MedCrave
Step into the World of Research

MOJ Orthopedics & Rheumatology

Research Article

Open Access

Adult telomerase positive stem cells: differential cryopreservation, cell surface marker profiles, and cell sorting

Abstract

The adult human body is composed of trillions and trillions of cells. These cells can be divided into three categories: functional differentiated cells, maintenance progenitor stem cells, and healing stem cells. Healing stem cells were discovered in 1975 residing within niches in the connective tissues of adult terrestrial salamanders undergoing complete limb regeneration. Since 1975, healing stem cells and maintenance progenitor stem cells have undergone extensive comparison and contrast analyses. The healing stem cells are uniquely different from maintenance progenitor stem cells. Healing stem cells are telomerase positive which gives them an essentially unlimited proliferation potential. Maintenance progenitor stem cells are telomerase negative, thus having a defined lifespan before pre-programmed senescence and cell death. In toto, healing stem cells will form all the somatic cells of the body, gametes, and the nucleus pulposis of the intervertebral disc. Maintenance progenitor cells will only form somatic cells within their respective cell lineages. The first report described a high throughput screening-assay, termed ELICA, to assess the differentiation potential of proliferative, progressive, inductive, and anti-differentiative biological agents on healing stem cells and maintenance progenitor cells. The second report introduced endogenous healing stem cells and described their location in the body. The third report described the methodologies developed to optimize the viability of mixed cultures of cells of differentiated cells, maintenance progenitor cells and healing stem cells during their isolation, segregation, plating, and propagation. This report describes in detail the separation of the mixed cultures of functional differentiated cells, maintenance progenitor stem cells, and healing stem cells based on differential centrifugation, cell surface markers, and cell sorting.

Keywords: differentiated cells, progenitor cells, stem cells, telomerase, differential centrifugation, cd markers, cell sorting

Volume 17 Issue 6 - 2025

Henry E Young[1–7]

[1]Dragonfly Foundation for Research & Development, USA
[2]Henry E Young PHD Regeneration Technologies, USA
[3]Division of Basic Medical Sciences, Mercer University School of Medicine, USA
[4]Department of Surgery, Mercer University School of Medicine, USA
[5]Department of Pediatrics, Mercer University School of Medicine, USA
[6]Department of Obstetrics and Gynecology, Mercer University School of Medicine, USA
[7]Department of Anesthesiology, Mercer University School of Medicine, USA

Correspondence: Henry E. Young, Dragonfly Foundation for Research and Development, 101 Preston Court, Suite 100, Macon, GA 31210 USA, 1+ (478) 319-1983, 1+ (478) 743-0280

Received: October 28, 2025 | **Published:** November 21, 2025

Introduction

The adult human body is composed of three categories of cells: telomerase negative functional differentiated cells (i.e., erythrocytes, osteocytes, chondrocytes, adipocytes, neurons, glial cells, fibrocytes, medicinal signaling cells, etc.), telomerase negative maintenance progenitor stem cells (i.e., hematopoietic stem cells [multipotent], mesenchymal stem cells [tripotent], neural stem cells [bipotent], and erythroblasts, osteoblasts, chondroblasts, adipoblasts, fibroblasts, neuroblasts, glioblasts [unipotent]), and telomerase positive healing stem cells (i.e., adult telomerase positive stem cells, aTPSCs).[1–8] The 220+ functional differentiated cells comprise approximately 40% of the cell types in the body. They are composed of functional parenchymal cells and connective tissue stroma.[8] The 230+ maintenance progenitor stem cells compose approximately 59% of the cells of the body and are the immediate precursor cells to the functional/ differentiated cells.[8] Healing stem cells comprise approximately 1% of the ~450+ cell types (~220+ differentiated/functional cells and ~230+ maintenance/ progenitor cells) of the body.[1,7]

There are 8 distinct subcategories of healing stem cells (aTPSCs) based on multiple parameters, including size, Trypan blue staining characteristics, unique cell surface markers, expressed genes, culture characteristics, response to biological agents, and differentiation potentials.

When differentiation of the aTPSCs occurs to form lineage-committed cell and tissue specific maintenance progenitor stem cells, the aTPSCs lose the telomerase enzyme and assume all the characteristics of the maintenance progenitor stem cells, including a biological clock defined by the number of population doublings before programmed senescence and death, e.g., 6 to 8 for rodents[11] and 70 for humans;[12] unique cell surface markers; expressed genes, culture characteristics, response to biological agents, and differentiation potentials (Figure 1).

The 8 categories are telomerase positive totipotent stem cells are composed of totipotent stem cells (TSCs) that are 0.1 to 2.0- microns in size and form all somatic cells of the body, gametes, cells of the placenta, and the nucleus pulposis of the intervertebral disc (the only functional adult structure formed by the notochord); telomerase positive halo-like stem cells (HLSCs) that are >2 to 4 -microns in size and form all somatic cells of the body; telomerase positive corona-like stem cells (CLSCs) hat are >4 to <6 microns in size and form all somatic cells of the body; telomerase positive pluripotent stem cells (PSCs) that are 6 to 8 microns in size and form all somatic cells of the body; telomerase positive germ layer lineage stem cells (GLSCs) that are 8 to 10-microns in size and form all somatic cells of the body; telomerase positive ectodermal stem cells (EctoSCs) that are 10 to 12-microns in size and form all somatic cells of the embryonic ectodermal germ layer lineage; telomerase positive mesodermal stem cells (MesoSCs) that are 10 to 12-microns in size and form all somatic cells of the embryonic mesodermal germ layer lineage; and telomerase positive endodermal stem cells (EndoSCs) that are 10 to 12-microns in size and form all somatic cells of the embryonic endodermal germ layer lineage (Figure 2).[7,9,10]

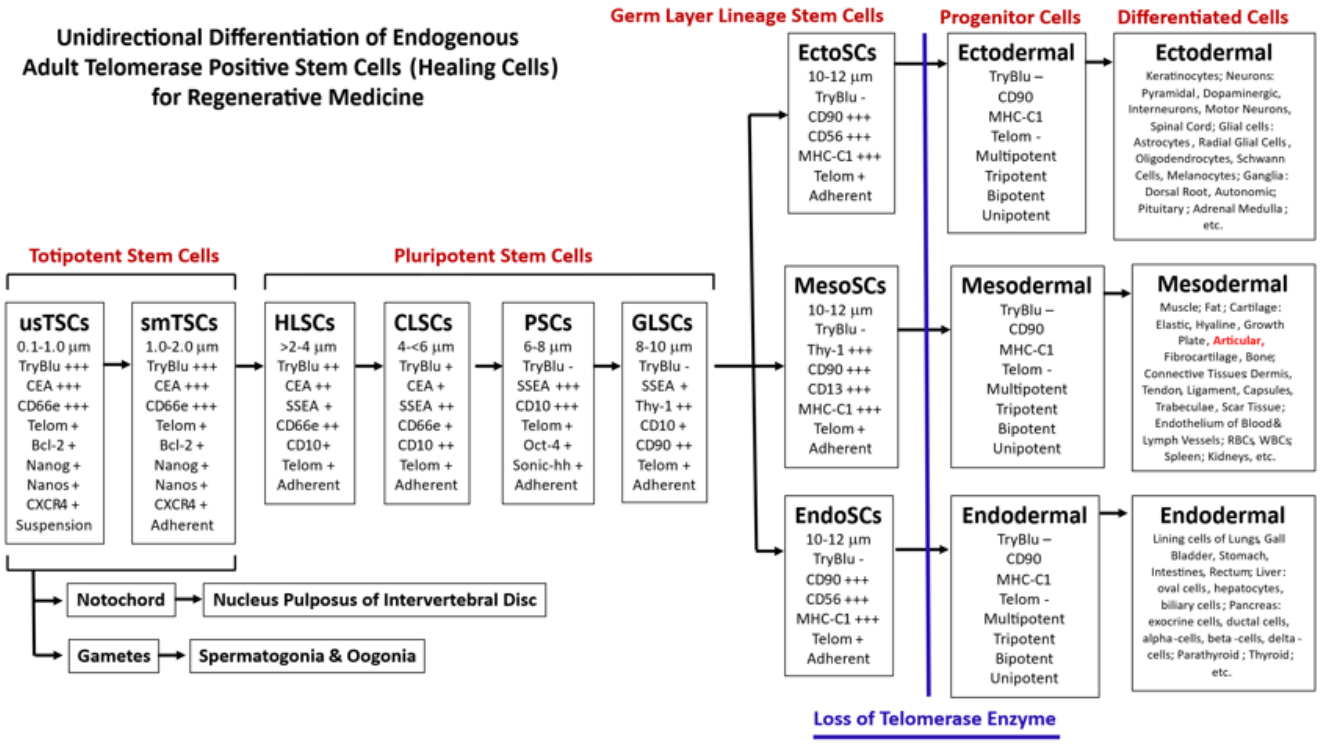

Figure 1 Identification of eight categories of adult telomerase positive stem cells and their unidirectional differentiation potentials into telomerase negative maintenance progenitor cells and telomerase negative functional differentiative cells using specific objective assays of Trypan blue staining, cell surface markers, expressed genes, inherent phenotypic expression markers, culture characteristics, response to biological agents, and differentiation potentials. 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.

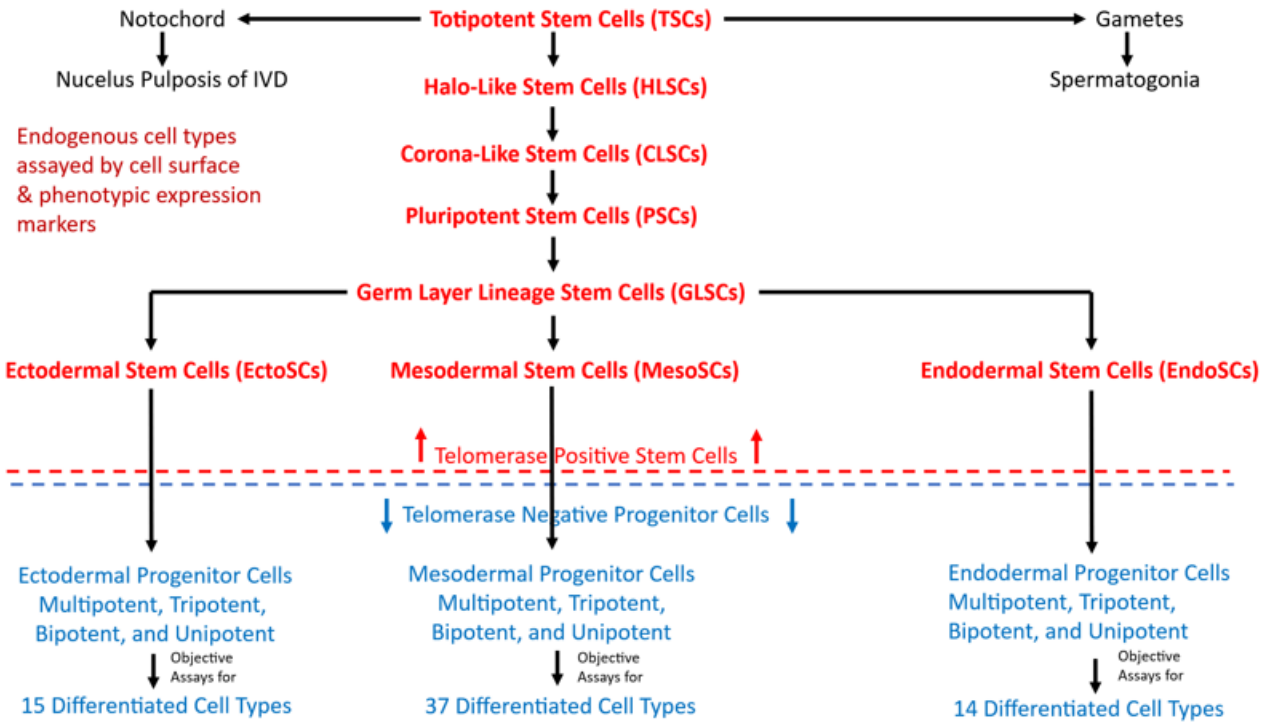

Figure 2 Identification of eight categories of aTPSCs and their unidirectional differentiation potentials into telomerase negative progenitor cells and telomerase negative differentiated cells using specific objective assays of cell surface markers and inherent phenotypic expression markers [13]. Reprinted 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.

