GSC Advanced Research and Reviews
eISSN: 2582-4597 CODEN (USA): GARRC2
Cross Ref DOI: 10.30574/gscarr
Journal homepage: https://gsconlinepress.com/journals/gscarr/

(RESEARCH ARTICLE)

Check for updates

Adult Telomerase Positive Stem Cells: Isolation, Plating and Propagation

Henry E. Young [1, 2, 3, 4, 5, 6, 7]

[1] *Dragonfly Foundation for Research and Development, Macon, GA 31210 USA.*
[2] *Henry E Young PHD Regeneration Technologies, Macon, GA 31210 USA.*
[3] *Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, GA, 31210, USA.*
[4] *Department of Surgery, Mercer University School of Medicine, Macon, GA, 31210, USA.*
[5] *Department of Pediatrics, Mercer University School of Medicine, Macon, GA, 31210, USA.*
[6] *Department of Obstetrics and Gynecology, Mercer University School of Medicine, Macon, GA, 31210, USA.*
[7] *Department of Anesthesiology, Mercer University School of Medicine, Macon, GA, 31210, USA.*

GSC Advanced Research and Reviews, 2025, 25(02), 407-440

Publication history: Received 06 October 2025; revised on 20 November 2025; accepted on 22 November 2025

Article DOI: https://doi.org/10.30574/gscarr.2025.25.2.0355

Abstract

The adult human body is composed of trillions and trillions of cells. These cells can be divided into three categories: functional [differentiated] cells comprising 40%, maintenance [progenitor] cells comprising 59%, and healing [stem] cells comprising 1% of the cells of the body. Healing stem cells were discovered in 1975 residing within the connective tissue stroma of adult terrestrial salamanders undergoing complete limb regeneration. They were shown to form all damaged or lost tissues of an amputated limb, thereby restoring function to the limb. Since 1975, these cells have been extensively characterized. The healing stem cells are uniquely different from functional cells and maintenance cells. Healing cells are telomerase positive which gives them an essentially unlimited proliferation potential. There are 8 distinct subcategories of healing stem cells based on multiple parameters, including differentiation potentials. In vivo, they do not synthesize and secrete their own substratum, and therefore one must be provided for them. They can form multiple cell layers in vitro. In situ, they reside in connective tissue niches interspersed throughout the body as dormant, hibernating, quiescent cells. They can be stimulated to proliferate and mobilize into the blood stream as non-activated cells, but they need to be activated to function to replace damaged cells and tissues. Activation occurs by unmasking homing receptors on their cell surfaces and unmasking cell surface receptors for exosomes containing biological agents. Due to their uniqueness, special methodologies were developed for their isolation, plating, and propagation. This report describes those methodologies in detail.

Keywords: Differentiated Cells; Progenitor Cells; Stem Cells; Isolation; Propagation; Segregation; Telomerase Positive; Adult

1. Introduction

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 cells, and healing stem cells [1]. The 220+ functional differentiated cells comprise approximately 40% of the cell types in the body. Functional differentiated cells are composed of parenchyma (functioning cell) and stroma (it's connective tissue stroma). Examples of functional differentiated cells are signal transmitting neurons [2,3], hormonal secreting pancreatic islet cells [4-6], ambulatory skeletal muscle cells [7-9], and detoxifying hepatocytes [10-12].

* Corresponding author: Henry E. Young

Table 1 Attributes of Endogenous Adult Telomerase Positive Healing Stem Cells

Attributes	**TSCs[1]**	**HLSCs[2]**	**CLSCs[3]**	**PSCs[4]**	**GLSCs[5]**	**EctoSCs[6]**	**MesoSCs[7]**	**EndoSCs[8]**
Size, microns	0.1-2.0	>2-4	>4-<6	6-8	>8-<10	10-12	10-12	10-12
0.4% Trypan blue	Entire Cell Positive[9]	Halo[10] Positive, Negative	Corona[11], Positive Negative	Entire Cell Negative [12]	Entire Cell Negative	Entire Cell Negative	Entire Cell Negative	Entire Cell Negative
Animal Cell Surface Markers	CEA-CAM-1[13]	CEA-CAM-1^{high} SSEA-4^{low}	CEA-CAM-1^{low} SSEA-4^{high}	SSEA-4[14]	SSEA-4, Thy-1[15]	Thy-1	Thy-1	Thy-1
Human Cell Surface Markers	CD66e[16]	CD66e^{high} CD10^{low}	CD66e^{low} CD10^{high}	CD10[17]	CD10 CD90[18]	CD56, CD90 MHC-1[19]	CD13, CD90 MHC-1	??? CD90 MHC-1
Expressed Genes	Telom +Bcl-2+ Nanog + Nanos + CXCR4 +	Telom +NYD	Telom +NYD	Telom + Oct-3/4 +Sonic- Hedge Hog +	Telom + NYD	Telom +NYD	Telom +NYD	Telom + NYD
Culture Conditions	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion	Substrate Adhesion
Differentiation Capabilities[22]	Somatic Cells, Gametes, NP of IVD	Somatic Cells Only[24]	Somatic Cells only	Somatic Cells only	Somatic Cells only	Ectoderm Lineage Only[25]	Mesoderm Lineage Only[26]	Endo-derm Lineage Only[27]
Maximum Proliferation To Date	>300 Rat Population Doublings	>300 Rat Population Doublings	>300 Rat Population Doublings	>400 Rat Population Doublings	>400 Rat Population Doublings	>400 Rat Population Doublings	>690 Human Population Doublings	>400 Rat Population Doublings
Doubling Time	12-14 hours	13-15 Hours	13-15 hours	14-16 hours	16-18 hours	18-24 hours	18-24 hours	18-24 hours

Table 1. TSCs[1], totipotent stem cells; HLSCs[2], halo-like stem cells; CLSCs[3], corona-like stem cells; PSCs[4], GLSCs[5], germ layer lineage stem cells; EctoSCs[6], ectodermal stem cells; MesoSCs[7], mesodermal stem cells; EndoSCs[8], endodermal stem cells; entire cell demonstrating Positive[9] staining for 0.4% Trypan blue; Halo[10], complete peripheral rim of positive trypan blue staining with central area of cell negative for Trypan blue staining; Corona[11], crown of positive Trypan blue staining, remainder of cell is negative for Trypan blue staining; entire cell demonstrating Negative[13]staining for 0.4% Trypan Blue; CEA-CAM-1[13], carcinoembryonic antigen-cell adhesion molecule-1; SSEA-4[14], stage-specific embryonic antigen-4; Thy-1[15], N-glycosylated glycophosphatidylinositol (=CD90); CD66e[16], carcinoembryonic antigen; CD10[17], common acute lymphoblastic leukemia antigen (CALLA); CD90[18], N-glycosylated glycophosphatidylinositol (=Thy-1); MHC-1[19], self-recognition molecule Major Histocompatibility Complex-Class-1; Suspension[20], cells grow in suspension culture only; Substrate Adhesion[21], only grows attached to a substrate; Differentiation Capabilities[22], differentiation capabilities; All Cells[23], will form all somatic cells of the body, the gametes (spermatogonia and oogonia), and the nucleus pulposus of the intervertebral disc (the only tissue derived from the notochord in adults); Somatic Cells Only[24], will only form somatic cells of the body, will not form gametes, will not form nucleus pulposus of the intervertebral disc; Ecto Lineage Only[25], will only form cells of the ectodermal germ layer lineage, will NOT form cells of either the mesodermal or the endodermal germ layer linages; Meso Lineage Only[26], will only form cells of the mesodermal germ layer lineage, will NOT form cells of either the ectodermal or the endodermal germ cell lineages; Endo Lineage Only[27], will only form cells of the endodermal germ layer lineage, will NOT form cells of either to ectodermal or mesodermal germ layer lineages; NYD[28] , not yet determined [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.

The 230+ maintenance progenitor cells compose approximately 59% of the cells of the body and are the immediate precursor cells to the functional differentiated cells. There are four subcategories of maintenance progenitor cells: multipotent, tripotent, bipotent, and unipotent [13]. An example of a multipotent maintenance progenitor cell is the "hematopoietic stem cell" [14-16]. It will form 18+ cell types all within the hematopoietic lineage. An example of a tripotent maintenance progenitor cell is the "mesenchymal stem cell" identified by Caplan [17]. It will form three cell types, e.g., fat, cartilage, and bone [13,17-19]. An example of a bipotent maintenance progenitor cell is the adipofibroblasts. It will form only two cell types: fat cells and fibroblasts/fibrocytes [20,21]. Examples of unipotent maintenance progenitor cells are osteoblasts forming osteocytes (bone), cardiac myoblasts forming cardiac myocytes, and fibroblasts forming fibrocytes [22].

Healing stem cells (e.g., adult telomerase positive stem cells, aTPSCs) comprise approximately 1% of the ~450+ cell types (~220+ differentiated functional cells and ~230+ maintenance progenitor cells) of the body. There are three major categories and eight subcategories of healing stem cells (aTPSCs). The first major category of aTPSCs are the totipotent stem cells (TSCs) which will form all somatic cells of the body, gender-specific gametes, placental cells (that synthesize and secrete chorionic gonadotrophin), and the nucleus pulposus of the intervertebral disc (the only adult derivative of the notochord). The second major category of aTPSCs are the pluripotent stem cells which will form all somatic cells of the body, but will not form gametes, placental cells, or the nucleus pulposis of the intervertebral disc. Pluripotent stem cells are composed of multiple populations of cells in transition, e.g., halo-like stem cells (HLSCs), transitioning to corona-like stem cells (CLSCs), transitioning to pluripotent stem cells (PSCs), and transitioning to germ layer lineage stem cells (GLSCs). The third major category of aTPSCs are the germ layer lineage ectodermal stem cells (EctoSCs) forming all somatic cell types within the ectodermal germ layer lineage; the germ layer lineage mesodermal stem cells (MesoSCs) forming all somatic cell types within the mesodermal germ layer lineages; and the germ layer lineage endodermal stem cells (EndoSCs) forming all somatic cell types within the ectodermal germ layer lineage, (Table 1, Figs. 1-3) [13,23].

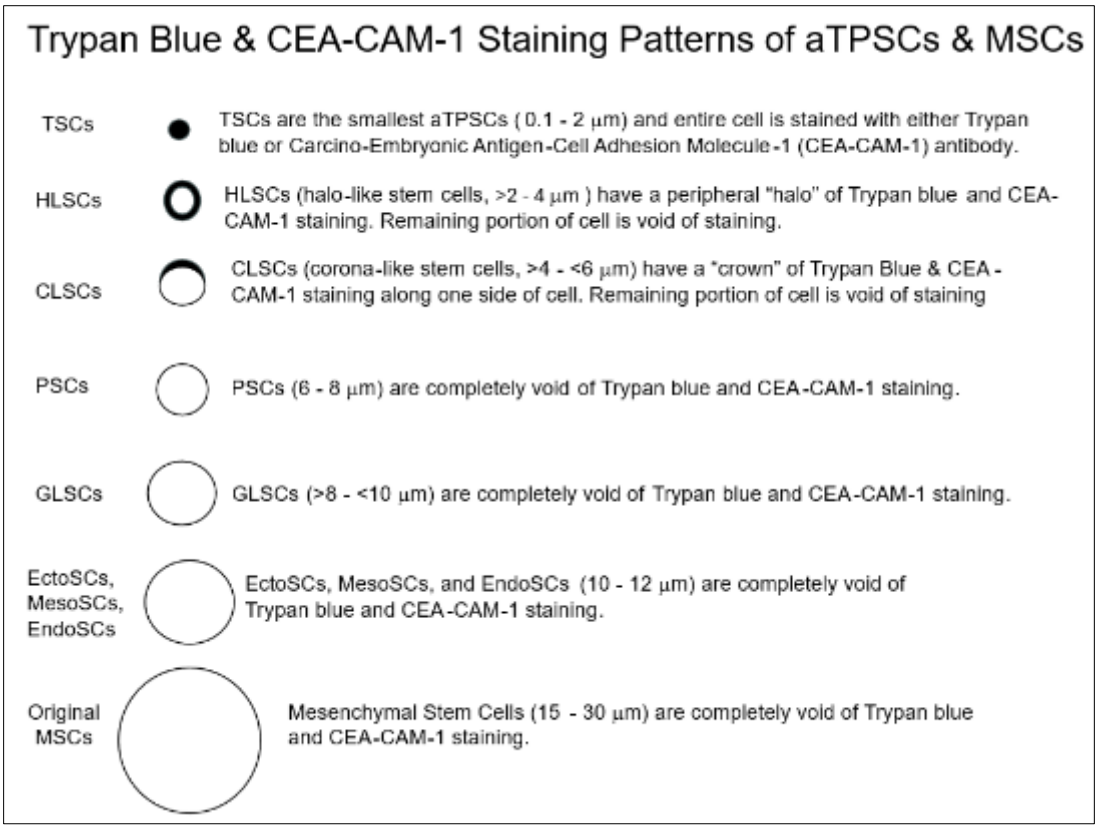

Figure 1 Diagrammatic representation of size comparison and visual staining patterns viewed by brightfield microscopy of aTPSCs (e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, and EndoSCs) and Progenitor Mesenchymal Stem Cells (MSCs) when stained with either 0.4% Trypan Blue or Carcino-Embryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1) antibody [24]. Reprinted with permission from Young HE. Adult telomerase positive stem cells: introduction and location. GSC Advanced Research and Reviews. 2025; 25(02): 296-331.

