat testis (RM 1021). A, chromosome spread and B, 20 pairs of somatic chromosomes, X chromosome and Y chromosome, for 42 chromosomes total, which is normal for a rat. Thy-1 positive cells from male, as well as female CEA-CAM-1 +, SSEA-4 +, and Thy-1 + cells all demonstrated an equivalent number of 42 chromosomes for each cell type examined (data not shown) [31]. Reprinted with permission from Henson NL, et al. Karyotypic analysis of adult pluripotent stem cells. Histology and Histopathology, 20: 769-784, 2005

Figure 11 Adult rat testis SSEA-4 karyotype

4. Discussion

Telomerase negative precursor (progenitor) cells have been derived from multiple tissues and organs throughout the body of mammals, including humans, e.g., bone marrow, adipose tissue, umbilical cords, amniotic fluid, placenta, etc. [5-26]. Telomerase positive precursor (stem) cells have been identified in 15 adult animals, including humans, from 36 organs and 50 tissues [2,3]. Based on immunocytochemical staining with selected antibodies to phenotypic expression markers coupled with isolation and characterization studies, five categories of telomerase positive precursor stem cells have been identified: totipotent stem cells (TSCs), pluripotent stem cells (PSCs), ectodermal stem cells (EctoSCs), mesodermal stem cells (MesoSCs), and endodermal stem cells (EndoSCs) [4].

This study was undertaken to test the hypothesis that CEA-CAM-1 positive cells and SSEA-4 positive cells identified previously in adult male and female rats [2], were totipotent stem cells and pluripotent stem cells, respectively, based on multiple characterization procedures.

To determine and verify the existence of totipotent stem cells and pluripotent stem cells in the tissues of adult rat reproductive organs, cells were released from their respective organs utilizing mincing of the tissues followed by enzymatic digestion with collagenase and dispase. The resultant cells underwent segregation by Miltenyi antibody magnetic columns using antibodies CEA-CAM-1, SSEA-4, and Thy-1 (Table 1), with subsequent characterization studies using biological agents, consisting of human recombinant proteins and inductive agents, present within conditioned medium from differentiated cells, to induce specific cells types derived from all three germ layer lineages and spermatogonia (Table 2). Antibodies to the phenotypic expression markers were used to identify of specific cell types (Table 3). And as shown in Table 4, CEA-CAM-1 and SSEA-4 positive cells demonstrate individual characteristics identical to TSCs and PSCs, respectively, in Table 1 as determined in previous studies [4]. For example, totipotent stem cells (TSCs) are ultra-small (< 2 micron) spherical entities that are Trypan blue positive throughout (Fig. 7A), stain for the CEA-CAM-1 epitope along their cell surface (Fig. 7C), and have a log phase doubling time of 12-14 hours. Pluripotent stem cells (PSCs) are small spherical entities (6-8 microns) that are Trypan blue negative and stain for the SSEA-4 epitope (Fig. 7D).

The Miltenyi magnetic separations with CEA-CAM-1, SSEA-4, and Thy-1, isolated two populations of Thy-1 positive cells from the reproductive organs. Both cell types formed a single confluent layer of cells when grown in SFDM. However, during the cryopreservation studies, it was noted that the two Thy-1 positive cell populations could be separated from each other based on their optimum cryopreservation and storage temperatures. One population preferentially froze at -70^{o}C with a >95% recovery, while the other population preferentially froze at -196^{o}C with an ~95% recovery. When both Thy-1 positive cell populations were frozen at their reverse temperatures, the -70^{o}C population frozen at -196^{o}C demonstrated a less than 5% recovery. When the -196^{o}C population was frozen at -70^{o}C, it fared a bit better, with a recovery of approximately 15%. The -70^{o}C Thy-1 positive cells characterized in this study matched the characteristics for mesodermal stem cells (MesoSCs) characterized in previous studies, compare Table 4 to Table 1 [4]. The -196^{o}C Thy-1 positive cells matched the characteristics for mesenchymal stem cells (MSCs), compare Table 4 to Table 1, [4]. For example, the -70^{o}C Thy-1 positive cells are comparatively large cells (10-12 microns), compared to CEA-CAM-1 positive cells ($\leq$ 2 microns) or SSEA-4 positive cells (6-8 microns), are Trypan blue negative, and have a log phase doubling time of 18-24 hours (Table 4). The -196oC Thy-1 positive cells are an even larger cell (15-30 microns), Trypan blue negative, and have a log phase doubling time of days to weeks (Table 4).

Populations of precursor cells from adult rat reproductive organs underwent G-banding karyotypic analysis to determine if there were any genomic differences with respect to the different precursor cell types or their respective genders. No discernible difference was noted for CEA-CAM-1 positive cells (Fig. 12) and SSEA-4 positive cells (Fig. 13) generated from the adult rat testis, nor from similar cells derived from adult rat ovaries or fallopian tubes.

No observable or significant differences were noted between genders, e.g., precursor cells derived from adult rat testes versus precursor cells derived from adult rat ovaries versus precursor cells derived from adult rat fallopian tubes, with respect to immunocytochemical staining, cell surface markers, size, Trypan blue staining, morphology, cultivation attributes, ELICA, phenotypic expression markers, potential for differentiation, and karyotypic analysis (Table 4).

Therefore, with respect to the precursor cells in the adult rat reproductive organs, the CEA-CAM-1 positive cells fit the profile of telomerase positive totipotent stem cells (TSCs); the SSEA-4 positive cells fit the profile of telomerase positive pluripotent stem cells (PSCS); the -70^{o}C Thy-1 positive cells fit the profile of telomerase positive mesodermal stem cells (MesoSCs); and -196^{o}C Thy-1 positive cells fit the profile of telomerase negative tripotent progenitor mesenchymal stem cell (MSC), compare Table 4 to Table 1 [4].

5. Conclusion

The original intent of this study was to test the hypothesis that the CEA-CAM-1 positive cells and SSEA-4 positive cells present in the connective tissues of adult mammal reproductive organs were, respectively, totipotent stem cells and pluripotent stem cells. This was based on their isolation from the tissues and subsequent characterization of their respective inherent characteristics, e.g., size, staining, isolation parameters, morphology, cultivation attributes, ELICA, phenotypic expression markers, differentiation potentials, and karyotypic analysis. In the process of this study, four populations of precursor cells were identified within adult rat reproductive organs. One population of precursor cells consisted of maintenance tripotent progenitor cells, e.g., Thy-1 positive mesenchymal stem cells. The remaining three populations consisted healing stem cells, e.g., CEA-CAM-1 positive totipotent stem cells, SSEA-4 positive pluripotent stem cells, and Thy-1 positive mesodermal stem cells.

Compliance with ethical standards

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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 [30].

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