Citation: Young HE. Adult telomerase positive stem cells: differential cryopreservation, cell surface marker profiles, and cell sorting. *MOJ Orthop Rheumatol.* 2025;17(6):155–173. DOI: 10.15406/mojor.2025.17.00726

This report deals with detailed explanations of the rationale, materials, methods, results, and discussion with nuances for the separation of the adult telomerase positive stem cells from adult maintenance progenitor stem cells and adult functional differentiated cells which compose cell/tissue isolates from various regions of the body. These techniques were designed to be universal (with particular nuances to species) and have been used with aTPSCs derived from Komodo Dragon,[13] avians,[13–15] mice,[16] rats,[17,18] cats,[19], dogs,[13,19] sheep,[19] goats,[19] pigs,[19,20] cows,[19] spectacled bear,[13] horses,[19] and humans,[13,21–24] for *in vitro* characterization studies, pre-clinical animal models of disease, and human clinical studies and trials of individuals with traumatic, chronic, and/or terminal diseases. This is the fourth in a series of technique papers, e.g., 1. enzyme-linked immunoculture assay (ELICA); 2. introduction and location within the body; 3. isolation from solid tissues, plating, and propagation; 4. differential cryopreservation, cell surface markers, and cell sorting (this article); 5. repetitive single cell clonogenic analysis; 6. generation of cell-specific inductive exosomes; and 7. Lac-Z gene genomic labeling of clonal populations of aTPSCs utilizing lipofectin; outlining in detail the technologies (step by step protocols) developed to either prove or disprove the existence and capabilities of endogenous adult telomerase positive healing stem cells.

Rationale

Differential cryopreservation

Following established protocols for cryopreservation,[25,26] commercially available 70% Dimethyl Sulfoxide (DMSO)[25] was added to a cell suspension of mixed cultures of functional differentiated cells, maintenance progenitor cells, and healing stem cells,[7] aliquoted into polypropylene cryopreservation vials, flash frozen, and stored in liquid nitrogen (-196°C) for up to 48 hours.[7,27] At selected intervals thereafter, aliquots of the mixed cultures were thawed and assayed using the ELICA procedure[27] with known inductive agents to ascertain differentiation potentials. Fifty percent of the functional differentiated cells and maintenance progenitor stem cells survived this process. However, less than 5% of the aTPSCs survived initial flash freezing and no aTPSCs survived longer than 48 hours at the liquid nitrogen storage temperature (-196°C),[27] The loss of the aTPSCs could have been due to either the composition and/or percentage of the cryoprotectant or the freeze thaw conditions. Therefore, a more extensive analysis of freeze / thaw conditions for functional differentiated cells, maintenance progenitor stem cells, and healing stem cells was undertaken.

Cell surface marker profiles

Every cell in the body, be it a functional differentiated cell, a maintenance progenitor stem cell, or a healing stem cell contains to unique profile of cell surface markers that identifies the type of cell, as well as the cell containing HLA markers to distinguish self from non-self.[28] With respect to human-derived healing stem cells[7,24,29] and a maintenance progenitor cell (e.g., the tripotent mesenchymal stem cell that would form fat, cartilage, and bone),[3–5] a battery of human cluster of differentiation (CD) markers was coupled with flow cytometry to identify cell surface markers of individual cells.[21,22,30] Then, utilizing antibody descriptions from the Developmental Studies Hybridoma Bank, Dr. Douglas Hixson, and commercially available antibodies, animal cell equivalent antibodies to the human CD markers were matched, located and obtained.

Cell sorting

There are multiple methods that can be utilized to sort cells into their respective categories of telomerase negative functional differentiated cells, telomerase negative maintenance progenitor stem cells, and healing adult telomerase positive stem cells, e.g., fluorescent activated cell sorting (FACS) utilizing fluorochromes attached to human CD markers or animal antibodies; Miltenyi magnetic column purification utilizing iron particle attached to animal antibodies or human CD markers; panning with antibodies or human CD markers attached to a substrate; Ficoll density gradient centrifugation; size, zeta potential, and density gradient centrifugation with plasma and saline; and water-induced lysis. We used FACS;[21,29,30] Miltenyi columns;[31] Ficoll density gradient centrifugation;[32] time, temperature, gravity, size, zeta potential;[24] density gradient centrifugation with plasma and saline;[24,33] and water-induced lysis[34] (to separate aTPSCs from exosomes); in our subsequent studies to separate cells for *in vitro* characterization studies,[29] IACUC-approval[35] for pre-clinical studies of animal models of disease, and IRB-approval[36] for human clinical studies and trials.

Materials and methods

Materials

A. Reagents:[10,14,16–22,32,37–41]

1. Species-specific buffers:

a. Amphibians and Reptiles – 10% Holtfreter's Solution

b. Avians – Tyrode's balanced salt solution, #T-2145 (Sigma)

c. Avian Ca+2, Mg+2-free – Tyrode's balanced salt solution, #T-2145 (Sigma)

d. Non-human mammals – Phosphate Buffered Saline, PBS (Sigma)

e. Non-human mammals – Ca+2, Mg+2-free Phosphate Buffered Saline, PBS (Sigma)

f. Humans – Dulbecco's Phosphate-Buffered Saline (10X), DPBS #310-4080AJ (GIBCO)

g. Humans – Dulbecco's Phosphate-Buffered Saline (10X) DPBS #310-4080AJ (GIBCO)

2. Medium

a. Eagle's Minimal Essential Medium with Earle's salts, #410-1100EB, GIBCO

b. OptiMEM + GlutaMax, GIBCO

c. Heat Inactivated Serum, Atlas Biologicals

d. HS7 Horse Serum, #H7889, Sigma

e. Antibiotic, Penicillin/Streptomycin, #600-514AG, GIBCO

3. Primary Probes

a. CEA-CAM-1, DH (D. Hixson, Providence, RI)

b. MC-813 (SSEA-4), DSHB

c. Thy-1 (CD90), DSHB

d. CEA, #111518, Sigma

e. CD66e, BD (Beckton-Dickinson)

f. MC-631 (SSEA-3), DSHB

g. CD10, BD

h. CD90, BD

i. Thy-1, DSHB

4. Secondary Probes

a. Goat anti-rat osteocalcin, #BT-413, Biomedical Technology

b. Biotin, goat anti-mouse IgG, #OB1126-21, Fisher

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

d. Biotin, goat anti-rabbit IgG, #OB1316-21, Sigma

e. Biotin-SP, goat anti-mouse IgG, #JGM-065003, Accurate

f. Biotin-SP, goat anti-mouse fab-specific, #JGM-066003, Accurate

g. Biotinylated affinity purified, rat adsorbed anti-mouse IgG (H + L) (BA-2001, Vector Laboratories)

5. Tertiary Probe

a. Iron particle – Avidin complex, Miltenyi

6. Miscellaneous Supplies

a. Propidium Iodide, Fisher-Scientific

b. 70% DMSO, ATCC

c. 100% Pure Dimethylsulfoxide (DMSO), Alfa Catalog Chemicals, Morton Thiokol

d. 99-100% Pure DMSO, Allied Chemical

e. 99-100% Pure DMSO, Sigma

f. Glycerin, Tissue Culture Grade, #830-951, Curtain Matheson Scientific

g. Sterile Tyrode's buffer powdered 1L, Sigma

h. Sodium Bicarbonate, Sigma

i. Sodium Hydroxide, Sigma

j. Penicillin/Streptomycin, GIBCO

k. Hydrochloric Acid concentrated 12N, Sigma

l. Phosphate Buffered Saline, 500-ml, Sigma

m. Dulbecco's Phosphate Buffered Saline, GIBCO

n. Eagle's MEM with Earle's Salts, powdered 1L, Sigma

o. Pre-Selected Horse Serum (HS7), Sigma

p. OptiMEM + GlutaMax, 500-ml, GIBCO

q. UltraCulture, #12-725B, #OMO455, Whittaker Bioproducts

r. Perfix, #CS514-1, Fisher

s. ELICA Fixative [4]:

i. Paraformaldehyde, powdered, Sigma

ii. Glutaraldehyde, Sigma

iii. Sodium Azide, Sigma

iv. D-Glucose, Sigma

v. HPLC Water, Sigma

t. Calf Thymus DNA, #D-1501, Sigma

u. Phenol, #2859-1, Baker

v. Saboraud Dextrose Browth, #0382-17-9, Curtain Matheson Scientific

w. Brain, Heart infusion medium, #0037-01-06, Curtain Matheson Scientific

x. Fluid thioglycolate medium, #0256-01-0, Curtain Matheson Scientific

y. Diaminobenzoic acid, #11,383-2, Sigma-Aldrich

z. Heat-Inactivated Serum, Atlas Biologicals

aa. Parafilm, #13-374-12, Diagger

ab. Ethanol, Sigma

ac. HPLC Water, Sigma

ad. Isopropyl alcohol, Sigma

ae. Nitrile Gloves, Diagger

af. 0.1-micron sterile bottle-top filter, Thermo-Fisher

ag. 0.2-micron sterile bottle-top filter, Thermo-Fisher

ah. 50-ml polypropylene centrifuge tubes, Falcon, Thermo-Fisher

ai. 15-ml polypropylene centrifuge tubes, Falcon, Thermo-Fisher

aj. 1.2-ml polypropylene cryopreservation tubes, Corning

ak. Trypan Blue, C.I. 23850, Kodak

al. Tissue Culture Bottles, Corning

am. Pipets: 1-ml, 5-ml, 10-ml, 25-ml, Diagger

an. Hemocytometer, Thermo-Fisher

ao. 96-well plates, Costar

ap. 6-well, 24-well, and 48-well plates, Corning

aq. T-25 and T-75 flasks, Falcon, Thermo-Fisher

ar. Gelatin, # GX0045-3, EM Sciences

as. 20-micron & 90-micron Nitex, Tetco

at. Fertilized White Leghorn Chick Eggs, Cagle's

au. Stir bars, Diagger

av. Aluminum foil, Diagger

aw. Sterilization bags, #1310-L75, Thomas Scientific

ax. Autoclave Tape, Diagger

ay. Miltenyi Magnetic Separation Columns

az. Boxes for low temperature storage, Diagger

ba. Cryopreservation canes, Diagger

bb. Ranin micro-pipettors and micropipette tips, Mettler Toledo

7. Equipment

a. Class-2 Biosafety Cabinet, Thermo-Fisher

b. Centrifuge, floor model #J21, Beckman

c. Blender, #7011G, Waring, Thermo-Fisher

d. Blender Cylinders, #MC-1 & MC-3, Thermo-Fisher

e. -4°C Refrigerator, Thermo-Fisher

f. -70°C Ultralow Freezer, Forma Scientific

g. -80°C Ultralow Freezer, Forma Scientific
h. Liquid Nitrogen Dewer, Thermo-Fisher
i. Liquid Nitrogen, AirGas
j. Stir Plate, Diagger
k. Steam Autoclave, Thermo-Fisher
l. Freezing Chamber, #9001, Biotech Research
m. Pipet-Aid #148, Drummond Scientific
n. Vortex Mixer, #S8223, Scientific Products
o. Watchmaker's Forceps, #72700D, Electron Microscopy Sciences
p. Hemostats, scissors, forceps, Diagger
q. Bunsen Birner, Diagger
r. Weigh Balance, Mettler Toledo
s. Crushed ice machine
t. Variable temperature water bath, Fisher Scientific
u. Variable temperature glass bead heating unit, Fisher-Scientific
v. FACS unit, Fisher Scientific
w. Flow Cytometer, Fisher Scientific