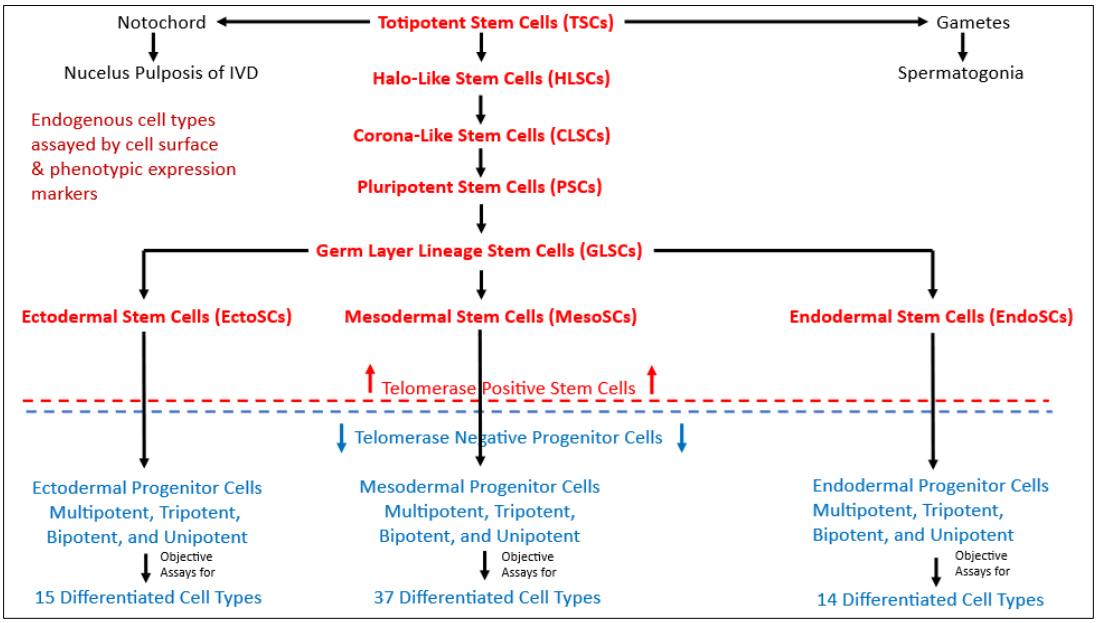

Figure 2 Identification of eight categories of aTPSCs and their unidirectional differentiation potentials into telomerase negative progenitor cells and telomerase negative differentiated cells using specific objective assays of cell surface markers and inherent phenotypic expression markers [13]. Reprinted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

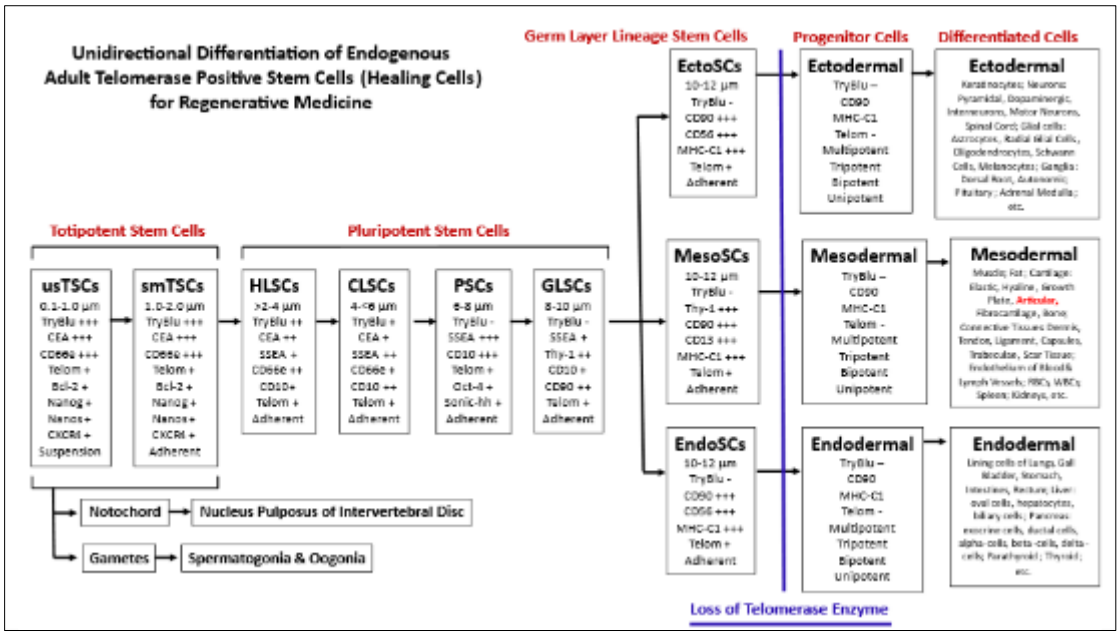

Figure 3 Diagrammatic representation of unidirectional transitioning of aTPSCs into maintenance/progenitor cells and subsequently into functional/differentiated cells with respect to size, Trypan blue staining, cell surface markers, expressed genes, and growth in culture [13]. Reprinted with permission of 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.

Healing stem cells were discovered in 1975 residing within the connective tissue stroma of adult terrestrial salamanders undergoing complete limb regeneration [25,26]. Healing stem cells were shown to form all damaged or lost tissues of an amputated limb, thereby restoring function to the limb [27-30]. Since 1975, healing stem cells have been extensively characterized utilizing multiple parameters [13]. They are composed of telomerase positive 0.1-2-micron cells totipotent stem cells (TSCs), >2.0 - <10.0-micron pluripotent stem cells (HLSCs, CLSCs, PSCs, and GLSCs), and 10-12-micron germ layer lineage stem cells (EctoSCs, MesoSCs, and EndoSCs). The 0.1-2.0-micron TSCs and 6-8-micron PSCs are seen at the 8-cell stage embryo (Fig. 4), suggesting that they probably arise at the 4-cell stage embryo through asymmetrical division of the blastomeres [31,32].

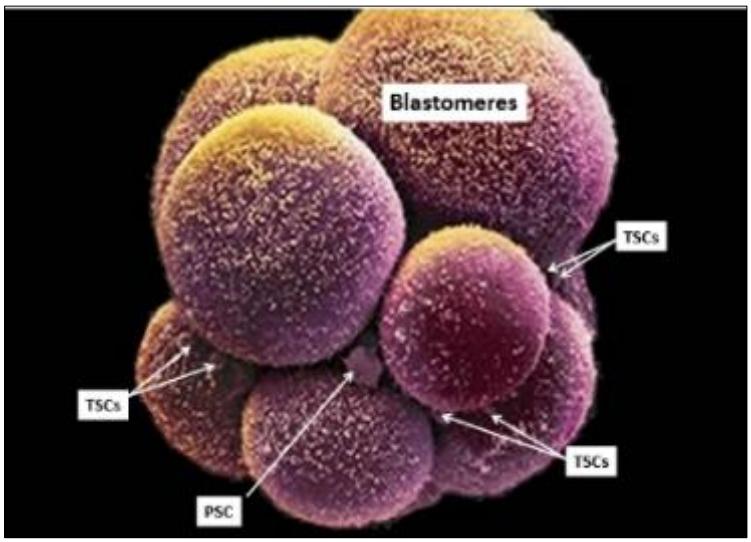

Figure 4 Scanning electron micrograph of a male embryo, somewhere between 8-cell stage and morula. Note presence of at least three pairs of totipotent stem cells (TSCs) and at least one pluripotent stem cell (PSC). Size approximation same as seen with flow cytometry (TSCs are 0.1 to 2 microns and PSC are 6 to 8 microns in size) [31]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol. 2014; 1(4): 00019

The aTPSCs, specifically PSCs and TSCs, and progenitor cell seen in a plated mixed cell population derived from fresh isolates using type-1 collagenase and dispase enzymatic digestion of solid tissues (Fig. 5).

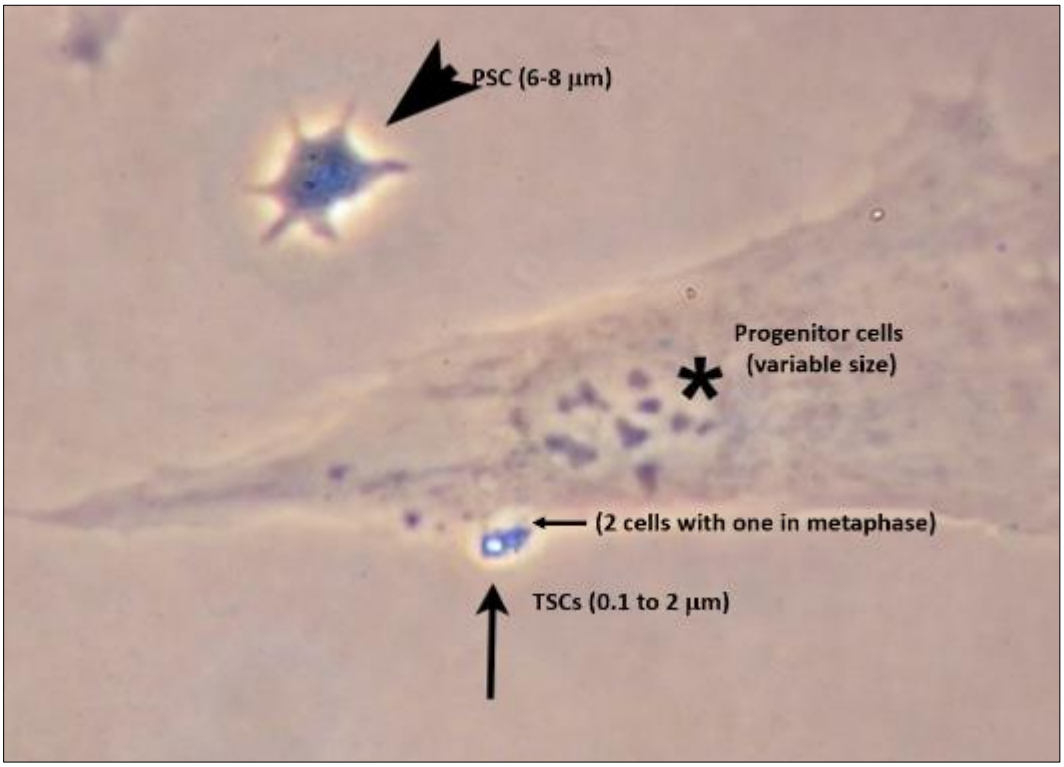

Figure 5 Fresh mixed isolate of aTPSCs and progenitor cells. Note presence of single PSC (large arrowhead), a portion of a progenitor cell (asterisk), and two TSCs (small arrow) in photograph. Of the two TSCs in the photograph one is in prophase (left) and the other (right) is in metaphase (see chromosomes along metaphase plate). Magnification of original photograph, 800x, electronically enlarged to 1600x [33]. 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. [Labelling differences between original paper and above Figure 5. PSCs = ELSCs and TSCs = BLSCs]

Table 2 Comparison / Contrast of Functional Cells, Maintenance Cells, and Healing Cells

Attributes	**Differentiated Cells**	**Maintenance Cells**	**Healing Cells**	**Healing Cells**	**Healing Cells**
			EctoSCs, MesoSCs, EndoSCs	PSCs	TSCs
% in adults	40	59	0.9	0.09	0.01
Telomerase Enzyme	Absent	Absent	Present	Present	Present
Location	Throughout the body	Adjacent to Differentiated Cells	Connective Tissues (CT) Niches	CT Niches	CT Niches
Age Range	Newborn to Geriatric	Newborn to Geriatric	Newborn to Geriatric	Embryonic to Geriatric	Embryonic to Geriatric
Numbers with Aging	Decline with age	Decline with age	Remain Constant	Remain Constant	Remain Constant
Native Naïve State	NA	Quiescent	Quiescent	Quiescent	Quiescent
Teratoma Formation	Absent	Absent	Absent	Absent	Absent
Growth In vitro	Single Layer Confluent	Single Layer Confluent	Single Layer Confluent	Multi-Layered Confluent	Multi-Layered Confluent
Respond to Inhibitory Factors	Yes	Yes	Yes	Yes	Yes
Respond to Proliferative Factors	Yes	Yes	Yes	Yes	Yes
Proliferation Potential	50-70 PDs in Humans 6-8 PDs Rodents	50-70 PDs in Humans 6-8 PDs Rodents	Unlimited Humans & Rodents	Unlimited Humans & Rodents	Unlimited Humans & Rodents
Respond to Progression Factors	NA	Yes	No	No	No
Respond to Inductive Factors	No	Only in Committed Lineage	Only in Committed Lineages	Yes	Yes
Cell Types Formed	NA	Only in Committed Lineage	Ectodermal, Mesodermal, Endodermal	All Somatic Cells	All Somatic Cells, NP of IVD, Gametes, Placenta

Time Period Fresh Isolate to In Vivo Use	4 hours	4 hours	4 Hours	4 hours	4 hours
Time Period Fresh Isolate to Ex Vivo Use	1-24 hours	6-8 hours	24 hours	24 hours	24 hours
Treatment Number Potential	Thousands	Millions	Billions	Billions to Trillions	Billions to Trillions
Express Self-Recognition Molecules	Yes	Yes	Yes	No	No
Immuno-Protected	No	No	No	Yes	Yes
Autologous Treatments	Yes	Yes	Yes	Yes	Yes
Allogeneic Treatments	No Induces GvHD[1]	No Induces GvHD	No Induces GvHD	Yes	Yes

Table 2. Attributes of functional differentiated cells, maintenance progenitor cells, and healing stem cells. [1]GvHD, Graft versus host disease. If recipient has competent immune system, donor cells will be destroyed. If recipient has compromised immune system, donor cells will try to kill the donor [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.