8. Suppliers

a. Biotech Research Laboratories, Rockville, MD
b. Drummond Scientific, Broomall, PA
c. Scientific Products, Stone Mountain, GA
d. Beckman Instrument Company, Fullerton, CA
e. Fisher Scientific Co., Norcross, GA
f. Thermo-Fisher Scientific, Waltham, MA
g. Curtain-Matheson Scientific, Marietta, GA
h. Tetco Inc., Elmsford, NY
i. Cagle's Strain Hatchery, Forsyth, GA
j. Electron Microscopy Sciences, F. Washington, VA
k. Thomas Scientific Co., Swedesboro, NJ
l. Sigma, Sigma Chemical Co., St Louis, MO
m. Aldrich, Aldrich Chemical Co., Milwaukee, WI
n. Morton Thiokol Inc., Danvers, MA,
o. GIBCO, GIBCO, Grand Island, NY
p. Kodak Co., Rochester, NY
q. EM Sciences, Cherry Hill, NJ
r. Whittaker Bioproducts, Walkersville, MD
s. J.T. Baker Chemical Co., Phillipsburg, NJ
t. 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
u. ICN, ICN Biomedicals, Costa Mesa, CA
v. SBA, Southern Biotechnology Associates, Birmingham, AL
w. Chem, Chemicon, El Segundo, CA
x. BTH, Biomedical Technology Incorporated, Cambridge, MA
y. Accurate, Accurate Chemical & Scientific Corporation, Westbury, NY
z. Vector, Vector Laboratories, Burlingame, CA
aa. KP, Kirkegaard & Perry Laboratories, Gaithersburg, MD
ab. BDH, BDH Chemicals Ltd, Poole, England
ac. DFRD, Dragonfly Foundation for Research and Development, Macon, GA
ad. ChemPure, Curtain Matheson Scientific, Houston, TX
ae. DH, Douglas Hixson, Department of Medicine, Brown University, Providence, RI
af. Jackson, Jackson Laboratories, Bar Harbor, ME
ag. AirGas, AirGas, Local Sore
ah. Atlas, Atlas Biologicals, Fort Collins, CO
ai. 96-well plate reader, R & D Systems, Minneapolis, MI
aj. Diagger Scientific, Vernon Hills, IL
ak. RB, Ray Biotech, Atlanta, GA
al. R&D Systems, Minneapolis, MN
am. Abcam, Cambridge, MA
an. Prospec, Prospec, Rehovot, Israel
ao. Antibodies Inc, Davis, CA
ap. Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA
aq. Diagger Scientific, Buffalo Grove, IL
ar. Mettler Toledo, Columbus, OH

Methods

Differential cryopreservation

Preparation of sterile supplies and reagents: Sterilization of Supplies. Wrap all glassware, funnels, gauze, 20-micron and 90-micron Nitex, instruments, stir bars, 250-ml centrifuge bottles, and blender cylinders in aluminum foil or place in sterilization bags and staple shut. Mark with autoclave tape. Sterilize at 250°C for 30-min in a steam autoclave. After autoclaving, remove instruments from foil and soak in 70% ethanol and flame over a Bunsen burner before contact with any living tissue.

Preparing Sterile 1% Gelatin/Collagen solution for coating tissue culture vessels. Follow detailed direction as described.[9]

Coating tissue culture plates: Follow detailed direction as described.[9]

Avian physiological saline: Sterile Tyrode's buffer with antibiotic (Sigma). Dissolve 1 package of powdered Tyrode's powder mix and 0.2 g sodium bicarbonate in 990-ml distilled (or HPLC) water. Add 10-ml penicillin/streptomycin antibiotic, then pH to 7.6 with 1.0M sodium hydroxide (Sigma). Filter sterilization with 0.2-micron bottle-top filter (Thermo-Fisher) into pre-sterilized 1000-ml culture bottle (Corning), pH should be 7.4 without further adjustment. Vacuum filtration through a bottle-top filter will cause a 0.2-unit decrease in

the pH of the solution. Therefore, it is imperative before filtration/sterilization begins to have a starting pH that is 0.2 units greater than the final desired pH of the solution.

Non-human mammal physiological saline: Phosphate Buffered Saline (PBS, Sigma) with antibiotic. 500-ml bottle of sterile PBS, withdraw 10-ml into separate sterile bottle. Inside Class-2 Biosafety cabinet, using sterile technique, add 10-ml of penicillin/streptomycin.

Human physiological saline: Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO) with or without antibiotic.

a. With antibiotic, 500-ml bottle of DPBS, Inside Class-2 Biosafety Cabinet, using sterile technique, withdraw 10-ml into separate bottle. Add 10-ml of penicillin/streptomycin.

b. Without antibiotic, use 500-ml DPBS without antibiotic.

Sterilize incomplete avian culture medium with antibiotic: Dissolve 1 package of Eagle's Minimal Essential Medium (MEM) with Earle's Salts powdered mix and 2.2-g sodium bicarbonate in 990-ml of distilled (or HPLC) water; add 10-ml of penicillin/streptomycin antibiotic; pH to 7.6, with 1N hydrochloric acid and/or 1.0M sodium hydroxide; filter sterilize as above, and pH should be 7.4 without further adjustment.

Preselected horse serum: Prescreen lots of bovine, fetal calf, porcine, goat, human, and equine for absence of inhibitory, proliferative, progressive, and/or inductive agents, and the ability to support myogenic growth and differentiation, but not support fibroblastic growth and differentiation. The 7th lot of horse serum tested met the above criteria and was designated as HS7. The entire lot was purchased and used with the avian cultures.

Mammalian culture medium: OptiMEM + GlutaMax (GIBCO):

1. With antibiotic: 500-ml bottle of OptiMEM + GlutaMax; inside Class-2 Biosafety Cabinet, using sterile technique, withdraw 10-ml into separate bottle. Add 10-ml of penicillin/streptomycin.

2. Without antibiotic, use 500-ml bottle of OptiMEM + GlutaMax without antibiotic.

Medium supplements

A. For mammalian cultures:

1. Use 10% Heat-Inactivated Serum

2. Serum-free Defined Medium

B. Avian Cultures:

1. Stage specific embryo extract: prepare as previously described in detail.[38, p.276]

2. Sterility testing of embryo extract: test as previously described in detail.[38, p.276]

3. Serum supplemented with EC-HS7: consists of Eagle's MEM with Earle's salts containing 10% v/v preselected horse serum and 5% vol/vol stage-specific embryo extract.

4. Serum-free defined medium (EC-SSIII): consists of Eagle's MEM with Earle's salts containing 15% v/v UltraCulture (Whittaker Bioproducts).

0.4% Trypan Blue in Tyrode's buffer, pH 7.4: Dissolve 1 package of Tyrode's powder mix and 0.2-g sodium bicarbonate in 990-ml distilled (or HPLC) water. Add 10-ml penicillin/streptomycin; pH to 7.6 with 1.0M sodium hydroxide. Dissolve 0.4-g Trypan blue (Kodak) w/v in 100-ml of this buffer; filter sterilize; pH should be 7.4 without further adjustment.

0.4% Trypan Blue in PBS, pH 7.4: In Class-2 Biosafety Cabinet, using sterile technique, withdraw 100-ml of sterile PBS, pH 7.4, into pre-labeled, pre-sterilized brown bottle. Add 0.4-g of Trypan blue w/v in 100-ml of PBS, swirl to mix contents.

0.4% Trypan Blue in DPBS, pH 7.4: In Class-2 Biosafety Cabinet, using sterile technique, withdraw 100-ml of sterile DPBS, pH 7.4, into pre-labeled, pre-sterilized brown bottle. Add 0.4-g of Trypan blue w/v in 100-ml of PBS, swirl to mix contents.

Glycerin-jelly: Prepare as described by Humason.[38]

Cell harvests

1. Avian Cells: Prepare as described in detail.[9,14,24,25]

2. Non-Human Mammalian Cells: Prepare as described in detail.[9,16,17]

3. Human Cells: Prepare as described.[13,21,22,24]

Cryopreservation:[27]

For each of three species, e.g., avians, non-human mammals, and humans. Prepare aliquots of 1, 5, 7, 10, and 11 x 10^6 cells per ml of each species in serum-supplemented medium or serum-free medium. Pipette 1.0-ml aliquots into 1.2-ml polypropylene cryovials (Corning). Various combinations and percentages of dimethyl sulfoxide (DMSO) with and without percentages of glycerol were examined for the following:

1. Add dimethyl sulfoxide (DMSO) at concentrations of 0.0, 0.1, 2.5, 5.0, 7.5, and 10% to separately to cryovials containing cell suspensions. To negate the concentration of commercially available 70% DMSO, ultra-pure DMSO (99.99%-100%) was diluted with HPLC water at percent concentrations of 0.0, 1.0, 2.5, 5.0, 7.5, and 10%.

a. 70% DMSO (ATCC), make up as if 100% concentration

b. 100% DMSO (Morton Thiokol)

c. 99-100% DMSO (Sigma)

d. 99-100% DMSO (Allied Chemical)

1. Add Glycerin (Curtain-Matheson) at concentration of 0, 1, 5, 10, 15, and 20% separately to cryovials containing cell suspensions and various percentages of DMSO. To negate the concentration of Glycerin, ultra-pure Glycerin (99.99%-100%) was diluted with HPLC water at percent concentrations of 0.0, 1.0, 5.0, 10.0, 15.0 and 20.0%.

2. Various combinations and percentages of dimethyl sulfoxide (DMSO) with and without percentages of glycerin were examined.

3. The suspensions were mixed by three methods to ascertain which procedure will allow for optimal equilibration of the cryopreservative(s) with the least cellular fragmentations.

a. Vortex for 60-sec using a setting of 3.5 with 6 x 10-sec pulses.

b. Triturating 20 times with a 10-ml sterile disposable pipette using Pipet-Aid.

c. Gentle inversion 5 times by hand.

4. Two freezing regimens were used:

a. "Fast" freezing consists of direct immersion of cryovial into liquid nitrogen (-196°C) for 18 hours and then storage in liquid nitrogen thereafter for 35 days maximum.

b. "Slow" freezing consists of placing cryovials into an ambient temperature freezing chamber containing absolute isopropyl alcohol and then into -70°C and -80°C freezers for 18 hours, and then removal of the cryovials from the freezing chamber and stored at their respective temperatures for 35 days maximum.

5. Two thawing procedures were tested:

a. Rub a frozen vial of cells between two hands in a back-and-forth motion until the frozen cell suspension (yellow) just thaws (red [Eagle's MEM] or salmon [OptiMEM]), and then placed on crushed ice for further processing.

b. Immerse a vial of frozen cell suspension (yellow) into a 37°C: water bath or glass bead bath, just thaws (red [Eagle's MEM] or salmon [OptiMEM]), and immediately process cell for plating.

6. The time allowed for cell suspension to be in presence of liquid cryopreservative was examined, from 0 to 6 hours, at increments of 5 min.

a. Remove 100-micoliter aliquots of cell suspensions to three recovery media per species (avian, non-human mammal, and human):

i. Cell suspension medium containing 7.5% DMSO

ii. Serum-supplemented 10% heat-inactivated serum

iii. Serum-free defined medium (UltraCulture)

7. Examine six separate dilution ratios of recovery medium per species and expressed as ratio of cell suspension volume to medium volume: 1:5, 1:10, 1:15, 1:20, 1:25, and 1:30.

8. Test three temperatures of recovery medium: 4°C, ambient temperature, and 37°C.

9. Examine seven centrifugation speeds: 46, 104, 184, 738, 1660, 2952, and 3254 x RCF (relative centrifugation force).

10. Examine four temperatures for centrifugation: 4°C, 10°C, 15°C, and 22°C.

11. Examine centrifugation times from10 to 17 minutes: 10, 11, 12, 13, 14, 15, 16, and 17.

12. Examine cell attachment to collagen-coated plates in serum-free defined medium vs

a. Eagle's MEM with Earle's Salts + 10% HS7 + 5% embryo extract[9]

b. OptiMem + GlutaMax + 10% Heat-Inactivated Serum[19]

13. Follow assessment of recovery as outlined previously[9]

14. Each of the above and combinatorial parameters were repeated a minimum of 8 times by each of three different investigators (PI, Research Associate, and Research Assistant) to verify results. Therefore, n=24 for each parameter tested.

Unique cell surface markers

Differential CD (cluster of human differentiation) cell surface marker staining was coupled with flow cytometry to ascertain the cell surface profile "fingerprint" unique for each subcategory of adult telomerase positive stem cells (i.e., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, and EndoSCs) and the tripotent telomerase negative mesenchymal stem cell (MSC). This was initially accomplished using a battery of human CD-markers (Table 1).[7,9,21,24,29] Then animal cell surface molecules were matched to their human counterparts.

Table 2 Positive human and animal CD-marker fingerprints and flow cytometry for aTPSCs and MSCs

Attributes	TSCs	HLSCs	CLSCs	PSCs	GLSCs	EctoSCs	MesoSCs	EndoSCs	Tri-MSCs	Med-MSC
Positive CD Markkers	CD66e	CD66e high	CD66e low	CD10	CD90	CD56,	CD13,	??,	CD90	
		CD10low	CD10high			CD90,	CD90,	CD90,	CD105	CD70
						MHC-	MHC-	MHC-	CD117	CD90
	CEA--CAM-1	CEA-CAM-1 high	CEA-CAM-1 low	SSEA-4	Thy-1	Class-1	Class-1	Class-1	CD123	CD105
		SSEA-4 low	SSEA-4 high						CD166	MHC-Class-1
						Thy-1	Thy-1	Thy-1	MHC--Class-1	
										Thy-1
									Thy-1	

Table 2. Human Cluster of Differentiation markers (CD) and the equivalent animal cell surface recognition markers were utilized to ascertain specific cell surface profile "fingerprints" to discern particular cell types. CEA-CAM-1, carcinoembryonic antigen-cell adhesion molecule-1, equivalent to CD66e; SSEA-4, stage-specific embryonic antgen-4, equivalent to CD10;Thy-1, glycosylphosphatidylinositol (GPI)-anchored protein found in cell membranes, equivalent to CD90. Reprinted with permission in part from Young, et al. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys. 2004; 40:1-80.

Cell sorting

Miltenyi magnetic isolation columns

An alternative to flow cytometry/FACS for the isolation of aTPSCs was to use Miltenyi Magnetic Antibody columns (Figure 3) to isolate and purify subcategories of aTPSCs prior to repetitive single cell clonogenic analysis. CD-markers were initially used for human cells. Then the animal equivalents cell surface markers were used to isolate subcategories of animal cells in this study: CD66e human = CEA-CAM-1 (carcinoembryonic antigen-cell adhesion molecule-1)

animal; CD10 human = SSEA-4 (stage-specific embryonic antigen-4) animal; CD90 human = Thy-1 (heavily N-glycosylated glycophosphatidylinositol anchored cell surface protein) animal, etc (Table 1).[7,10,28] Antibodies to the cell surface markers were bound to iron particles, and the non-antibody-bound cells separated from the antibody-bound cells (Figure 2). The best method to perform magnetic Miltenyi Antibody column separation was to perform two negative sorts, followed by a positive sort. If TSCs were to be purified, the first negative sort would use the antibody Thy-1. Thy-1 would then remove GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs being bound to the column from the initial mixed population of cells, leaving TSCs, HLSCs, CLSCs, and PSCs in the eluent. The second negative sort with the eluant from the 1st negative sort with a new Miltenyi column would entail using the SSEA-4 antibody. This would remove PSCs, CLSCs, and HLSCs bound to the column from the mixed population, leaving TSCs in the eluent. Then the positive sort would use the eluant from the second negative sort with CEA-CAM-1 to obtain a purified population of TSCs. If sorting with CEA-CAM-1 was performed first, this would have removed TSCs + HLSCs + CLSCs from the population and bound to the column, with PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs in the eluent. Therefore, one is still dealing with a mixed population of cells, those that were pure CEA-CAM-1 positive (TSCs) and those that were CEA-CAM-1 positive and SSEA-4 positive (HLSCS and CLSCs). For purified PSCs, the two negative Miltenyi antibody column sorts would utilize Thy-1 (1st) and CEA-CAM-1 (2nd). The positive sort would utilize SSEA-4. For semi-purified GLSCs, EctoSCs, MesoSCs, and EndoSCs, the two negative sorts would entail CEA-CAM-1 (1st) and SSEA-4 (2nd). The positive sort would entail Thy-1.

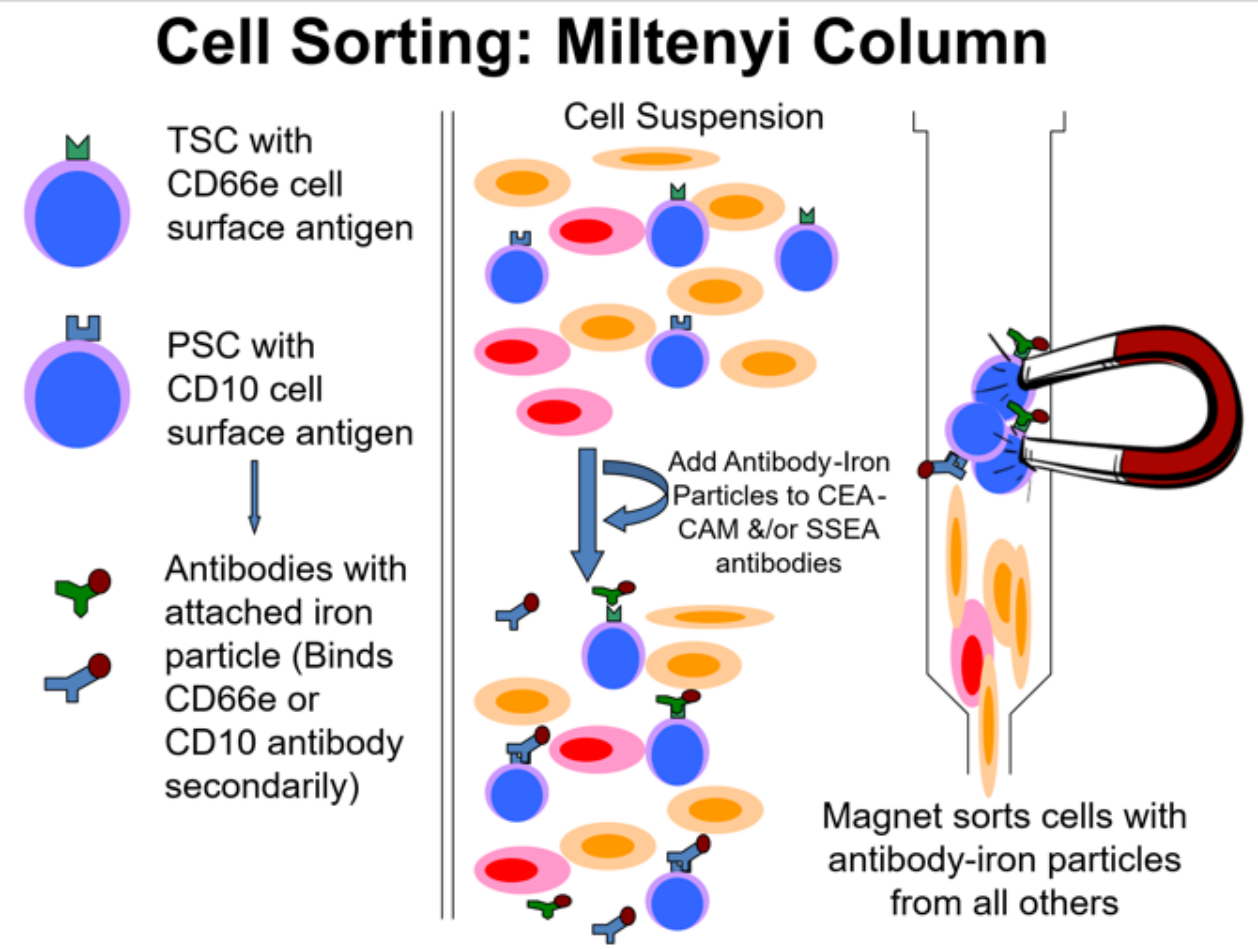

Figure 3 Miltenyi antibody column purification of telomerase negative mesenchymal stem (progenitor cell) and telomerase positive stem cells (e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, and EndoSCs) isolated from remaining cell types from stem cell harvest. Yields routinely in the 95-98% purity range. 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): 149-147; Young HE. Totipotent stem cells and pluripotent stem cells are present in the reproductive organs of an adult mammal. GSC Advanced Research and Reviews. 2025; 23(03): 158-180.