Due to their limited number in the body and their uniqueness in not conforming to standard tissue culture practices [34], special methodologies were developed to examine adult telomerase positive stem cells both in vitro and for their use in vivo. Previous reports have been published demonstrating their in vitro characterization [13,23,35] and in vivo use in both pre-clinical IACUC-approved animal models [35-40] and in IRB-approved compassionate use human clinical diseases [36,37,40-55]. This begins a series of articles, explaining in step-by-step detail, the methodologies (e.g., rationale, standardization, reagents, manufacturers, catalog numbers, step-by-step instructions, and examples of expected results via text, tables, and figures) that were developed for in vitro characterization of aTPSCs and their use in vivo. The current report describes the methodologies developed to optimize the viability of the aTPSCs during their isolation, plating, and propagation for in vitro analyses.

The hypothesis tested was "methodologies could be developed to isolate, plate, and propagate adult telomerase positive stem cells".

2. Materials and methods

2.1. Reagents: [13,23]

2.1.1. Species-specific buffers:

- Amphibians & Reptiles – 10% Holtfreter's solution [46]
- Amphibians & Reptiles – Ca+2, Mg+2-free 10% Holtfreter's solution [46]
- Avians – Tyrode's balanced salt solution, #T-2145 (Sigma)
- Avian Ca+2, Mg+2-free – Tyrode's balanced salt solution, #T-2145 (Sigma)
- Non-human mammals – Phosphate Buffered Saline, PBS (Sigma)
- Non-human mammals – Ca+2, Mg+2-free Phosphate Buffered Saline, PBS (Sigma)
- Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)
- Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)

2.1.2. Enzymes

- Trypsin, Sigma
- Collagenase type-1, Worthington Biochemicals
- Dispase, R&D Laboratories

2.1.3. ELICA Fixative Reagents

- Species-Specific Buffers
- Paraformaldehyde, #P6146, Sigma
- Glutaraldehyde, #G5882, Sigma
- Sodium Azide, #S2002, Sigma
- D-Glucose, # G-6138, Sigma

2.1.4. Inhibition and Exhaustion of Endogenous Peroxidases

- 5% Sodium Azide, #S2002 Sigma
- 30% Hydrogen Peroxide, #H-1009, Sigma

2.1.5. Blocking Agents for ELICA

- Horse serum, #H7889, Sigma
- Goat serum, #200-6210-AG, DSHB
- Porcine Serum, #P9783 Sigma
- Bovine serum albumin, #A-7906, Sigma
- Neonate Bovine Serum, #2002, BioCell

- Fetal Calf Serum, #F0392, Sigma
- Human Serum, #H4522, Sigma
- Tween 20 (polyoxyethylenesorbitan), ChemPure, Curtain Matheson Scientific, Houston, TX
- Nonidet P-40, P-40, #56007, BDH
- Triton-X-100, #T-6878 (Sigma)
- Powdered non-fat dry milk, Kroger's
- Gelatin (cold water fish soluble collagen), #935425, Sigma

2.1.6. Positive Standards

- Calf thymus DNA type-I, #D-1501, Sigma
- Myosin from chicken muscle, #M-7266, Sigma
- Rabbit anti-myosin (skeletal-smooth), #M-7648, Sigma

2.1.7. Primary Probes

- CEA-CAM-1, DH (D. Hixson, Providence, RI)
- MC-813 (SSEA-4), DSHB
- Thy-1 (CD90), DSHB
- CEA, #111518, Sigma
- CD66e, BD (Beckton-Dickinson)
- MC-631 (SSEA-3), DSHB
- CD10, BD
- CD90, BD
- Thy-1, DSHB
- IA4, R&D Systems

2.1.8. Secondary Probes

- Goat anti-rat osteocalcin, #BT-413, Biomedical Technology
- Biotin, goat anti-mouse IgG, #OB1126-21, Fisher
- Biotin, goat anti-mouse IgG (fab specific), #B-7151, Sigma
- Biotin, goat anti-rabbit IgG, #OB1316-21, Sigma
- Biotin-SP, goat anti-mouse IgG, #JGM-065003, Accurate
- Biotin-SP, goat anti-mouse fab-specific, #JGM-066003, Accurate
- Biotinylated affinity purified, rat adsorbed anti-mouse IgG (H + L) (BA-2001, Vector Laboratories)

2.1.9. Tertiary Probes

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

2.1.10. Reagents to Quantify or Visualize Tertiary Probes

- ABTS kit, #506201, Kirkegaard & Perry
- 4-Cloro-1-napthol, #C-8890, Sigma
- 3,5-Diaminobenzoic acid dihydrochloride, #11-383-2, Aldrich
- Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA

2.1.11. Histochemical Stains

- Von Kossa, Chroma-Gesellschaft
- AlkPhos, Sigma
- Mallory Heidenhain One-Step reagents, Chroma-Gesellschaft
- Alcian blue, Chroma-Gesellschaft, pH 1.0 and pH 2.5
- Alcec blue, Chroma-Gesellschaft, pH 1.0 and pH 2.5
- Safranin-O, Chroma-Gesellschaft, pH 1.0 and pH 2.5
- Sudan Black-B, 199664, Sigma

- Oil Red-O, 00625, Sigma

2.1.12. Verification of Histochemical Staining

- Toluene, removes hydrocarbons from fat cells, Sigma
- Hyaluronidase, Sigma
- Chondroitinase-AC, Sigma
- Chondroitinase-ABC, Sigma
- Keratanase, Sigma
- Heparanase, Sigma
- EDTA, divalent cations (Ca^{+2}, Mg^{+2}, Zn^{+2}, Ba^{+2}), Sigma
- EGTA, univalent cation (Ca^{+2} only), Sigma

2.1.13. Miscellaneous Supplies

- Parafilm, #13-374-12, Diagger
- Aqua-Mount (mounting coverslips), Vector Laboratories
- Ethanol, Sigma
- Isopropyl alcohol, Sigma
- Nitrile Gloves, Diagger
- 0.1-micron sterile bottle-top filter, Thermo-Fisher
- 50-ml polypropylene centrifuge tubes, Falcon, Thermo-Fisher
- 15-ml polypropylene centrifuge tubes, Falcon, Thermo-Fisher
- 2-ml polypropylene centrifuge tubes, Falcon, Thermo-Fisher
- Hemocytometer, Thermo-Fisher
- Dissection Microscope, Nikon
- Class-2 Biosafety Cabinet
- Eppendorf Refrigerated Centrifuge, 5810R
- -4oC Refrigerator
- 96-well plates, Costar
- Corning 6-well, 24-well, and 48-well plates, Corning
- T-25 and T-75 flasks, Falcon, Thermo-Fisher

2.1.14. Suppliers

- Sigma, Sigma Chemical Co., St Louis, MO
- Aldrich, Aldrich Chemical Co, Milwaukee, WI
- GIBCO, GIBCO, Grand Island, NY
- 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
- ICN, ICN Biomedicals, Costa Mesa, CA
- SBA, Southern Biotechnology Associates, Birmingham, AL
- Chem, Chemicon, El Segundo, CA
- BTH, Biomedical Technology Incorporated, Cambridge, MA
- Fisher, Fisher Scientific Co., Norcross, GA
- TF, Thermo-Fisher Scientific, Waltham, MA
- Accurate, Accurate Chemical & Scientific Corporation, Westbury, NY
- Vector, Vector Laboratories, Burlingame, CA
- KP, Kirkegaard & Perry Laboratories, Gaithersburg, MD
- BDH, BDH Chemicals Ltd, Poole, England
- Kroger's, Kroger's, Macon, GA
- DFRD, Dragonfly Foundation for Research and Development, Macon, GA
- ChemPure, Curtain Matheson Scientific, Houston, TX
- DH, Douglas Hixson, Department of Medicine, Brown University, Providence, RI
- Morton Thiokol, Morton International, Rohm and Haas, Philadelphia, PA
- Kodak, Kodak, Rochester, NY
- Aldrich, Aldrich Chemical Co., Sigma-Aldrich, St. Louis, MO
- Jackson, Jackson Laboratories, Bar Harbor, ME
- AirGas, AirGas, Local Sore

- Atlas, Atlas Biologicals, Fort Collins, CO
- Fisher-Biotech, Waltham, MA
- 96-well plate reader, R & D Systems, Minneapolis, MI
- Vortex Mixer, Thermo-Fisher, Waltham, MA
- Diagger Scientific, Vernon Hills, IL
- RB, Ray Biotech, Atlanta, GA
- R&D Systems, Minneapolis, MN
- Abcam, Cambridge, MA
- Prospec, Prospec, Rehovot, Israel
- Antibodies Inc, Davis, CA
- Chroma-Gesellschaft, Roboz Surgical Co, Washington, DC
- Developmental Studies Hybridoma Bank (DSHB)

2.2. Procedure - Rationale

2.2.1. Harvesting Cells from Solid Tissues

The established procedure for harvesting cells from solid tissues is to macerate the tissue and then treat with trypsin for 15 min at 37^{o}C to release the cells [34]. Trypsin cleaves proteins between adjacent lysine and arginine residues [56]. Therefore, trypsin will disrupt the core proteins of proteoglycans and glycoproteins within the extracellular matrix, thus releasing the cells. Unfortunately, aTPSC cell membranes of also contain adjacent lysine and arginine residues [22,56], and are disrupted causing loss of cell integrity and decreased viability of released cells. While release of aTPSCs with trypsin is possible, the resultant release of non-viable cells would not be good if one wanted to propagate viable cells.

Instead of using trypsin, we utilized enzymes that would disrupt their attachment substratum. The cells within an individual are attached to their extracellular matrices through glycoprotein moieties in their basement membranes [57-59]. The basement membrane is a non-cellular structure which contains type-4 collagen, heparan sulfate proteoglycan, laminin, fibronectin, etc. [22,59]. Basement membrane fibronectin connects to type-12 collagen which is the bridge molecule to type-1 collagen, the predominant extracellular matrix glycoprotein in connective tissues [22,59]. Instead of using trypsin, collagenase type-1 and dispase were used in combination to disrupt/disperse the extracellular matrix releasing the cells from their extracellular matrices resulting in increased numbers of viable cells released from the tissues [60-65].

2.2.2. Counting Cells

Cell counting occurred using sterile 0.4% Trypan blue in species-specific buffer [13,23]. The commercially available 0.4% Trypan blue is pre-made in either a standardized buffer or water. The standardized buffer may not be optimal due to non-species specificity, while using water and due to water's low osmotic pressure [64], will destroy a majority of the cells by lysis. It is far better to make sterile 0.4% Trypan blue in species-specific buffer, pH to 7.4, and store in an opaque/brown bottle at ambient temperature [65].

2.2.3. Electrostatic Charge on Surface Plastic of Culture Vessels

The aTPSCs have a high net negative charge, that increases in charge density as the cells become smaller, compare Fig. 6 A to C [66]. This is due to the overall number of charge entities per volume ratio. Plastic culture vessels have an inherent electrostatic charge, dependent on the plastic utilized [65]. The electrostatic charge can be either negative (D.), neutral (E.) or positive (F.). While the charge density may not seem important with respect to either progenitor cells or differentiated cells, it is very important with respect to the zeta potential of aTPSCs being plated. Net negative aTPSCs will not attach to any plastic with a net negative charge due to electrostatic repulsion (think magnet where two similar charge groups repel each other) and they float on the surface of the medium. In contrast, aTPSCs with a net negative electrostatic charge (zeta potential) will irreversibly attach to a plastic with a positive charge (think magnet where positive and negative adhere to each other) and they will permanently attach to the flask surface [65]. Using trypsin, other proteolytic enzymes, or scaping would only remove bits and pieces of the cells, but any resultant released "cells" would be non-viable. Therefore, it is imperative that aTPSCs are seeded onto plastic vessels having a neutral charge, in which, without a substratum (see below), they float just above the plate surface. In some respects, they appear to be attached, but simple movement of flask will dislodge the cells as they float just above the surface of the culture vessel. The culture vessels used with aTPSCs, circa. 1988-2008, were 96-well plates (Costar); 48-well plates, 24-well plates, and 6-well plates (Corning); and T-25 flasks and T-75 flasks (Falcon) [23]. It is unknown at this time whether the manufacturers have changed the composition of the plastics for culture vessel construction. However, the easiest way to test is to add a suspension of cells/medium to culture vessels from the same manufacturers. Allow to settle for 10

min. then look at culture. If the cells are floating at the surface of the medium, the floor of the plastic is composed predominantly of polypropylene, with a net negative electrostatic charge. If the cells are attached to the floor (with no amount of shaking the cells loose) the plastic is predominantly composed of polystyrene, with a net positive electrostatic charge. If the cells appear to float just above the vessel floor, and if gentle shaking of the flask dislodges the cells, the floor plastic is composed of a mixture of both polypropylene and polystyrene, having a net neutral electrostatic charge. The culture vessels with the neutral electrostatic charge are the culture vessels of choice for culturing aTPSCs.

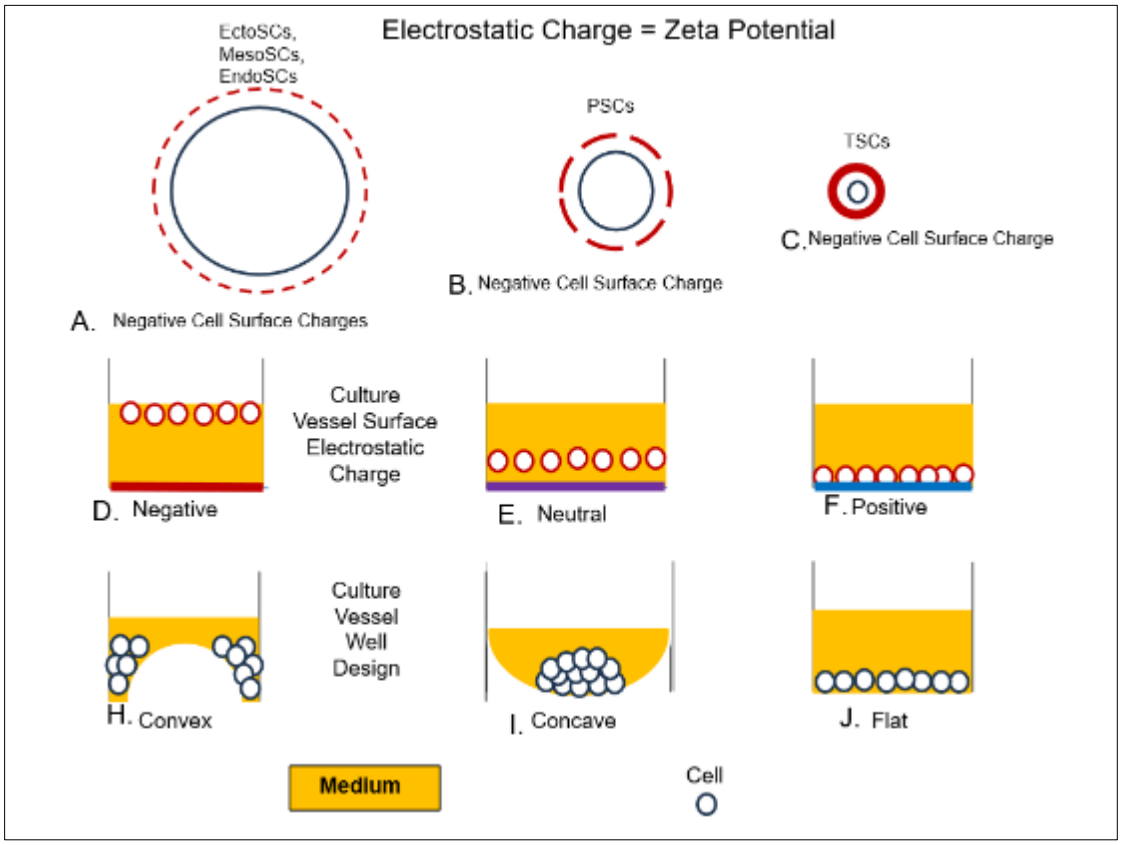

Figure 6 Electrostatic charge on aTPSCs demonstrating that as aTPSCs decrease in size, the net negative charge density increases (Fig. 5 A-C). Culture vessels are composed of either D. predominantly polypropylene plastic having a net negative charge; E. composed of both polypropylene- polystyrene mixture having a net neutral charge; or F. predominantly polystyrene with a net positive charge. Culture vessels, especially 96-well plates can have either a H. convex bottom; I. concave bottom; or J. a flat bottom

2.2.4. Substratum for Attachment

Unlike progenitor mesenchymal stem cells, other progenitor cells, or differentiated cells, the aTPSCs will not attach to bare plastic, even neutral bare plastic, because they do not synthesize and secrete their own substratum for attachment [65]. In contrast to not binding to bare plastic, aTPSCs will irreversibly bind to poly-L-lysine-coated glass or bare plastic culture vessels composed of polystyrene. This is due to the permanent positive electrostatic charge of poly-L-lysine. Therefore, a substratum needed to be provided for their attachment. As shown [24], aTPSCs reside within connective tissue niches within the body. Since the predominant extracellular matrix glycoprotein associated with aTPSCs within their connective tissue niche is type-1 collagen [22,39], it was theorized that the cells would prefer that as a substratum for their attachment [59,65].

To test that hypothesis, multiple percentages, e.g., 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5, and 10%, of type-I collagen solution were examined to prepare a suitable substratum for attachment. Percentages of collagen were added w/v dissolved in purified (HPLC) water. HPLC-grade water is superior to double-distilled water because of its higher purity and absence of organic residues, which helps maintain the stability of the collagen structure and cell compatibility. The aTPSCs preferred a solution of 1% collagen as a substratum for their attachment [65].

2.2.5. Preferred Medium for aTPSCs

While Eagle's Minimal Essential Medium with Earle's salts is the preferred medium for culturing animal cells [34], the included pH indicator is too concentrated to allow slight changes in lactic acid buildup to be detected visually. Therefore, we switched to OptiMEM + GlutaMax (GIBCO) as the base medium. It contains 1/10th the amount of pH indicator as Eagle's MEM, thus allowing visual inspection to approximate amount of lactic acid in the cultures based on the color of the medium before medium change [23,65].

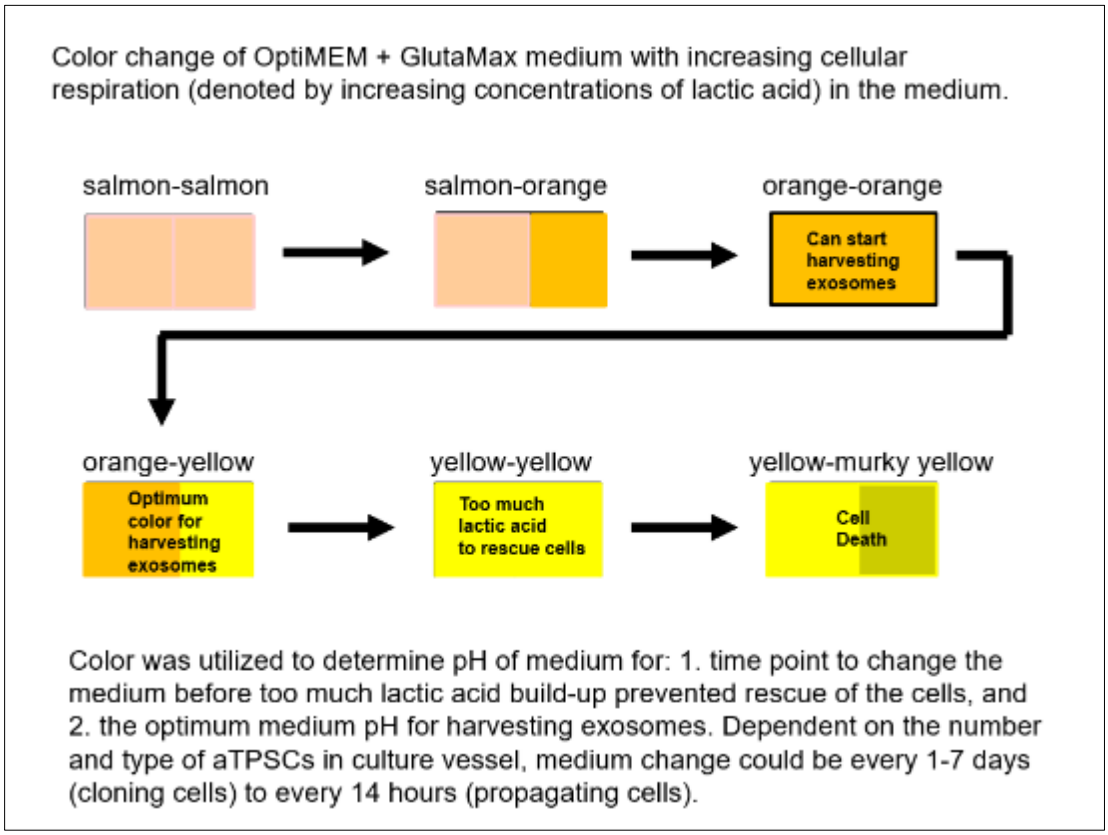

Figure 7 Colour change of plating medium, propagation medium, cloning medium, etc., allowed the opportunity to maintain optimal growth conditions by changing medium based on the "wants" of the cells. The sequential change of colour from salmon to yellow meant an increase in lactic acid build-up in the medium. Too much lactic acid in the medium, denoted by yellow-yellow of the medium, meant that the cells could no longer be rescued by adding fresh medium. Dead cells in the medium, denoted by yellow-murky yellow colour, meant mass die off of the cells

2.2.6. Anerobic Environment

The aTPSCs, specifically TSCs and PSCs, exist in a dormant, quiescent, hibernating anaerobic state [65], whereas GLSCs, EctoSCs, MesoSCs, and EndoSCs exist in a dormant, hibernating aerobic state [65]. To mimic the environment for characterization of TSCs and/or PSCs, beta-mercaptoethanol and/or putrescine was added to the culture medium to reduce oxygen tension for in vitro propagation studies [65,67]. However, if human TSCs or PSCs were to be utilized for human clinical treatments, they were grown within a BioSpherix unit where gases were regulated to simulate an anaerobic environment, e.g., oxygen 5%, carbon dioxide 5%, and nitrogen 90% [68].

2.2.7. Serum

Non-processed serum contains multiple and varied concentrations of biological agents, e.g., proliferation agents, progressive agents, inductive agents, and/or anti-differentiation agents, that can alter the outcome of any experiment [23,69,70]. To do any type of repetitive testing for biological activity, one needs to maintain the cells in their own undifferentiated state. To do this, one needs a serum that is either void of any biological agents or one where the activity of the biological agents within the serum have been destroyed. After screening over 400 lots of serum from bovine, goat, pig, horse, and human, and only finding one lot of horse serum (HS7) that was void of any biological agents, we determined that heat-inactivation proved to be the best low-cost method to achieve that goal of serum without any active biological agents. Brute force testing with regular serum from bovine, goat, pig, horse, and human, with and

without the addition of recombinant proliferative factors (e.g., PDGF-BB), progressive factors (e.g., insulin), inductive factors (e.g., TGF-beta, basic-FGF), and/or anti-differentiative factors (e.g., Leukemia Inhibitory Factor, LIF; Anti-Differentiative Factor, ADF), was examined through ranges of temperatures, times, centrifugation speeds, and filtration. Optimum parameters for heat-inactivation were 56^{o}C incubation for 8 hours, followed by high-speed centrifugation at 200,000 RCF (relative centrifugation force), followed by ultrafiltration using 0.1-micron positive pressure filtration. This heat inactivation procedure was found to be sufficient to destroy any of the aforementioned activities, e.g., inhibitory, proliferative, progressive, or inductive activities, within the serum. I gave the heat-inactivation procedure to Atlas Biologicals, Fort Collins, CO, for them to make for us. Atlas Biologicals also sells the heat inactivated serum commercially. Ten percent heat-inactivated serum (Atlas Biologicals) v/v culture medium was used for the studies reported herein for mammalian cells [23,24,35-39,65,67,69].

2.2.8. Doubling Rate, Propagation, and Potential for Mutations

The majority of the naturally-occurring adult cells used for testing, e.g., progenitor cells and differentiated cells, have a doubling rate from days to weeks to months, depending on the particular cell type examined [71,72]. Telomerase positive TSCs require 12-14 hours to double in number; HLSCs and CLSCS require 13-15 hours; PSCs require 14-16 hours; GLSCs require 16-18 hours; EctoSCs, MesoSCs, and EndoSCs require 18-24 hours; while telomerase negative MSCs require days to double in number (Table 1) [13]. The time period for a cell to double in number is inherent to the length of time it takes the cell to progress through its cell cycle + $\geq$ 2 hours [65]. During this time period the newly formed DNA strands are checked and rechecked for aberrant DNA match(es) that can lead to mutations [73-75]. Also within the doubling time frame is the amount of time necessary to replicate the cellular organelles, e.g., nuclear membrane, plasma membrane, and cytosolic organelles, such as additional mitochondria, free ribosomes, endoplasmic reticulum (SER and RER), golgi, centrosome, microtubules, etc. prior to mitotic division [22,56]. Therefore, with respect to cell propagation, it is imperative that the time for cells to double in number not be any shorter than their respective doubling rate + $\geq$ 2 hours. If the propagation rate is shorter than the doublings rate, mutations will occur, usually every 10^9 cell doublings [65].

2.2.9. Cell Release Protocol from Tissue Culture Vessel

Release of telomerase negative progenitor cells or differentiated cells from a culture vessel is usually performed using scraping cells from the surface of the vessels and/or enzymatic digestion with trypsin [34]. Scraping lyses cell membranes, leaving a majority of the cells either dead and/or dying. In addition, trypsin cleaves proteins associated with paired lysine/arginine amino acids [56]. Plasma cell membranes of aTPSCs contain a plethora of paired lysine/arginine amino acids. Thus, as trypsin degrades the plasma membranes of aTPSCs to get the cells released from the surface of the vessel, a majority of the cells are either dead and/or dying after trypsin digest. This was tested by two different approaches. First, by culturing the released "cells" from a trypsin digest in complete medium with a proliferation agent, 2-ng/ml PDGF-BB [13,76,78]. Within a six-day time period with two fresh medium changes, no observable proliferation had occurred. Second, we tested the established trypsin release procedure with plated human aTPSCs. The human cells that had not lysed showed apoptotic figures and stained with the CD95, the cluster of differentiation marker for cell death [77].

Telomerase positive stem cells prefer a type-1 collagen substratum for cell attachment and growth, utilizing two binding sites, a calcium-dependent binding site [79] and an RGD-fibronectin to basement membrane dependent binding site [80]. This knowledge was used to design a cell releasing protocol for the TPSCs from the plate surface.