Results

Cryopreservation

Table 3 Conditions Examined for Cryopreservation and Storage of Functional Differentiated Cells, Maintenance Progenitor Cells, and Healing Stem Cells

Testing parameters	**Items tested**	**Functional differentia-ted cells optimum**	**Main-tenance progenitor stem cells (MSCs) optimum**	**Healing stem cells (GLSCs, EctoSCs, MesoSCs, EndoSCs) optimum**	**Healing stem Cells (PSCs, CLSCs, HLSCs, TSCs) optimum**
# of Cells/ml/ Aliquot	1, 5, 7, 10, 11 x 10^6	1 to 11x10^6	1 to 11x10^6	1 to 11x10^6	1 to 11x10^6
DMSO %	0.1, 2.5, 5, 7.5, 10	10%	10%	7.5%	7.5%
Glycerol %	0, 1, 5, 10, 15, 20	0%	0%	0%	0%
Mixing	Vortexing Triturating Inversions	5-6 Inversions	5-6 Inversions	5-6 Inversions	5-6 Inversions
Freezing	Fast (Liquid N2) Slow (1°/min)	Fast (Liquid N2)	Fast (Liquid N2)	Slow (1°/min)	Slow (1°/min)
Cryopreservation & Storage	Liquid N2 -70°C -80°C	Liquid N2	Liquid N2	-70°C	-80°C
Thawing	Rub cryovial between hands 37°C Water Bath Heating Unit	37°C Water Bath Heating Unit	37°C Water Bath Heating Unit	37°C Water Bath Heating Unit	37°C Water Bath Heating Unit
Cell Suspension Max Time in Liquid DMSO	0 to 3 hours, at 5 min increments	5 min	5 min	5 min	5 min
Ratio of Cell Suspension to Recovery Medium	1:5, 1:10, 1:15, 1:20, 1:25, 1:30	1:15	1:15	1:15	1:15
Temperature of Recovery Medium	4°C, Ambient (22-25°C), 37°C	Ambient (22-25°C)	Ambient (22-25°C)	Ambient (22-25°C)	Ambient (22-25°C)
Centrifugation Speed, RCF	46, 104, 184, 738, 1660, 2952, 3254	3254	3254	3254	3254
Centrifugation Temperature	4°, 10°, 15°, 22°	4°	4°	4°	4°
Centrifugation Time	10, 11, 12, 13, 14, 15, 16, 17	15-17 min	15-17 min	15-17 min	15-17 min

Citation: Young HE. Adult telomerase positive stem cells: differential cryopreservation, cell surface marker profiles, and cell sorting. *MOJ Orthop Rheumatol.* 2025;17(6):155–173. DOI: 10.15406/mojor.2025.17.00726

Table 3 Continued....

Plating Medium	Serum-free defined Medium, Medium with Supplement	Medium with Supplement	Medium with Supplement	Medium with Supplement	Medium with Supplement

Parameters examined for optimal cryopreservation, storage, and thawing of functional differentiated cells, maintenance progenitor stem cells, and healing stem cells. The number of samples tested for each parameter was n=8 for each of three investigators (PI, Research Associate, and Research Assistant) for a total of n=24 for each separate parameter examined.

Unique cell surface marker profiles: Animal cell surface markers were matched to their equivalent human CD markers (e.g., CEA-CAM-1 matched to CD66e; SSEA-4 matched to CD10; and Thy-1 matched to CD90) and were utilized for cell sorting of animal aTPSCs (Table 4).

Table 4 Attributes of Adult Telomerase Positive Cells & Adult Telomerase Negative Cells

Attributes	**Size, microns**	**0.4% Trypan blue**	**Telomerase**	**Human Cell Surface Markers**	**Animal Cell Surface Markers**
TSCs	0.1-2.0	All Positive	Present	CD66e	CEA-CAM-1
HLSCs	>2-4	Halo Positive Remainder Negative	Present	$CD66e^{high}$ $CD10^{low}$	$CEA\text{-}CAM\text{-}1^{high}$ $SSEA\text{-}4^{low}$
CLSCs	>4-<6	Corona Positive Remainder Negative	Present	$CD66e^{low}$ $CD10^{high}$	$CEA\text{-}CAM\text{-}1^{low}$ $SSEA\text{-}4^{high}$
PSCs	6-8	All Negative	Present	CD10	SSEA-4
GLSCs	>8-<10	All Negative	Present	$CD10^{high}$ $CD90^{low}$	$SSEA\text{-}4^{high}$ $Thy\text{-}1^{low}$
EctoSCs	10-12	All Negative	Present	CD90 CD56 MHC Class-1	Thy-1
MesoSCs	10-12	All Negative	Present	CD90 CD13 MHC Class-1	Thy-1
EndoSCs	10-12	All Negative	Present	CD90 MHC Class-1	Thy-1
Tripotent MSC-PCs	15-30	All Negative	Absent	CD90 CD105 CD117 CD123 CD166 MHC Class-1	Thy-1

Table 4 Continued....

Medicinal MSC-PCs	15-30	All Negative	Absent	CD70 CD90 CD105 MCH Class-I	Thy-1

Attributes of telomerase positive healing stem cells[1,7] and telomerase negative mesenchymal progenitor stem cells, both the tripotent MSCs that will form fat, cartilage and bone,[4–6] and the medicinal MSCs that will secrete agents to modulate the immune system.[2,3]

Cell Sorting

Flow cytometry/Fluorescent activated cell sorting (FACS): Figure 4 is an example from flow cytometry/FACS of human (HM0001) totipotent stem cells (TSCs), transitional totipotent stem cells (HLSCs) to pluripotent stem cells (CLSCs), and pluripotent stem cells (PSCs). Flow cytometry/FACS demonstrates ultra-small totipotent stem cells (us-TSCs), small totipotent stem cells, transitional totipotent to pluripotent stem cells (halo-like stem cells and corona-like stem cells), and pluripotent stem cells. The ultra-small TSCs are located in an area of the flow diagram normally assumed to contain cell debris (X-axis, 10^{o} to 10^{1} and Y-axis 10^{o} to 10^{1}). Yet when this material was stained with propidium iodide, ≥ 95% of the material stained for intact nucleated cells. Size range in this area is 0.1 to 1.0 microns (Figure 4).

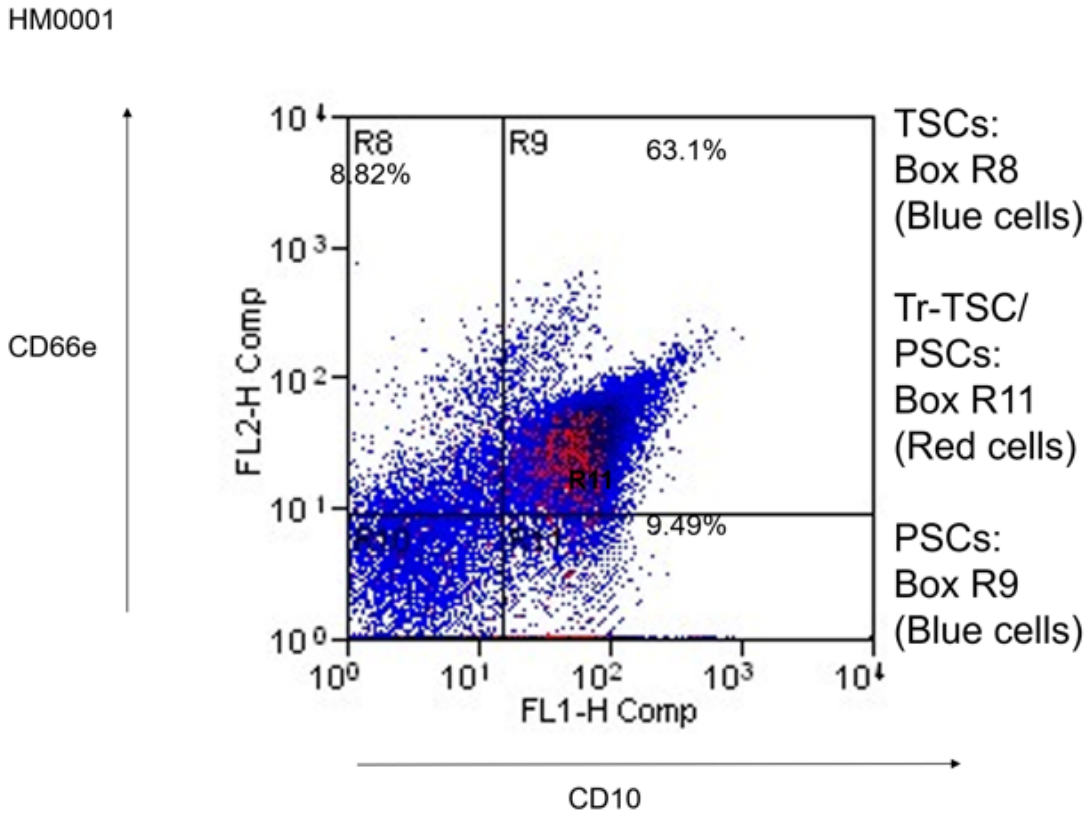

Figure 4 Endogenous adult human TPSCs were sorted using CD antibodies, CD66e and CD10. As shown, TSCs (CD66e) were found in the grid R10 (blue cells), while PSCs (CD10) were found in grid R9 (blue cells). Transitional-totipotent stem cells/pluripotent stem cells, e.g., HLSCs and CLSCs (Tr-TSC/PSCs, red) gate R9, CD66e+/CD10+, represent an intermediate population of cells between the totipotent stem cells (TSCs, blue) gate R8, CD66e+/CD10, and the pluripotent stem cells (PSCs, blue) gate R11, CD66e-/CD10+.

Miltenyi magnetic antibody column separation

Table 5 Miltenyi antibody column purification of mixed population of telomerase positive healing stem cells versus telomerase negative maintenance progenitor cells

TSCs	Type of Cells	Cell Surface Marker	Cells in Eluant	Cells Bound
1st Sort (Negative)	Mixed Culture	Thy-1	TSCs, HLSCs, CLSCs, PSCs	GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs
2nd Sort (Negative)	Cells from 1st Run Eluant	SSEA-4	TSCs	HLSCs, CLSCs, PSCs
3rd Sort (Positive)	Cells from 2nd Run Eluant	CEA-CAM-1	TSCs	Any residual Cells
PSCs	Type of Cells	Cell Surface Marker	Cells in Eluant	Cells Bound
1st Sort (Negative)	Mixed Culture	Thy-1	TSCs, HLSCs, CLSCs, PSCs	GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs
2nd Sort (Negative)	Cells from 1st Sort Eluant	CEA-CAM-1	PSCs	TSCs, HLSCs, CLSCs
3rd Sort (Positive)	Cells from 2nd Sort Eluant	SSEA-4	PSCs	Any Residual HLSCs, CLSCs
GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs	Type of Cells	Cell Surface Marker	Cells in Eluant	Cells Bound
1st Sort (Negative)	Mixed Culture	CEA-CAM-1	PSCs GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs	TSCs, HLSCs, CLSCs
2nd Sort (Negative)	Cells from 1st Sort Eluant	SSEA-4	GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs	PSCs

Table 5 Continued....

3rd Sort (Positive)	Cells from 2nd Sort Eluant	Thy-1	None	GLSCs, EctoSCs, MesoSCs, EndoSCs, Tri-MSCs & Med-MSCs

Results from Miltenyi Antibody Column Purification of Mixed Population of Telomerase Positive Healing Stem Cells versus Telomerase Negative Maintenance Progenitor Cells. Two negative sorts are performed to rid population of contaminating cells before final purification sort. For TSCs, first negative sort removes cells that are Thy-1 positive, e.g., GLSCs, EctoSCs, MesoSCs, EndoSCs, tripotent MSC, and medicinal MSC. The second negative sort removes cells that are SSEA-4 positive, e.g., PSCs, CLSCs, and HLSCs. The third run is final purification sort for solely CEA-CAM-1 positive cells, e.g., ultra-small TSCs (0.1 to 1.0-microns) and small TSCs (1.0 to 2.0-microns). Thy-1 positive healing stem cells, e.g., GLSCs, EctoSCs, MesoSCs, and EndoSCs, were separated from maintenance progenitor cells, e.g., tripotent MSCs (forming fat cartilage and bone) and medicinal MSCs, by differential cryopreservation, cell sorting, and single cell repetitive clonogenic analysis.

Discussion

Preparation of solutions, pH

Vacuum filtration (negative pressure) using bottle top filter unit lowers the pH of any solution by 0.2 pH units. Therefore, when making up any solution requiring vacuum filtration/sterilization, always pH the solution 0.2 pH units higher than required. Alternatively, using a positive pressure filtration system will usually raise the pH of a solution by 0.2 pH units. Therefore, when making up any solution requiring positive pressure filtration/sterilization, always pH the solution 0.2 pH units lower than required.

Differential cryopreservation

Optimal recovery of cryopreserved cells using commercially available 70% DMSO resulted in cell recoveries approximating 50%.[13] We chose to examine DMSO from various vendors to determine if it was possible to increase recoveries of viable cells above 50%. The testing procedure entailed using 10^6 to 10^7 (avian, non-human mammal, and human) cells suspended in complete species-specific culture medium (avian: Eagle's MEM with Earle's salts with 10% HS7 and 5% Embryo Extract, and non-human mammalian and human cells: OptiMEM + GlutaMax with 10% Heat-Inactivated Serum) and then 1-ml of cell suspension pipetted into 1.2-ml polypropylene cryovials. Based on the listed ingredients for the commercially available 70% DMSO, it was unsure what constituted the other 30% of the formulation. The other 30% may have accounted for the apparent 50% viability of the thawed cells. Therefore, multiple percentages of DMSO were formulated with either sterile reverse osmosis double-distilled water (avian cells) or sterile HPLC water (non-human mammalian cells and human cells), utilizing 99% and 100% DMSO, from three different vendors, as the starting material.

Testing DMSO with glycerin at different percentages did nothing to enhance viability after cryopreservation.[14] Actually, it was discovered that if ultra-pure (99.99% to 100%) DMSO was used as the sole cryoprotectant, one could obtain maximized viability recoveries of telomerase negative functional differentiated cells, telomerase negative maintenance progenitor stem cells, and telomerase positive healing stem cells, based on their respective freezing and storage parameters. The parameters entailed using (-196°C) liquid nitrogen for flash freezing and storage of telomerase negative functional differentiated cells and maintenance progenitor stem cells versus slow freeze and storage of healing stem cells at -70°C for GLSCs, EctoSCs, MesoSCs, and EndoSCs, and -80°C for TSCs, HLSCs, CLSCs, and PSCs. Viable recoveries were >95% for functional differentiated cells and maintenance progenitor stem cells and >98-99% for adult telomerase positive healing stem cells.

During the course of these studies, it was discovered that functional differentiated cells, maintenance progenitor stem cells, and telomerase positive healing stem cells were extremely fragile in the presence of the ultra-pure (99-100%) form of liquid DMSO. This was due to the exothermic nature of the ultra-pure DMSO. The exothermic nature of 99-100% DMSO can be tested by putting 50-ml of water into a beaker (with stir bar), place onto a stir plate, and insert a thermometer into the water above the stir bar. Stir the water and allow it to acclimate to ambient temperature. Then add 25-ml of 99-100% DMSO and measure change in temperature.

Therefore, mixing 99-100% liquid DMSO with the cell suspension was imperative to dilute the effects of the exothermic reaction. However, vortexing was NOT an option for mixing the contents of the cryovial, even at the slowest speed. Vortexing was tested with healing stem cells, maintenance progenitor stem cells, and functional differentiated cells at one-second bursts with speeds from 1-10. Most of the cells were killed at any speed (assayed by proliferation in culture in the presence of a known proliferation agent, e.g., 2-ng per ml platelet-derived growth factor-BB (PDGF-BB), regardless of whether they were functional differentiated cells, maintenance progenitor stem cells, or healing stem cells. It was determined that slow inversion of the cryovials for 5-6 times was sufficient to generate an even distribution of the contents of the cryovial (cell suspension with ultra-pure liquid DMSO).

The methods used for an optimal thawing procedure were also examined in detail. Frozen aliquots of healing stem cells, maintenance progenitor stem cells, and functional differentiated cells were thawed at either refrigeration temperature (4°C), ambient temperature (22-25°C) or at 37°C using either a water bath or glass bead heating unit. The key to determining optimum thawing was not the temperature of thawing, but rather when the medium changed from its frozen state to its liquid state. This was relatively easy to ascertain by looking at the color of the cell suspension medium in the cryovial. When the frozen culture medium (for either Eagle's MEM with Earle's Salts or OptiMEM + GlutaMax) was yellow and then changed to the liquid form (red for Eagle's MEM with Earle's Salts or salmon-colored for OptiMEM + GlutaMax), the cell suspension had thawed. Then the liquified cell suspension/DMSO was immediately removed from the cryovial and placed into14-ml of respective complete medium in a 15-ml polypropylene centrifugation tube. The tubes were spun to pellet the cells. The supernatant was discarded to bleach, and the cell pellet reconstituted in their respective complete medium.

Throughout the cryopreservation/thawing process, one had a total maximum of 5 minutes in the liquid form of DMSO before massive cell death occurred. Therefore, it was imperative that once DMSO was added to the cell suspensions, that the processing time in liquid DMSO prior to freezing be added to the processing time in liquid DMSO after thawing, and that that time not to exceed 5 minutes. During thawing, it was noted that 37°C in either a water bath or heating provided the quickest time and less exposure to liquid DMSO.

Utilizing reciprocal freezing and storage parameters, it was noted that the recoveries of viable telomerase negative maintenance stem progenitor cells and functional differentiated cells slow frozen and then stored at temperatures from -70°C to -80°C approximated less than 5%. Similarly, it was noted that if telomerase positive healing stem cells were flash frozen and stored in liquid nitrogen, then percent viable recoveries also approximated less than 5%. In both cases, greater than 95% of the cells died under these reciprocal cryopreservation conditions. Therefore, telomerase negative functional differentiated cells and telomerase negative maintenance progenitor stem cells could be partially separated from the telomerase positive healing stem cells using differential cryopreservation at their respective observed temperatures.

After exhaustive comparative studies across multiple parameters, optimal procedures were identified, e.g., formulating cell suspension, adding DMSO, mixing contents, cryopreservation & storage temperatures based on cell type, thawing, reconstitution, cell counting, plating, and standardized. Following reconstitution in species-specific medium, 50-microliters of cell suspension was added to 50-microliters of sterile species-specific 0.4% Trypan blue, and triturated. Then 50-microliters of stained cell suspension was removed, placed on a hemocytometer, and counted.

The following are the optimal procedures identified for cryopreservation and reconstitution of telomerase negative functional differentiative cells, telomerase negative maintenance progenitor stem cells, and telomerase positive stem cells and their resultant viabilities. From avian, non-human mammals, and human sources, the procedures are as follows (Table 6).

Table 6 Optimum Conditions for Cryopreservation of Healing Stem Cells versus Maintenance Progenitor Cells or Functional Differentiated Cells for Avian, Non-Human Mammal, and Human Cells

Parameters	Avian	Non-Human Mammals	Humans
Species-Specific Buffer	Tyrode's Buffer	Phosphate Buffered Saline	Dulbecco's Phosphate Buffered Saline
Species-Specific Medium	Eagle's MEM with Earle's Salts	OptiMEM + GlutaMax	OptiMEM + GlutaMax
Species-Specific Supplements	10% HS7 + 5% Embryo Extract	10% Heat Inactivated Serum	10% Heat Inactivated Serum
Cells per aliquot	No difference 1X10^6 to 11x10^6	No difference 1X10^6 to 11x10^6	No difference 1X10^6 to 11x10^6
Cryopreservative: Healing Stem Cells	7.5% DMSO	7.5% DMSO	7.5% DMSO
Cryopreservative: Maintenance Progenitor Stem Cells or Functional Differentiated Cells	10% DMSO	10% DMSO	10% DMSO
Equilibration: Healing Stem Cells, Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	Gentle Inversion 5-6 times	Gentle Inversion 5-6 times	Gentle Inversion 5-6 times
Freezing Temperature: Healing Stem Cells	-70°C to -80°C	-70°C to -80°C	-70°C to -80°C
Freezing Temperature: Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	Liquid Nitrogen, -196°C	Liquid Nitrogen, -196°C	Liquid Nitrogen, -196°C
Freezing Time: Healing Stem Cells	Slow Freeze, 1 degree per minute	Slow Freeze, 1 degree per minute	Slow Freeze, 1 degree per minute

Table 6 Continued....

Freezing Time:			
Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	Fast Freeze	Fast Freeze	Fast Freeze
Storage Temperatures for	-70°C to	-70°C to	-70°C to
Healing Stem Cells	-80°C	-80°C	-80°C
Storage Temperature for	Liquid Nitrogen,	Liquid Nitrogen,	Liquid Nitrogen,
Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	-196°C	-196°C	-196°C
Thawing Temp:	37°C	37°C	37°C
Healing Stem Cells,	Water Bath or	Water Bath or	Water Bath or
Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	Glass Bead Heating Unit	Glass Bead Heating Unit	Glass Bead Heating Unit
Time in Liquid DMSO	≤ 5 min	≤ 5 min	≤ 5 min
Species-Specific Recovery Medium	Eagle's MEM with Earle's Salts	OptiMEM + GlutaMax	OptiMEM + GlutaMax
Species-Specific Recovery Supplements	10% HS7 + 5% Embryo Extract	10% Heat Inactivated Serum	10% Heat Inactivated Serum
Recovery Dilution	1:15	1:15	1:15
Recovery Temp	37°C	37°C	37°C
Centrifugation Speed	3254 x RCF	3254 x RCF	3254 x RCF
Centrifugation Temperature	No Difference between 4°C to 15°C	No Difference between 4°C to 15°C	No Difference between 4°C to 15°C
Centrifugation Time	15 to 17 min	15 to 17 min	15 to 17 min
Species-Specific Attachment Medium	Eagle's MEM with Earle's Salts	OptiMEM + GlutaMax	OptiMEM + GlutaMax
Species-Specific Attachment Supplements	10% HS7 + 5% Embryo Extract	10% Heat Inactivated Serum	10% Heat Inactivated Serum
Recovery Percentages: Healing Stem Cells	98-99%	98-99%	98-99%
Recovery Percentages: Maintenance Progenitor Stem Cells, and Functional Differentiated Cells	>95%	>95%	>95%

The optimum parameters discovered for the cryopreservation of healing stem cells, maintenance progenitor stem cells, and differentiated cells from avian, non-human mammal (e.g., mouse, rat, cat, dog, sheep, goat, pig, horse), and human cell sources; encompassing: species-specific buffer, species-specific medium, species-specific supplements, cells per aliquot frozen, source and percentage of cryopreservative, equilibration of DMSO with cell suspension, freezing temperatures, freezing times, storage temperatures, thawing temperature, maximal time in liquid DMSO, recovery dilution, centrifugation speed, centrifugation temperature, centrifugation time, and percent cell recovery.