2.2.10. Plating Cells

The mixed population of cells, e.g., aTPSCs, progenitor cells, and differentiated cells, were plated onto various sized 1% collagen-coated tissue culture vessels (Fig. 8) [13,23]. The number of cells plated, as number of cells per ml of plating medium, was dependent on the surface area of the vessel utilized.

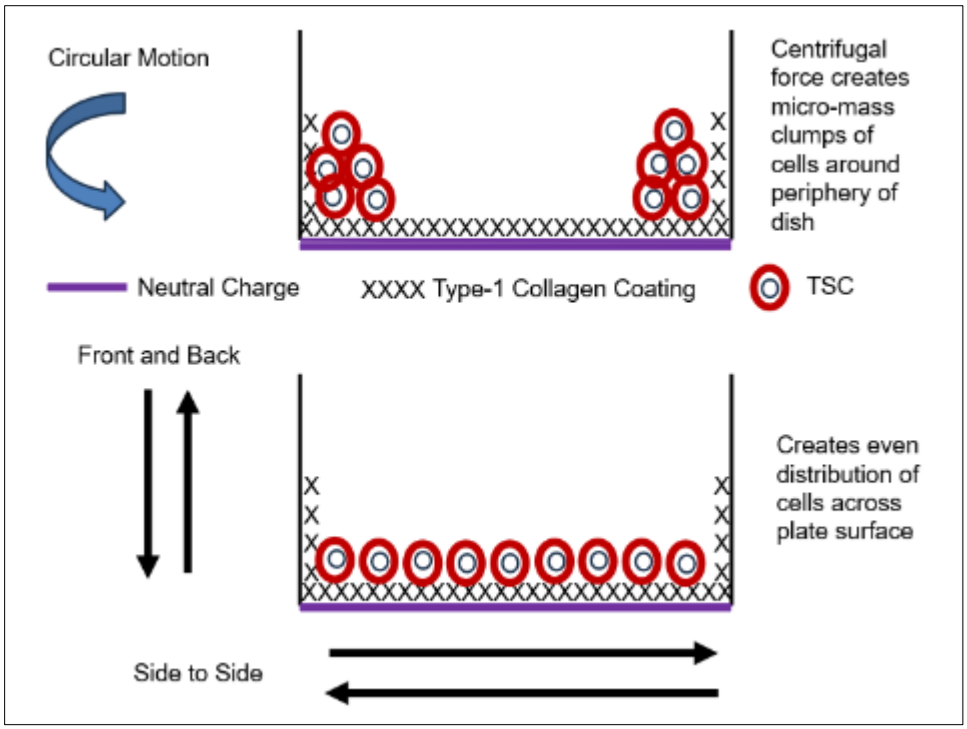

Figure 8 When plating aTPSCs always perform front to back and side to side movements of culture vessel to obtain an even coating of cells across plate surface. Due to centrifugal force, a circular motion will create micro-mass clumps of cells around the periphery of the flask which will alter the final results of the experiment

2.2.11. Propagation

Cultures were fed whenever the color of the medium changed from salmon-colored to orange-yellow in color (Fig. 7) [65]. To maintain a standardized propagation time (doubling time + ≥ 2 hours), 2-ng per ml platelet-derived growth factor-BB was added to the in-house medium [76].

3. Procedures - methodologies

3.1. 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 [83].

3.2. Tissue Harvest from Skeletal Muscle

Utilizing sterile procedures with universal precautions, postnatal male and female Sprague-Dawley rats (200-300 g) were euthanized using carbon dioxide inhalation [81,82]. The rats were soaked with 70% ethanol and transferred to a Class-2 Biosafety Cabinet. The hind limbs were washed with a Betadine solution, a circular incision made around the juncture of the thigh to the trunk of the body, and the skin pulled down over the foot. The bellies of the anterior compartment leg muscles, e.g., tibialis anterior, extensor digitorum longus; lateral compartment muscles, e.g., fibularis longus and brevis; and posterior compartment muscles, e.g., soleus, gastrocnemius, tibialis posterior, and flexor digitorum longus, were separated from their tendon attachment sites [83]. The removed skeletal muscle bellies were pooled in species-specific buffer in sterile pre-tared 50-ml polypropylene centrifuge tube and weighed. The skeletal muscle in species-specific buffer was then aliquoted into glass petri dishes, no more than 5-g per dish, and minced to the consistency of orange marmalade using sterile watchmaker's forceps with a pre-sterilized (wiping down outside surfaces with 70% ethanol) stereo-dissection microscope [65]. The resultant minced tissue was returned to sterile 50-ml polypropylene centrifuge tubes and enzymatically digested with collagenase/dispase [78]. The enzymatic solution consisted of 250 units per ml of type-I collagenase, 33.3 units per ml of dispase, 1% antibiotic-antimycotic, in calcium^{+2}-free, magnesium^{+2}-free Opti-MEM + GlutaMax, pH 7.4 [64]. 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°C. After 18 hours of digestion the tubes were removed from the incubator/shaker, the outside of the tubes 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 pelleted cells were re-suspended by agitation and trituration. The dispersed cell pellets were reconstituted with 2-ml of basal medium. The basal medium consisted of Opti-MEM + GlutaMax, 10% Heat Inactivated Serum (Atlas Biologicals), 1% antibiotic-antimycotic (Sigma), pH 7.4. Ten ng/ml PDGF-BB (R&D Systems) was added to the basal medium to form the propagation+ medium [13,23]. Cell counts were performed as described below.

3.3. 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). Fifteen microliters of sterile 0.4% Trypan Blue solution (filter-sterilized) 0.4 g Trypan blue (Kodak) in sterile PBS 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, small Trypan blue-positive/negative cells, and Trypan blue negative cells (Fig. 1) 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. The isolated cells were counted on a hemocytometer by four separate individuals, the counts averaged and standardized per gram of tissue. The final cell number calculations were based on number of cells per gram of tissue.

The 0.4% Trypan blue that is commercially available is pre-made in water, which lyse a majority of the cells, due to water's low osmotic pressure. For accurate cell counting it is far better to make sterile 0.4% Trypan blue in species-specific buffer, pH to 7.4, and store in an opaque/brown bottle at ambient temperature [65].

The species-specific buffer of choice for amphibians and reptiles is 10% Holtfreter's solution [84,85]; for avians, it is Tyrode's buffer, pH 7.4 [60-61]; for non-humans it is Phosphate Buffered Saline [62]; and for humans, it is Dulbecco's Phosphate Buffered Saline, Ph 7.4 [63]. Sterilized 0.4% Trypan Blue is made by weighing out 0.4 grams of dry Trypan Blue powder, adding to 100-ml of species-specific buffer of choice, and sterilized by filtering through a 0.1-micron bottle top filter (Thermo-Fisher). Sterilized Trypan blue staining solution should only be used in a sterile hood, and never outside the hood on the countertop. Otherwise, the possibility of bacterial contamination is very high, which can lead erroneous results with respect to the number of viable cells obtained [65].

3.4. Electrostatic Charge of Surface Plastic of Culture Vessels

The reason for the differences with respect to culture vessels from different manufacturers is that the manufacturers used plastics with different electrostatic charges for their various culture vessels. The plastic culture vessels examined were composed of plastic with a surface electrostatic charge of either negative, neutral, or positive (Fig. 6) [65]. Initially, we attempted to obtain the plastic electrostatic charge information from technical representatives from the various companies. Most either had no clue what we were asking or would not tell us the information, citing proprietary information that could not be divulged. Therefore, this side study was obtained through brute force testing. After testing various sized culture vessels, from a plethora of companies, the particular culture vessels chosen for use with aTPSCs, circa 1988-2008, were 96-well plates (Costar), 48-well plates (Corning, Corning, NY), 24-well plates (Corning), 6-well plates (Corning), T-25 flasks (Falcon, Thermo-Fisher Scientific), and T-75 flasks (Falcon) [13,23,65]. Knowledge of whether the plastic composition for these plates has changed is currently unknown. But given the above trouble-shooting notes, it is relatively easy to ascertain.

3.5. Substratum for Attachment

The original procedure for making the various percent concentrations of type-1 collagen for testing followed established protocols, e.g., measure designated gram quantities of type-1 collagen peptides (Sigma-Aldrich Chemical), was to add dry material w/v to 100-ml sterile reverse osmosis double-distilled water, stir contents for 48 hours, then sterile-filter through a 0.1-micron filter. 96-well plates were coated with resultant percentages of type-1 collagen, and allowed to dry for 24-hours. Cells suspended in plating medium at 10^3 cells/well were plated into each well containing the percentage collagen coating solutions, e.g., 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5, and 10%, with n=12 per percent coating. Cells were allowed to attach for 24 hours, and medium removed. The DNA content of each well, noting number of cells attached, was measured using the ELICA procedure [23].

For our studies, a less arduous and less time-consuming procedure for 1% collagen coating was developed. Add 1-g of type-1 collagen peptides to w/v to 100-ml HPLC water, but don't stir. Autoclave the solution on the liquid cycle for 15 min. Remove from the autoclave and let solution in bottle return to ambient temperature (22-39^{o}C, warm to the touch) on the countertop. The 1% collagen solution should be water crystal clear. If one tries to hasten the cooling process with refrigeration or an ice bath, the collagen peptides will precipitate out of solution (become cloudy) and the cells will not attach to the resultant substratum [65,76].

The culture vessels were coated with the 1% collagen solution at regular intervals at the same time and then stored in a closed cabinet at ambient temperature for future use. In a HEPA-filtered Class-2 Biosafety Cabinet, pipet a volume of 1% collagen solution that is ½ the maximum medium feeding volume for each vessel. For example, maximum feeding volume in each well of a 96-well plate is 200-microliters. Therefore, pipet 100-microliters of 1% collagen solution into each well. Place lids/caps on the vessels. Move vessels back and forth and side to side, the same as when plating cells, providing for an even distribution of the collagen solution across the vessel surface (Fig. 7). Allow it to sit undisturbed in the Biosafety Cabinet for a minimum of 30-min with UV-light turned on. Turn off UV light and aspirate residual 1% collagen solution into bleach using a sterile 9-inch glass pipet attached to a vacuum apparatus. Cover vessels with lids/caps and store in a cabinet at ambient temperature. Shelf life for 1% collagen coated culture vessels is approximately one year. However, due to the number of experiments that were performed, collagen coating usually occurred every month, depending on the vessels utilized. Usually, a case of each vessel was coated at a time. Multiple 100-ml batches of 1% collagen solution for coating flasks were generated, based on the number of vessels being coated and the volume of the solution to be used [63]. Any leftover sterile 1% collagen solution was saved at ambient temperature (never refrigerated), and used inside a sterile class-2 hood to negate collagenase/dispase enzymatic digestion of substrate to release the cells from tissues or culture vessels [13,23,65,76].

3.6. Preferred Medium for aTPSCs

Culture medium of choice for aTPSCs is OptiMEM + GlutaMax (GIBCO). When cells are cultured for in vitro testing using the ELICA procedures, antibiotic/antimycotic, e.g., penicillin, streptomycin, and fungizone, are added to maintain sterility in the cultures [65]. When cells were propagated for in vivo testing, antibiotic/antimycotic was withheld from the medium.

3.7. Anerobic Environment

Beta-mercaptoethanol was added to culture medium of propagating mammalian cells (TSCs, HLSCs, CLSCs, and PSCs) destined for characterization to reduce oxygen tension, e.g., 5% O2, 5% CO2, 90% N2, then pH to 7.4. In contrast, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs destined for characterization with biological agents (inhibition, proliferation, progression, and induction) preferred an ambient oxygenated environment (21% O2, 5% CO2, 74% N2), then pH to 7.4 [13,23,65]. If human cells are cultured for use in clinical studies/trials, the medium must be absent of any antibiotic/antimycotic, fungizone, beta-mercaptoethanol, and/or putrescine. Therefore, a sterile containment unit, BioSpherix, was utilized [68]. This unit contains a HEPA-filtered Class-2 hood for ingress and egress of supplies, multiple sterile chambers for culturing, cell processing, centrifugation, and a computer system that monitors and regulates CO_2, O_2, N_2, floor temperature, chamber temperatures, particle counts, volatiles, etc., in the various chambers.

3.8. Serum

Depending on the particular lot of serum that was used, there were differences within the cells with respect to resultant phenotypic expression markers. Most of the sera contained biological agents that stimulated the formation of fibroblasts in the TSC and PSC cultures [67]. And these particular serum (or recombinant protein) fibroblastic agents induced the TSC, PSC, and MesoSC cultures to form an ECM reminiscent of scar fibroblasts/fibrocytes [31,65]. When recombinant proteins were tested individually, it was noted that both TGF-beta and basic-FGF would induce similar scar fibroblastic phenotypes and scar-ECMs. TGF-beta and basic-FGF are two of the most prominent components in platelets, as assessed by SDS-PAGE of 46 bands of proteins from lysed platelets seen with Coomassie blue staining, transferred to nylon membranes, and probed with antibodies to inductive agents (data not shown) [66]. It is conceivable that platelets were lysed during the commercial collection of serum releasing these components into the serum.