Unique cell surface marker profiles

Human CD (cluster of differentiation) cell surface marker staining was used to ascertain the cell surface "profile / fingerprint" unique for each subcategory of telomerase positive healing stem cells and telomerase negative maintenance progenitor cells. This was initially accomplished using a battery of human CD-markers (Table 1).

Animal cell surface markers were then matched to their equivalent human CD markers (e.g., CEA-CAM-1 matched to CD66e; SSEA-4 matched to CD10; and Thy-1 matched to CD90) (Table 2) and were utilized for cell sorting of animal aTPSCs (Table 3).

Cell Sorting

We utilized differential cryopreservation at -70ºC, -80ºC, and -196ºC; fluorescent-activated cell sorting;[21,29,30] Miltenyi columns;[31] Ficoll density gradient centrifugation;[32] time, temperature, gravity, size, zeta potential;[24] density gradient centrifugation with plasma and saline;[24,33] and water-induced lysis[34] (to separate aTPSCs from exosomes); in our subsequent studies to separate telomerase positive healing stem cells from mixed populations of harvested cells. These mixed populations were composed of telomerase negative functional differentiated cells, telomerase negative maintenance progenitor stem cels, telomerase positive healing stem cells, platelets, and exosomes. This was performed for *in vitro* characterization studies,[29] IACUC-approved[35] pre-clinical studies of animal models of disease,[29] and IRB-approved[36] human clinical studies and trials for treatment of individuals with traumatic, chronic and/or terminal diseases, and orthopedic injuries.[24]

Telomerase positive healing stem cells were then separated into individual populations of TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and single vesicle exosomes. Single vesicle exosomes were separated from aTPSCs utilizing water-induced lysis of the exosome vesicles, followed by centrifugation. This purification procedure was accomplished prior to repetitive single cell clonogenic analysis with cell-specific exosome-conditioned medium, followed by Lac-Z gene beta-galactosidase genomic labeling of individual clones utilizing lipofectin,[29] to ensure purified populations of genomically-labeled individual healing stem cells were available for *in vitro* testing,[7] pre-clinical animal models,[17,18] and human clinical studies.[13,24]

Segregation of healing stem cells

With IRB approval, whole blood was obtained from HM0001 by venipuncture. Then using gravity, time, temperature, and inherent zeta potential,[24] blood elements, e.g., red blood cells, white blood cells, and platelets suspended in plasma, were separated from healing stem cells, e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and single inductive exosomes suspended in plasma. Differential density gradient centrifugation with plasma and saline was then used to segregate individual healing stem cells from each other. Sterile reverse osmosis double-distilled water or sterile high pressure liquid chromatography (HPLC) water was used to separate co-localized exosomes from each category of telomerase positive healing stem cells.

Flow cytometry/Fluorescent activated cell sorting (FACS)

Figure 4 is an example of flow cytometry of human (HM0001) healing stem cells demonstrating separation from totipotent stem cells (TSCs), transitioning to halo-like stem cells (HLSCs), transitioning to corona-like stem cells (CLSCS), and transitioning to pluripotent stem cells (PSCs). The four-quadrant flow diagram demonstrates ultra-small totipotent stem cells (us-TSCs). The ultra-small TSCs are located in an area of the flow diagram normally assumed to contain cell debris (X-axis, 10^{o} to 10^{1} and Y-axis 10^{o} to 10^{1}). Yet when this material was stained with propidium iodide (Figure 5), > 95% of the material stained for intact nucleated cells.

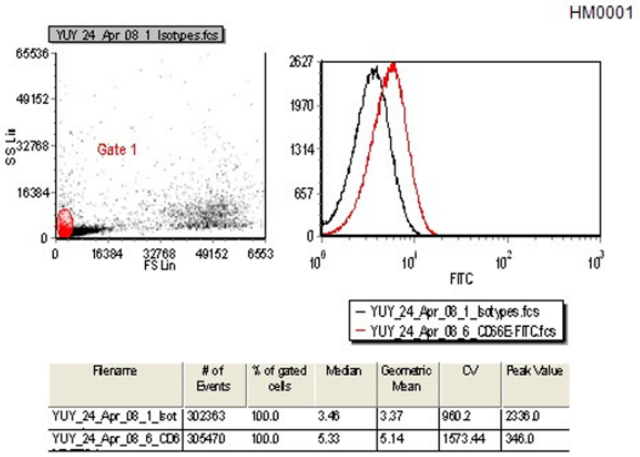

Figure 5 Flow cytometry of human (HM0001) ultra-small totipotent stem cells (us-TSCs), red; small totipotent stem cell (s-TSCs), black, both ultra-small and small, are located in an area normally assumed to contain cell debris (X-axis, 10^{o} to 10^{1} and Y-axis 10^{o} to 10^{1}). Yet when this material was stained with propidium iodide, ≥ 95% of the material stained for intact nucleated cells. Size range in this area is 0.1 to 1.0 microns.

Size range in this area of the X-axis and Y-axis, e.g., 10^{o} to 10^{1} and Y-axis 10^{o} to 10^{1}, is 0.1 to 1.0 microns. Sizing in this lowest end of the size range was verified using sterile 0.2-micron and 0.1-micron bottle top filters. A cell suspension of 0.1-2-micron of TSCs was applied to 0.2-micron and 0.1-microns bottle top filters. Three areas were tested for the presence of TSCs: above the 0.2-micron filter (0.2 to 2.0-micron TSCs), between the 0.2-micron and 0.1-micron filters (0.1 to <0.2-micron TSCs), and below the 0.1-micron filter (no cells present). Cells with the differentiation potential of TSCs, e.g., all somatic cells of the body + gametes + placenta + nucleus pulposus of the intervertebral disc, were only found in the fractions above the 0.1-micron filter. No nucleated entities (potential cells) with the ability to propagate in culture were located in the eluant below the 0.1-micron filter.

Miltenyi magnetic isolation columns

An alternative to flow cytometry for the isolation of aTPSCs was to use Miltenyi Magnetic Antibody columns (Figure 3) to isolate and purify subcategories of aTPSCs prior to repetitive single cell clonogenic analysis[21] utilizing cell-specific exosome-conditioned media. CD-markers were initially used for human cells (Table 1). Then the animal equivalents cell surface markers were used to isolate subcategories of animal cells: CD66e human = CEA-CAM-1 animal; CD10 human = SSEA-4 animal; CD90 human = Thy-1 animal, etc. (Table 2). Antibodies to the cell surface markers were bound to iron particles, and the non-antibody-bound cells separated from the antibody-bound cells as shown in Figure 3. The best method to perform magnetic Miltenyi Antibody column separation was to perform two negative sorts, followed by a positive sort. If TSCs were to be purified, the first negative sort would use the antibody Thy-1. Thy-1 would then remove GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs being bound to the column from the initial mixed population of cells, leaving TSCs, HLSCs, CLSCs, and PSCs in the eluent (Figure

5). The second negative sort with a new Miltenyi column would entail using the SSEA-4 antibody. This would remove PSCs, CLSCs, and HLSCs bound to the column from the mixed population, leaving TSCs in the eluent. Then the positive sort would use CEA-CAM-1 to obtain a purified population of TSCs (Figure 7A). If sorting with CEA-CAM-1 was performed first, this would have removed TSCs + HLSCs + CLSCs from the population and bound to the column, with PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs in the eluent. Therefore, with respect to the eluant, one would still be dealing with a mixed population of cells.

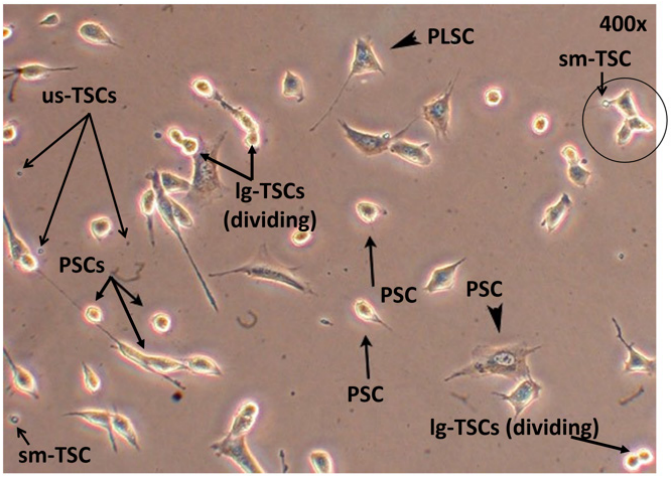

Figure 6 Eluant cells after first Miltenyi negative sort, using Thy-1 antibody. Cells were plated onto 1% collagen-coated flasks in OptiMEM + GlutaMax medium containing 10% Heat-Inactivated serum and 1% antibiotic. As shown eluant cells composed of dividing PSCs, large TSCs (lg-TSCs, 1.0-2.0-microns) and small TSCs (sm-TSCs, 0.1 to 1.0-microns), original magnification at 400X.

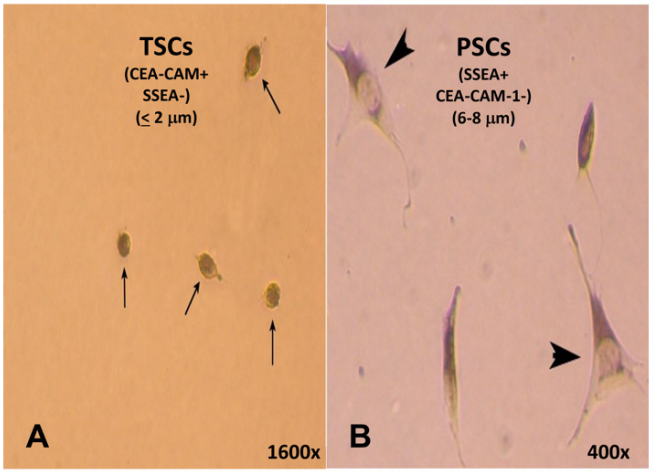

Figure 7 Plated animal TSCs and PSCs after Miltenyi cell sorting. A: Plated TSCs demonstrating small rounded (spherical) cells with minimal cytoplasm surrounding nucleus. Projections on either side of central nucleus are areas of attachment to the substrate. TSCs display positive staining for CEA-CAM-1 and are slightly less than 2 microns in size by flow cytometry of live cells. B: Plated PSCs demonstrating cells that are predominantly multipolar in appearance. PSCs display positive staining for SSEA-4 and are 6 to 8 microns in size by flow cytometry of live cells[28]. 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. [Labeling differences between original paper and above Figure 6. PSCs = ELSCs and TSCs = BLSCs, Pictures are reversed compared to original Figure].