To do any type of repetitive testing for biological activity, one needs to maintain the cells in their own undifferentiated state. Initially, when single cell repetitive serial dilution cloning was performed in chicken cells, over 400 lots of serum from bovine, calf, horse, pig, and human were screened. Only a single lot of serum (HS7, horse serum, 7th lot tested) was discovered to be void of any of the four biological agents affecting the cells, e.g., inhibition, proliferation, progression, or induction [13]. The entire lot was purchased and stored at -80^{o}C for testing. Horse serum HS7 was used for cloning avian cells and initial testing of human recombinant proteins for biological activity [13,23,38,65,67,69,70]. For cloning mouse

stem cells, a heat inactivated serum procedure was devised to negate the potential biological activities of factors inhibiting differentiation, proliferation, progression, and induction [69]. The process for heat inactivation was to incubate any serum from bovine, fetal calf, equine, porcine or human origin, at 56°C for 8 hours, high speed centrifugation at 200,000 RCF to remove precipitate, and then positive pressure filtration through a 0.1-micron filter to sterilize [13,23,65]. For cloning rat cells and culturing human cells for study, the heat inactivation technique was given to Atlas Biologicals, in Fort Collins, CO to generate the heat inactivated serum [13], which was/is commercially available. Ten percent Heat Inactivated serum (Atlas Biologicals) was added to the cultures to maintain growth of the cells [67].

3.9. Doubling Rate and Potential for Mutations

The doubling rate for telomerase positive TSCs require 12-14 hours; HLSCs and CLSCs require 13-15 hours; PSCs require 14-16 hours; GLSCs require 16-18 hours; EctoSCs, MesoSCs, and EndoSCs require 18-24 hours; while telomerase negative MSCs require days to double in number (Table 1) [13]. We titrated platelet-derived growth factor-BB (PDGF-BB, R&D Systems) to its physiological concentration (2-ng/ml) in basal medium and designated it as propagation+ medium to maintain established proliferation rates (doubling rate + $\geq$ 2-hours) for propagation of the cells without mutations [65,76].

3.10. Cell Release from Tissue Culture Vessel

The aTPSC release from culture vessels occurs sequentially in two phases. The first phase entailed incubation with EGTA (ethylene-glycol-tetraacetic acid, a specific calcium chelator, Sigma) dissolved in an electrolyte-free (calcium, magnesium, potassium, and sodium) buffer of choice [86]. This disrupted the calcium-dependent attachment to its surrounding ECM, allowing the cells to round up with minimal attachment sites to the substratum. The second phase consisted of 250-units of type-1 collagenase and 33.3-units of dispase suspended in electrolyte-free EGTA buffer of choice. The protocol for release from the surrounding ECM is to remove culture medium from the culture vessel; rinse cultures twice with species-specific buffer to remove any residual material; aspirate wash solution into bleach; add electrolyte-free EGTA-buffer to vessel at ½ volume of feeding medium. Look through a phase contrast microscope as cells round up as they are released from their calcium dependent binding sites. Provide a mild rocking motion if required. This EGTA incubation process takes from 1-5 minutes and is dependent on the cell types released. The smaller the cell type, the lesser the number of calcium-dependent binding sites, the shorter the time frame for release from calcium-dependent sites, the quicker the rounding up of the cells. The electrolyte-free EGTA-buffer solution is removed and replaced with electrolyte-free EGTA buffer containing type-1 collagenase and dispase. This solution usually takes approximately 30-60 seconds for cells to detach completely from the culture vessel surface either as single cells or as sheets of cells. Triturate the solution to suspend released cells in enzyme solution and remove cell suspension from culture vessel into a 50-ml polypropylene centrifuge tube. The 1% type-1 collagen solution remaining from coating tissue culture vessels dilutes the cell suspension 14:1 and used to neutralize any further activities of the enzymes. To remove enzymes from cells, centrifuge the cell suspension to form a cell pellet. Decant supernatant into 10% sodium hypochlorite solution (bleach) and then using repeated trituration, resuspend cells in complete culture plating medium of choice. Preferential culture plating medium for TSCs and PSCs in mammalian species is Opti-MEM plus GlutaMAX (stabilized form of glutamine, GIBCO), 10% Heat Inactivated Serum, 1% antibiotic/antimycotic, 0.009% beta-mercaptoethanol, at pH 7.4.

Preferential culture plating medium for GLSCs, EctoSCs, MesoSCs, and EndoSCs in mammalian species is Opti-MEM plus GlutaMAX (stabilized form of glutamine, GIBCO), 10% Heat Inactivated Serum, 1% antibiotic/antimycotic, at pH 7.4.

3.11. Plating Cells

The mixed populations of cells, e.g., aTPSCs, progenitor cells, and differentiated cells, were plated onto various sized 1% collagen-coated tissue culture vessels [13,23], based on the number of cells per ml of basal medium. Plating density for each well of 96-well plates was 10^3 cells per 0.1-ml. Plating density for T-25 and T-75 flasks was 2x10^5 cells per ml, with 5-ml plated into T-25 flasks and 10-ml plated into T-75 flasks. When plating cells, swirling the culture vessels after cell/medium deposition will cause the cells to move to the periphery by centrifugal force, forming uneven plating of cells across vessel surface (Fig. 8). The swirling action predominantly forms micro-mass cultures of cells, which will lead to erroneous differentiation results [87-90]. Therefore, once cell suspension/medium was added to culture vessels the vessels were rocked side to side and back to front (Fig. 8). This allows for an even distribution of the cells across the vessel surface [13,23].

Twenty-four hours after plating, the plating medium was switched to propagation medium. Propagation medium is plating medium with the addition of 2-ng/ml PDGF-BB. Thereafter, propagation medium changes were based on the color change of the medium from salmon-colored to orange-yellow (Fig. 7). The time between medium changes ranged

from days to hours, dependent on the number of cells being propagated. When cultures reached confluence (telomerase negative differentiated cells, telomerase negative progenitor cells and/or telomerase positive germ layer lineage stem cells) or multi-layered post-confluence (telomerase positive pluripotent stem cells and telomerase positive totipotent stem cells), the cells were released and replated onto fresh 1% collagen-coated dishes.

3.12. Propagation of Cells

Regardless of cell type, e.g., telomerase negative differentiated cells, telomerase negative progenitor cells, or telomerase positive germ layer lineage stem cells, telomerase positive pluripotent stem cells, or telomerase positive totipotent stem cells, as long as the cultures were fed with fresh medium when medium color changed from salmon-color to orange-yellow, the cells would survive (Fig. 7) [65].

To maximize cell numbers and dependent on the cells being propagated, cells were released and replated into fresh 1% collagen-coated dishes in plating medium, then switched to propagation+ medium 24-hours after plating. For differentiated cells, progenitor cells and germ layer lineage stem cells, when cultures reached single-layer confluence, they were released and replated (Fig. 9). For pluripotent stem cells (Fig. 10) and totipotent cells (Fig. 11), cultures were allowed to propagate past well confluence (600-800%) before release and replating.

3.13. Plating

Plating followed by replating continued until sufficient numbers of cells were obtained for segregation into individual populations of differentiated cells, progenitor cells, and aTPSCs, by differential cryopreservation and cell sorting.

3.14. Release from Culture Vessel

First, the medium was decanted into bleach and the vessel was rinsed with a magnesium-free and calcium-free species-specific buffer to remove any unattached constituents. Next, initially we used ethylenediamine-tetraacetic acid (EDTA), but later switched to ethylene glycol-tetraacetic acid (EGTA) and dissolved in magnesium-free, calcium-free species-specific buffer. While EDTA will chelate calcium, along with any other divalent cation, e.g., magnesium (Mg^{+2}), manganese (Mn^{+2}), zinc (Zn^{+2}), copper (Cu^{+2}), iron (Fe^{+2}), or lead (Pb^{+2}), EGTA is specific solely for calcium. The calcium chelator in species-specific calcium-free buffer [86] was used to disrupt the calcium-dependent binding site. Flattened cells would round up with only a few attachments to the substratum. Incubation took anywhere from 1-5 minutes depending on number of cells in the culture vessel, and directly proportional to the amount of disrupting buffer utilized. This was followed with incubation in an enzymatic solution containing type-1 collagenase and dispase to disrupt the ECM and basement membrane attachment sites to free the cells from the plate surface [13,23,65].

4. Results

Table 3 Species, Age and Location of Adult Telomerase Positive Stem Cells

Ch[1]	**Sa[2]**	**Rep[3]**	**Av[4]**	**Mo[5]**	**Rt[6]**	**Rb[7]**	**Fe[8]**	**Cn[9]**	**Ov[10]**	**Cp[11]**	**Pr[12]**	**Bo[13]**	**SB[14]**	**Eq[15]**	**HM[16]**	**HF[17]**
Pre[18]			+[19]												+	+
Mor[20]															SEM[21]	
Psn[22]	+			+	+	+	+	+	+	+	+	+		+	+	+
Nb[23]				I[24]	I										+	+
Ad[25]				I	I										+	+
SexM[26]	H[27], Hc[28]			I	I										+	+
Ge[29]				I[30]										I[31]	I[32]	I[33]
SkM[34]	H, Hc		H, Hc, I	H, Hc, I, Cr[35]	H, Hc, I, Cr	H, Hc	I	I	I	I	I	H, Hc, I			H, Hc, I	H, Hc, I

Der[36]	H, Hc		I	I, Cr	I, Cr						I, Cr				I	I
Hrt[37]	H, Hc		I	I	I, Cr											
SmM[38]	H, Hc			I	I										+	+
Gt[39]					I										I	I
Bone[40]	H, HC		H, HC	H, HC	H, HC							H, HC				
Pos[41]	Hh		I	I	I, Cr						Cr				I	I
HyCart[42]	H, HC		H, HC	H, HC	H, HC							H, HC				
ElasCar[43]			H, HC	H, HC	H, HC							H, HC				
ArtCart[44]	H, HC		H, HC	H, HC	H, HC							H, HC				
FibCart[45]			H, HC	H, HC	H, HC							H, HC				
GPCart[46]				H, HC	H, HC											
Pch[47]	H, HC		I	I	I, Cr						Cr				I	I
Ns[48]	H, Hc		I	I	I, Cr						Cr				I	I
Adip[49]	H, Hc		I	I	I, Cr						Cr				I	I
Lig[50]	H, Hc		I	I	I, Cr						Cr				I	I
Ten[51]	H, Hc		I	I	I, Cr						Cr				I	I
BV[52]	H, Hc		I	I	I, Cr						Cr				I	I
BoM[53]	H, Hc		I	I	I, Cr						Cr				I	I
Bld[54] Pre-GC[55]															I	I
Bld Post-GC[56]		I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Tra[57]					I, Cr						Cr					
Lng[58]					I, Cr						Cr					
Eso[59]					I, Cr						Cr					
Stm[60]					I, Cr						Cr					
Liv[61]					I, Cr						Cr					
SmI[62]					I, Cr						Cr					
LgI[63]					I, Cr						Cr					
SpI[64]					I, Cr						Cr					
Brn[65]					I, Cr						Cr					
Men[66]					I, Cr						Cr					
SpC[67]											Cr					
Pan[68]					I, Cr						Cr				I	I
Kid[69]					I, Cr						Cr					

Ub[70]					I, Cr						Cr					
Thy[71]					I, Cr						Cr					
Tng[72]					I, Cr						Cr				Cr	Cr
Tes[73]					I, Vi[74]											
Ov[75]					Cr											
Ft[76]					Cr											
Kar[77]				Dip[78]	Dip						Dip			Dip	Dip	Dip

Table 3. Ch[1], Characteristics; Sa[2], adult terrestrial salamanders, *Ambystoma maculatum, Ambystoma annulatum, Ambystoma texanum, Ambystoma tigranum;* Rep[3], reptile, Komodo Dragon; Av[4], avians, *Gallus domesticatus*, Wedel Crane; Mo[5], mouse; Rt[6], rat: outbred *Sprague-Dawley* and inbred *Wistar Furth*; Rb[7], rabbit; Fe[8], feline (cat); Cn[9], canine (dog); Ov[10], ovine (sheep); Cp[11], caprine (goat); Pr[12], porcine (pig); Bo[13], bovine (cow); SB[14], Spectacled Bear; Eq[15], equine (horse); HM[16], human male; HF[17], human female; Pre[18], pre-natal; +[19], presence of TSCs and PSCs; Mor[20], morula; SEM[21], scanning electron microscopy of Day 8+ blastomeres; Psn[22], Post-natal (after birth); Nb[23], Newborn; I[24], Isolation; Ad[25], Adolescent; SexM[26], sexually mature; H[27], histology; Hc[28], histochemistry; Ge[32], geriatric; I[30], isolation from NIH's aging model, 36-month old CBF-1 mouse; I[31], isolated from a 40 year old horse; I[32], isolated from a 80 year old male with Parkinson's disease; I[33], isolated from a 98 year old female with Alzheimer's disease; SkM[34], Skeletal Muscle; Cr[35], cryosection followed by immunocytochemistry using antibodies to IA4 (smooth muscle, positive control), CEA-CAM-1 (TSC) and SSEA-4 (PSC); Der[36], dermis of skin ; Hrt[37], heart; SmM[38], smooth muscle; Gt[39], granulation tissue; Bone[40], bone; Pos[41], periosteum; HyCart[42], hyaline cartilage; ElasCart[43], elastic cartilage; ArtCart[44], articular cartilage; FibCart[45], fibrocartilage; GPCart[46], growth plate cartilage; Pch[47], perichondrium; Ns[48], nerve sheaths; Adip[49], adipose tissue (unilocular white fat); Lig[50], ligament; Ten[51], tendon; BV[52], arterial and venous blood vessels; BoM[53], bone marrow (hematopoietic cells and stromal cells); Bld[54], blood pre-GC[55], before glacial cap ingestion if individual had pre-existing morbidity(ies); Bld post-GC[56], Blood after glacial cap ingestion; Tra[57], trachea; Lng[58], lung; Eso[59], esophagus (lamina propria, submucosa, adventitia); Stm[60], stomach (submucosa, serosa); Liv[61], liver; SmI[62], small intestine (lamina propria, submucosa, serosa); LgI[63], large intestine (lamina propria, submucosa, serosa); Spl[64], spleen (capsule, trabeculae, interstitial tissue); Brn[65], brain (white mater, gray mater); Men[66], meninges (dura mater, arachnoid mater, pia mater); SpC[67], spinal cord (white mater, gray mater); Pan[68], pancreas (exocrine and endocrine portions); Kid[69], kidney (capsule, interstitium); Ub[70], urinary bladder; Thy[71], thyroid; Tng[72], tongue; Tes[73], testis; Vi[74], vibratome sectioning followed by immunocytochemistry; Ov[75], Ovary; FT[76], fallopian tube; Kar[77], karyotype; Dip[78], respective diploid number of chromosomes for each species examined [31]. Four additional species were added to the Table: Komodo Dragon, Wedel Crane, Spectacled Bear, and German Shepard, see reference [45]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014 [31]; 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 [45]**.**

4.1. Plating a Mixed Cell Isolate

The aTPSCs, specifically PSCs and TSCs, and a progenitor cell are seen in a plated mixed cell population derived from fresh isolates using type-1 collagenase/dispase enzymatic digestion of solid tissues, 24-hours after plating (Fig. 5). With time, the progenitor cells reach confluence and are overlain with PSCs and TSCs (Fig. 9). With continued time in culture, and due to the doubling rate of PSCs and TSCs, the progenitor cell cultures can become overrun with PSCs and TSCs (Fig. 10) to a point that even the PSCs can become overrun with TSCs (Fig. 11).

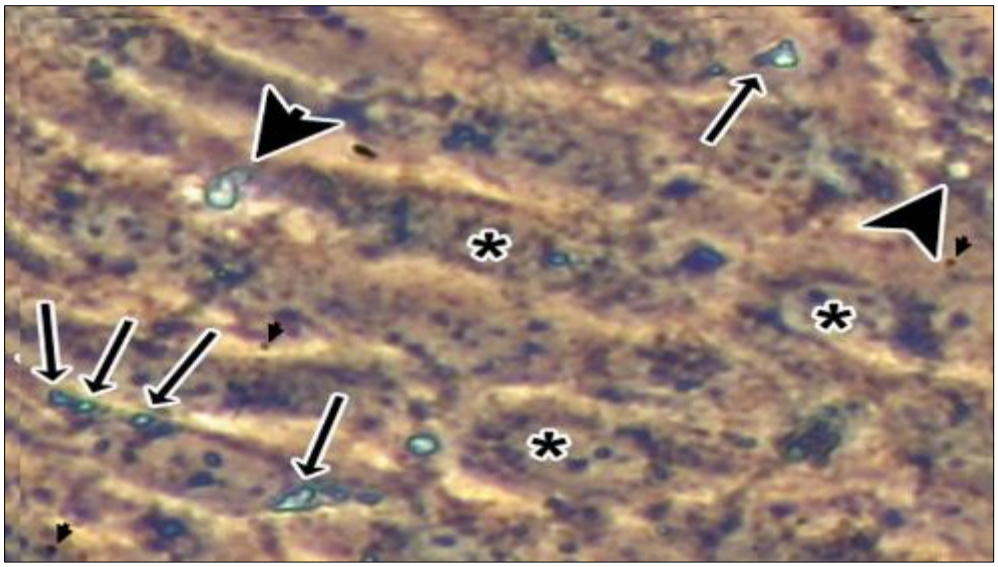

Figure 9 Single contact inhibited layer of progenitor cells (asterisk) overlain with pluripotent stem cells (PSCs, large arrowheads), halo-like stem cells (HLSCs, arrows), and TSCs (3 small arrowheads). Visible multiple cell types (contact inhibited progenitor cells, TSCs, HLSCs, and PSCs) suggest contaminating cells within a "pure" culture of contact inhibited progenitor cells [65]. Reprinted with permission from Young HE. Endogenous Adult Telomerase Positive Stem Cells and/or Combinatorial Nutraceuticals, 50 Years in the Making, 50 Years of Discovery; (In preparation*)*

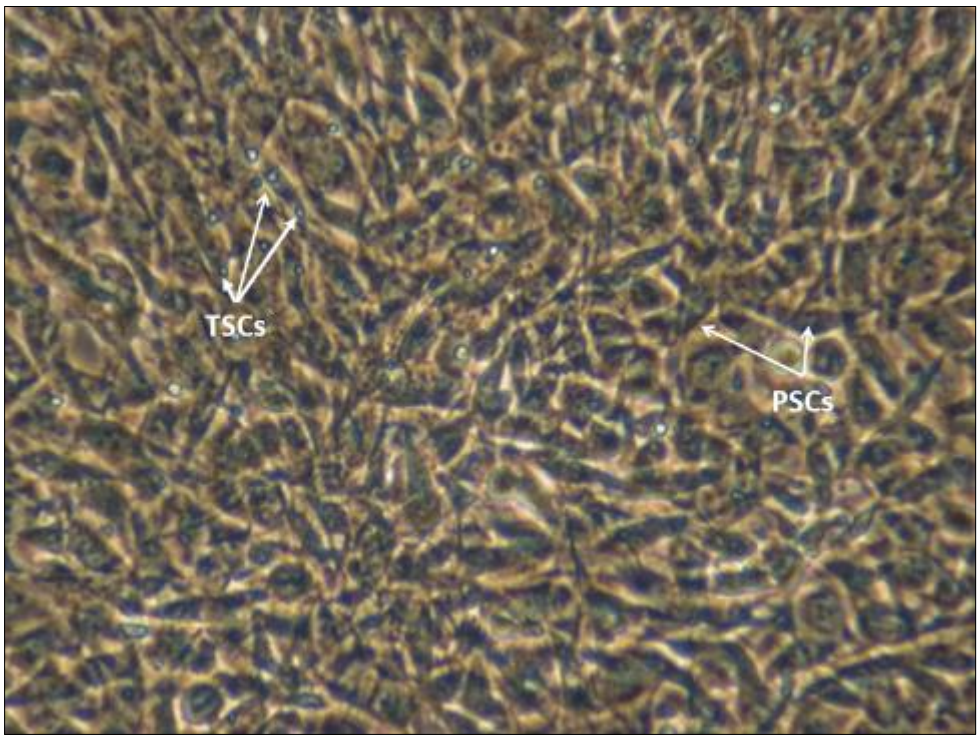

Figure 10 Initial plating of aTPSCs to form multi-layered confluent cells. Post-natal totipotent stem cells (TSCs) were initially plated onto a type-1 collagen substratum and allowed to grow to multi-layered confluence. Next, post-natal pluripotent stem cells (PSCs) were seeded onto layers of TSCs to form a bilaminar shell of cells [39]. Reprinted were permission from Young HE, Limnios JI, Lochner F, McCommon G, Cope LA, Black AC Jr. Pancreatic islet composites secrete insulin in response to a glucose challenge. J Stem Cell Res. 2017; 1(1) 001: 1-12

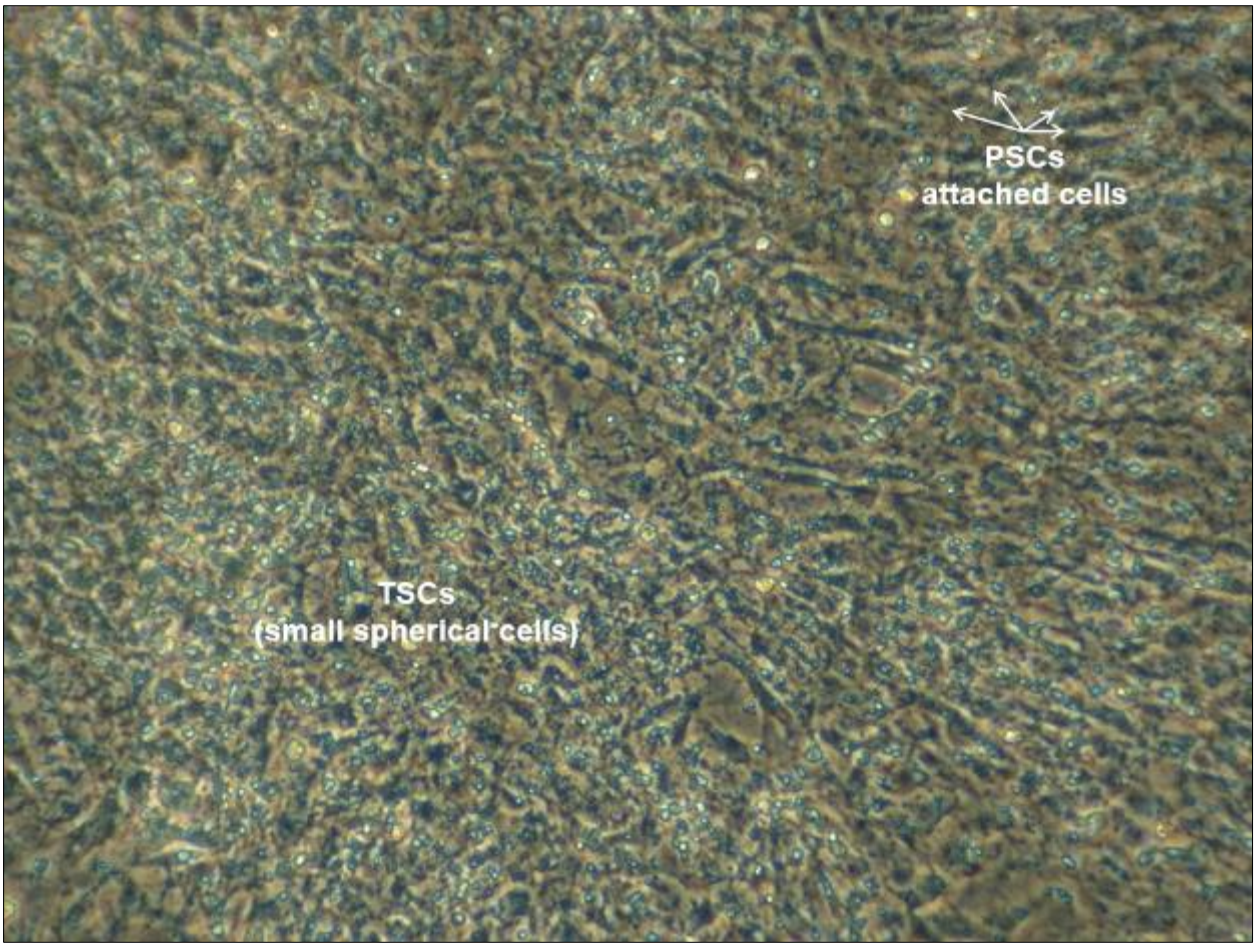

Figure 11 post-natal pluripotent stem cells (PSCs) plated on decellularized pancreatic collagen substratum (upper portion of photograph). Outer layer of 3D organoid consisted of post-natal totipotent stem cells (TSCs) (lower portion of photograph) plated on top of PSCs, eventually forming a bilaminar layer of cells [39]. Reprinted were permission from Young HE, Limnios JI, Lochner F, McCommon G, Cope LA, Black AC Jr. Pancreatic islet composites secrete insulin in response to a glucose challenge. J Stem Cell Res. 2017; 1(1) 001: 1-12

5. Discussion

The maintenance progenitor cells were originally designated as "adult stem cells", because they were from post-natal individuals and would form one or more cell types with a separate identity from themselves [13,91]. This is a misnomer in terminology for several reasons. 1. There is loss of the telomerase enzyme in maintenance progenitor cells at birth. Therefore, these cells have a finite lifespan in humans of 50-70 population doublings [92] and in rodents of 6-8 population doublings [93] before cell senescence and cell death [94]. 2. Maintenance progenitor cell numbers decrease with increasing age of the animal, including humans [19]. 3. Maintenance progenitor cells are pre-committed to distinct differentiated cell types [13,23]. 4. Maintenance progenitor cells are responsive to progression factors that accelerate their expression to their pre-determined cell types [65]. And 5. Maintenance progenitor cells are unresponsive to known inductive factors (human recombinant proteins) outside their pre-determined cell types [65].

Examples of the healing stem cells are the endogenous adult telomerase positive stem cells (aTPSCs) [13]. The eight populations of aTPSCs are uniquely different from each other as well as being uniquely different from both functional differentiative cells and maintenance progenitor cells as shown in Tables 1 and 2. These differences include, but not limited to, size, 0.4% Trypan blue staining patterns, cell surface markers, age range (Figs. 1-3), growth in culture (Figs. 10-13), cryopreservation temperatures, population doublings, and differentiation capabilities (Tables 1 and 2) [13]. Healing stem cells are telomerase positive which gives them an essentially unlimited proliferation potential [94]. There are eight distinct subcategories of healing stem cells based on multiple parameters, including differentiation potentials (Figs. 2,3) [13]. In contrast to maintenance progenitor cells: 1. Healing stem cells (aTPSCs) retain the telomerase enzyme at birth, giving the cells essentially an unlimited lifespan as long as they remain undifferentiated [95]. 2. Healing stem

cells remain constant throughout the lifespan of the organism, from four-cell stage embryogenesis to geriatric-aged individuals, as long as the cells remain undifferentiated (Fig. 4, Table 2) [13,31]. 3. Two categories of healing stem cells exist with different pre-commitments. The aTPSCs that will form all somatic cells of the body are composed of TSCs, HLSCs, CLSCs, PSCs, and GLSCs. Healing stem cells that are pre-committed to particular germ layer lineages are the EctoSCs, MesoSCs, and EndoSCs [13,23,65]. 4. Healing stem cells are not responsive to progression factors [65]. And 5. Healing stem cells are responsive to both known and "unknown" inductive factors, e.g., human recombinant proteins, morphogenetic proteins, and exosome-conditioned medium derived from differentiated cells [65].