For purified PSCs, the two negative Miltenyi antibody column sorts would utilize Thy-1 (1st) and CEA-CAM-1 (2nd). The positive sort would utilize SSEA-4 (Figure 7B). For semi-purified GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs, the two negative sorts would entail CEA-CAM-1 (1st) and SSEA-4 (2nd). The positive sort would entail Thy-1. Differential cryopreservation would be used separate EctoSCs, MesoSCs, and EndoSCs from MSCs; and/or repetitive single cell clonogenic analysis could be used to separate individual populations of EctoSCs, MesoSCs, EndoSCs, and MSCs.[31]

Water-induced lysis of exosomes

Due to the unique histoarchitecture of adult telomerase positive stem cells, the aTPSCs can withstand incubation in plain water (reverse osmosis double-distilled water or high-pressure liquid chromatography HPLC-water) without lysing This is in contrast telomerase negative functional differentiated cells, telomerase negative maintenance progenitor cells, and exosomes which will lyse in the presence of plain water, due to the difference in osmotic pressures between the increased content of cytosol and the water. While one could rinse whole blood with plain water to disintegrate telomerase negative functional differentiated cells, telomerase negative maintenance progenitor cells, and exosomes, it is not recommended as a method to isolate adult telomerase positive stem cells.

When telomerase negative nucleated cells disintegrate, they release nuclear membrane-disintegrated unencapsulated "naked DNA". This naked DNA is extremely sticky and will attach to anything, including aTPSCs. It is next to impossible to separate naked DNA attached to aTPSCs and retain viable aTPSCs after their separation. Therefore, it is far better for viability to separate nucleated differentiated cells and progenitor stem cell first, and then only dealing with non-nucleated exosomes and nucleated aTPSCs. The exosomes will lyse, while the aTPSCs remain relatively untouched. It is preferable to only use one incubation with water to lyse the exosomes. EctoSCs, MesoSCs, EndoSCs and GLSCs can withstand one 5-min water incubation before the cells disintegrate; HLSCs, CLSCs, and PSCS can withstand two 5-min water incubations before the cells disintegrate; and TSCs can withstand three 5-min water incubations before the cells disintegrate.

Conclusion

Methodologies were described in detail to separate telomerase negative functional differentiated cells and maintenance progenitor stem cells from telomerase positive healing stem cells. These methods include differential cryopreservation; flow cytometry; fluorescent-activated cell sorting; and Miltenyi magnetic antibody columns; gravity, time, temperature, and inherent zeta potential; differential density gradient centrifugation with plasma and saline; and water-induced lysis of exosomes using sterile reverse osmosis double-distilled water (avian cells) or sterile HPLC water-induced lysis (mammalian cells, both non-human and human). Healing stem cells were then separated into eight populations of adult telomerase positive healing stem cells, i.e., TSCs; HLSCs, CLSCs, PSCs; and GLSCs, EctoSCs, MesoSCs, and EndoSCs, utilizing cell-specific cell surface maker antibodies coupled with Miltenyi magnetic antibody columns. Future reports will explain in detail repetitive single cell clonogenic analysis with cell-specific exosome-conditioned medium and beta-galactosidase genomic labeling, to ensure purified populations of genomically-labeled individual healing stem cells for *in vitro* testing, pre-clinical animal models, and human clinical studies/trials.

Citation: Young HE. Adult telomerase positive stem cells: differential cryopreservation, cell surface marker profiles, and cell sorting. *MOJ Orthop Rheumatol.* 2025;17(6):155–173. DOI: 10.15406/mojor.2025.17.00726

Acknowledgements

This work was supported by grants from Rubye Ryle Smith Charitable Trust, Dragonfly Foundation for Research and Development, MedCen Community Health Foundation, Henry O. and Lucille M Young Estate Trust, and MorphoGen Pharmaceuticals, Inc. I would like to thank my long-time collaborator and medical school mentor, Dr. Asa C. Black Jr for his guidance, editing, and assistance in navigating the perils of academia. I would like to thank my clinical collaborators; research associates (J Floyd-Collins-Coleman, LW Blake, Donna Morris) and research assistants (GF Long-Black, NL Henson, Michelle Reeves, Kathi Davis); my multitude of co-authors, clinical attendings, clinical residents, foreign graduate students (Australia and South Korea), USA graduate students, medical students, and undergraduate students for their insight, strong work ethic, editing, and help in performing this research. The antibody CEA-CAM-1 was generously provided by D. Hixson (Providence, RI). The antibody to SSEA-4 was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biological Sciences, Iowa City, IA: MC813 (SSEA-4) antibody was developed by D. Solter.

Conflicts of interest

The authors declare that there are no conflict of interest in these studies.

Statement of ethical approvals

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.[35]

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).[36]

References

1. Young HE, Speight MO, Black AC Jr. Functional cells, maintenance cells, and healing cells. *J Stem Cell Res.* 2017;1(1):003:1–4.
2. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochemistry.* 2006;98(5):1076–1084.
3. Caplan AI. Mesenchymal stem cells: time to change the name. *Stem Cells Translational Medicine.* 2017;6(6):1445–1451.
4. Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 1991;9:641–650.
5. Caplan AI. Review: Mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng.* 2005;11:1198–1211.
6. Pittenger MF, Mackay AM, Beck SC, et.al. Multilineages potential of adult human mesenchymal stem cells. *Science.* 1999;284:143–147.
7. 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.
8. Kierszenbaum AL. Histology and Cell Biology, An Introduction to Pathology. Elsevier/Mosby, 11830 Westline Industrial Dr, St Louis, MO 63146, Copyright 2002, ISBN: 0-323-01639-1.
9. Young HE. Adult telomerase positive stem cells: isolation, plating, and propagation. *GSC Advanced Research and Reviews.* 2025;0285, In press.
10. 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.
11. Rohme D. Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts *in vitro* and erythrocytes in vivo. *Proc Natl Acad Sci USA.* 1981;78(8):5009–5013.
12. Hayflick L, Moorehead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res.* 1961;25(3):585–621.
13. Young HE, Speight MO. Osteoarthritis treated with telomerase-positive adult stem cells in animals and humans. *Stem Cells Regen Med.* 2020;4(2):1–11.
14. Young HE, Morrison DC, Martin JD, et.al. Cryopreservation of embryonic chick myogenic lineage-committed stem cells. *Journal of Tissue Culture Methods*. 1991;13:275–284.
15. Young HE, Wright RP, Mancini ML, et.al. Bioactive factors affect proliferation and phenotypic expression in pluripotent and progenitor mesenchymal stem cells. *Wound Repair and Regeneration*. 1998;6(1):65–75.
16. Rogers JJ, Adkison LR, Black AC Jr, et.al. Differentiation factors induce expression of muscle, fat, cartilage, and bone in a clone of mouse pluripotent mesenchymal stem cells. *The American Surgeon*. 1995;61(3):231–236.
17. Young HE, Black GF, Coleman JA, et.al. Pulmonary diseases and adult healing cells: from bench top to bedside. *J Stem Cell Res.* 2017;1(2):1–9.
18. Young HE, Limnios IJ, Lochner F, et.al. Cardiovascular disease and adult healing cells: From bench top to bedside. *J Stem Cell Res.* 2017;1(3):1–8.
19. Young HE, Lochner F, Lochner D, et.al. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine, and Equine Peripheral Blood. *J Stem Cell Res*. 2017;1(1):004.
20. Stout CL, Ashley DW, Morgan III JH, et.al. Primitive stem cells reside in adult swine skeletal muscle and are mobilized into the peripheral blood following trauma. *The American Surgeon.* 2007;73(11):1106–1110.
21. Young HE, Steele T, Bray RA, et.al. Human progenitor and pluripotent cells display cell surface cluster differentiation markers CD10, CD13, CD56, CD90 and MHC Class-I. *Proc Soc Exp Biol Med*. 1999;221:63–71.
22. Young HE, Steele T, Bray RA, et al. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *Anat Rec*. 2001;264:51–62.
23. Young HE, Lochner F, Lochner D, et al. Primitive stem cells in adult human peripheral blood. *J Stem Cell Res*. 1(2) 001:1-8, 2017
24. Young HE. Fresh isolate adult telomerase positive stem cells: an addition to embryonic stem cells (ESCs), Induced pluripotent stem cells (iPSCs), and/or Mesenchymal stem cells (MSCs) for regenerative medicine. *GSC Advanced Research and Reviews*. 2023;16(1):066–081.
25. ATCC Animal Cell Culture Guide, The Essentials of Life Sciences Research, American Type Culture Collection, Gaithersburg, Maryland.
26. ATCC Catalog of cell lines and hybridomas, 6th Ed. Rockville, MD; American Type Culture Collection: 1988.
27. Young HE, Morrison DC, Martin JD, et al. Cryopreservation of embryonic chick myogenic lineage-committed stem cells. *Journal of Tissue Culture Methods*. 1991;13:275–284.
28. Abbas AK, Litchman AH, Pillai S. Cellular and Molecular Immunology. 7th ED, 2012. ELSEVIER, Saunders.
29. Young HE, Duplaa C, Romero-Ramos M, et.al. Adult reserve stem cells and their potential for tissue engineering. *Cell Biochem Biophys*. 2004;40:1–80.

30. 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):147–149.

31. Young HE. Totipotent stem cells and pluripotent stem cells are present in the reproductive organs of an adult mammal. *GSC Advanced Research and Reviews*. 2025;23(03):158–180.

32. Young HE, Limnios JI, Lochner F, et al. Pancreatic islet composites secrete insulin in response to a glucose challenge. *J Stem Cell Res*. 2017;1(1):1–12.

33. Young HE, Speight MO. Allogeneic and autologous telomerase-positive stem cells as a potential treatment for systemic lupus erythematosus. *Stem Cells Regen Med*. 2020;4(2):1–9.

34. Young HE, Speight MO. Criteria to distinguish TSCs from exosomes as major players in regenerative medicine. *J Regen Med & Biol Res*. 2020;1(1):1–5.

35. National Research Council as outlined *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.

36. Federal Regulations for Protection of Human Research Subjects, HHS Office for Human Research Protections – 45 and 46 CFR: 46.102(I) [24].

37. Hamburger V. *A Manual of Experimental Embryology*. University of Chicago Press, Chicago, IL. 1969, pp. 221

38. Humason G. *Animal Tissue Techniques*, 3rd ED. San Francisco, CA. WH Freeman and Co. 1972;140–141.

39. Young HE, Carrino DA, Caplan AI. Initial characterization of small proteoglycans synthesized by embryonic chick leg muscle-associated connective tissues. *Connect Tissue Res*. 1988;17(2):99–118.

40. Young HE, Carrino DA, Caplan AI. Histochemical analysis of newly synthesized and accumulated sulfated glycosaminoglycans during musculogenesis in the embryonic chick leg. *J Morph*. 1989;201:85–103.

41. Young HE. Adult telomerase positive stem cells: repetitive single cell clonogenic analysis utilizing cell-specific exosome-conditioned media. 2025; In preparation.

42. Young HE, 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, 2013. pp. 63–93.