In situ, healing stem cells reside in connective tissue niches interspersed throughout the body as dormant, hibernating, quiescent cells [65]. They can be stimulated to proliferate and mobilize into the blood stream as quiescent non-activated cells [96], but they need to be activated to function for replacing damaged cells and tissues [97]. Activation occurs by unmasking homing receptors on their cell surface and unmasking cell surface receptors for biological agents that direct the ultimate fate of the cells. Within an individual, activation of homing receptors occurs with release of chemokines from damaged tissues [98,99], and activation of receptors for exosomes occurs with endogenous matrix metalloproteinases at the site of injury [100]. Due to their limited number in the body and their uniqueness in not conforming to established tissue culture practices [34], special methodologies were developed to examine them both in vitro and for their use in vivo. This begins a series of articles explaining in step-by-step detail the methodologies (e.g., rationale, standardization, reagents, manufacturers, catalog numbers, step-by-step instructions, expected results) that were developed for in vitro characterization and in vivo use. The current report describes the methodologies developed to optimize the viability of the aTPSCs during their isolation, plating, and propagation for in vitro analyses.

5.1. Location in the Body

As shown (Fig. 4), three pairs of small (0.1-2.0 micron) and one comparatively larger (6-8 micron) cellular structures appear interspersed within 8+-cell stage blastomeres of a developing embryo [31]. Their appearance early in development explains their location within all connective tissues of a post-natal individual, within the umbilical cord (data not shown), and within the placenta (data not shown). The latter two structures, e.g., umbilical cord and placenta, form after the 8-cell stage during embryogenesis [101]. In post-natal individuals, biopsy specimens from newborn to geriatric individuals were obtained, immersed in ELICA fixative for preservation, sectioned, and stained using the ELICA procedure for immunochemistry of tissue sections using antibodies for TSCs (CEA-CAM-1), PSCs (SSEA-4) [102]. Procedural controls were added to verify that the staining for TSCs and PSCs was real and not an artifact of preparation. Each ELICA staining run contained positive and negative controls to validate that the procedures were working properly. An antibody to smooth muscle alpha-actin (IA4) was used as the positive control [23,102]. Six negative controls, one for each step in the ELICA procedure [23,102], were utilized to demonstrate the potential for non-specific binding of the reagents to the tissue or to each other. Immuno-stained representative figures (Figs. 3-17 in [24]) show SSEA-4+ cells (PSCs) and CEA-CAM-1+ cells (TSCs) located within the connective tissue compartments of their associated organs, e.g., heart (pericardium, coronary vessels, intramural septum), brain, pancreas, dermis, adipose tissue, lung, spleen, bone marrow, kidney, skeletal muscle, testis, ovary, and fallopian tube [24]. In toto, biopsy specimens from newborn to geriatric-aged individuals, comprising multiple organs from 15 species of animals, including humans, were assayed for location of TSCs and PSCs within an individual. The results demonstrate that TSCs and PSCs are a conserved population of cells present within the connective tissues of all animals examined (Table 2) [31,45].

5.2. Harvesting Cells from Solid Tissues

The standard procedure for harvesting cells from solid tissues is to macerate the tissue and then treat with trypsin for 15 min at 37^{o}C to release the cells [34]. Trypsin cleaves proteins between adjacent lysine and arginine residues. Therefore, it will disrupt the core proteins of proteoglycans and glycoproteins within the extracellular matrix, thus releasing the cells. Unfortunately, cell membranes also contain adjacent lysine and arginine residues, and are disrupted causing loss of cell integrity and decreased viability of released cells [58-61,65].

An alternate method was developed to optimize viability of the aTPSCs during the harvesting procedure. This entailed using type-1 collagenase and dispase in combination to disrupt just the extracellular matrix constituents releasing the cells from their connective tissue niches [13,65].

5.3. Counting Cells

Cell counting occurred using sterile 0.4% Trypan blue in species-specific buffer [23]. The commercially available 0.4% Trypan blue is pre-made in water, which will lyse a majority of the cells, due to water's low osmotic pressure [65]. It is

far better to make sterile 0.4% Trypan blue in species-specific buffer, pH to 7.4, and store in an opaque/brown bottle at ambient temperature [65].

5.4. Electrostatic Charge on Surface Plastic of Culture Vessels

Plastic culture vessels have an inherent electrostatic charge, dependent on the plastic utilized [65]. The electrostatic charge can be either negative, positive, or neutral (Fig. 6). While this may not seem important for those culturing progenitor cells or differentiated cells, but it is very important with respect to the zeta potential of aTPSCs being plated. The aTPSCs have a zeta potential with a very high net negative charge. They will not attach to any plastic with a negative charge due to electrostatic repulsion. In contrast, they will irreversibly attach to a plastic with a positive charge (scraping will not even dislodge intact cells). Therefore, it is imperative that they are seeded onto plastic vessels having a neutral charge. The culture vessels used with aTPSCs were 96-well plates (Costar); 48-well plates, 24-well plates, and 6-well plates (Corning); and T-25 flasks and T-75 flasks (Falcon) [13,23,65].

5.5. Substratum for Attachment

The aTPSCs do not synthesize and secrete their own substratum for attachment [63]. Therefore, a substratum needed to be provided. Since the predominant extracellular matrix glycoprotein for aTPSCs is type-1 collagen, it was theorized that the cells would prefer that as a substratum for their attachment [13,23]. Multiple percentages of type-I collagen solution were examined to prepare a suitable substratum for attachment, e.g., 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5, and 10% w/v in purified (HPLC) water. The aTPSCs attached to a type-1 collagen substratum via two binding sites: a calcium dependent binding site [79] and an RGD-fibronectin dependent binding site [80]. The aTPSCs preferred a solution of 1% collagen as a substratum for their attachment [65,76].

5.6. Preferred Medium for aTPSCs

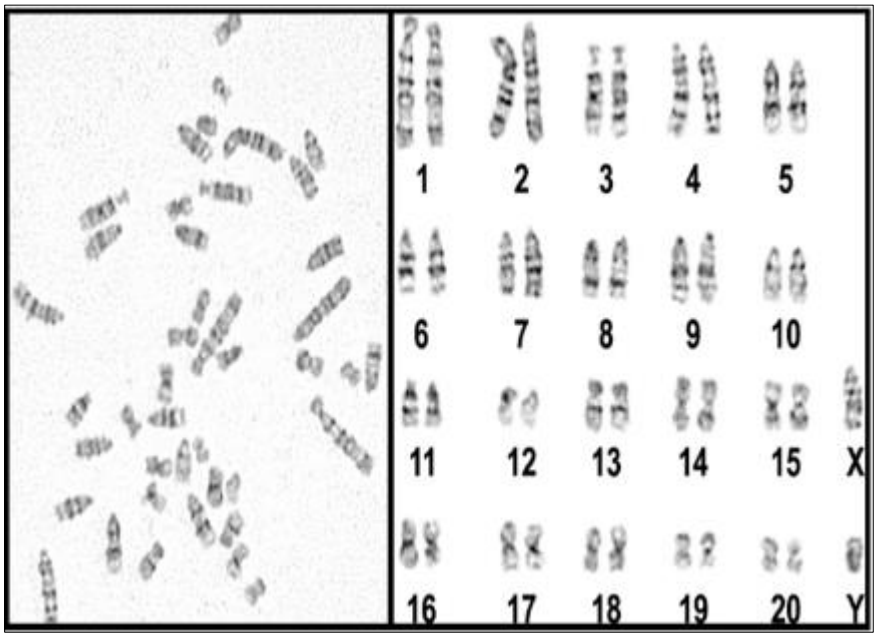

Figure 12 Karyotypic analysis of adult male rat TSCs demonstrating 42 chromosomes in the diploid state [103]. Reprinted with permission Henson NL, Heaton ML, Holland BH, Hawkins KC, Rawlings B, Eanes E, Bozof R, Powell S, Grau R, Fortney J, Peebles B, Kumar D, Yoon JI, Godby K, Collins JA, Sood R, Bowyer 3rd FP, Black Jr AC, Young HE. Karyotypic analysis of adult pluripotent stem cells. Histology and Histopathology. 2005; 20: 769-784

Culture medium of choice for aTPSCs is OptiMEM + GlutaMax (GIBCO). When cells are cultured for testing, antibiotic/antimycotic, e.g., penicillin, streptomycin, and fungizone, are added to maintain sterility in the cultures [65].

As shown (Fig. 5), isolation of cells from skeletal muscle biopsies resulted in a mixed collection of maintenance progenitor cells and healing stem cells (aTPSCs). It is relatively easy to discern that there are different cells present, solely based on size alone (Fig. 5). However, when the cultures become single layer confluent (Fig. 10), unless one knows what they are looking for, it is difficult to imagine and easy to miss the aTPSCs, especially the TSCs, which appear as small spherical dots within the field of cells. When others have viewed multi-layered confluent PSCs and TSCs (Figs.

10,11), their responses ranged from “must be cell trash” to “mycoplasma contamination” to “those are the strangest things I have ever seen, are they really cells?”. Cell trash has straight edges, cells do not; the aTPSCs are more spherical in appearance, rather than having straight edges. We sent representative cultures to three different independent companies to evaluate for the presence of mycoplasma. All three stated that there was no mycoplasma contamination of the cultures examined. And lastly, yes, they are cells. With respect to single cell clones from multiple species, TSCs (Fig. 12) and PSCs (Fig. 13) exhibited the correct number of diploid chromosomes from each species as assessed by karyotypic analysis, e.g., mouse, rat, pig, horse, and human (Table 2) [103].

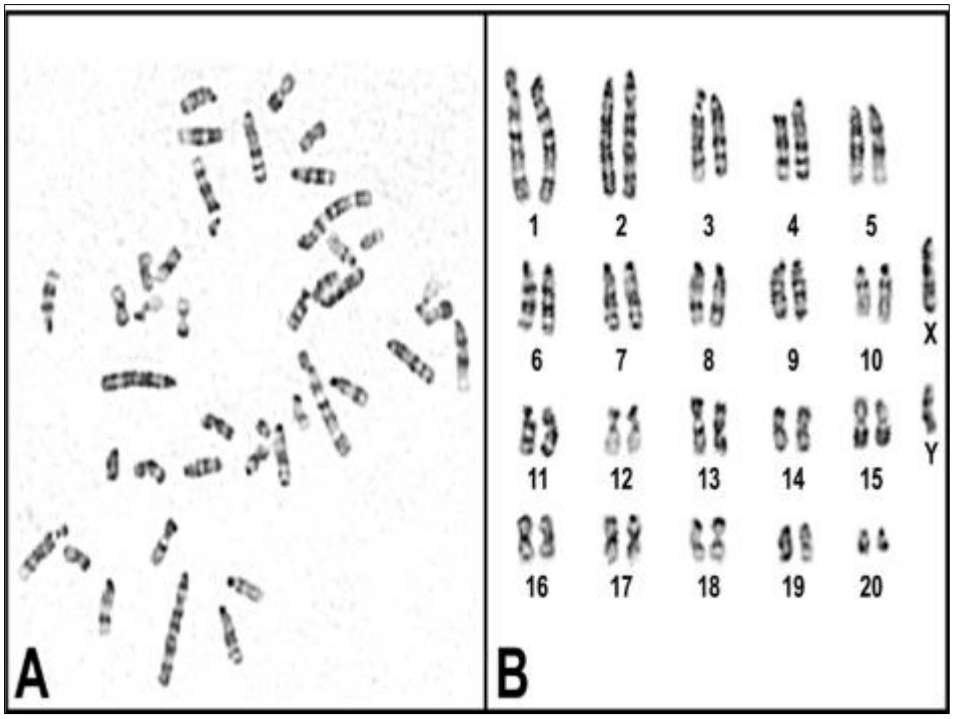

Figure 13 Karyotypic analysis of adult male rat pluripotent stem cells (PSCs) demonstrating 42 chromosomes in the diploid state [103]. Reprinted with permission Henson NL, Heaton ML, Holland BH, Hawkins KC, Rawlings B, Eanes E, Bozof R, Powell S, Grau R, Fortney J, Peebles B, Kumar D, Yoon JI, Godby K, Collins JA, Sood R, Bowyer 3rd FP, Black Jr AC, Young HE. Karyotypic analysis of adult pluripotent stem cells. Histology and Histopathology. 2005; 20: 769-784.

5.7. Serum

Non-processed serum contains multiple and varied concentrations of biological agents that can alter the outcome of an experiment [31,69,70]. Depending on the particular lot of serum that was used, there were differences within the cells with respect to resultant phenotypic expression markers. Most of the sera contained biological agents that stimulated the formation of fibroblasts in the TSC and PSC cultures [65,70]. And these particular serum (or recombinant protein) fibroblastic agents induced the TSC and PSC cultures to form an ECM reminiscent of scar fibroblasts/fibrocytes [65]. When recombinant proteins were tested individually, it was noted that both TGF-beta and basic-FGF would induce similar scar fibroblastic phenotypes. TGF-beta and basic-FGF are two of the most prominent exosome components in platelets, as assessed by SDS-PAGE of 46 bands of proteins from lysed platelets (data not shown). It is conceivable that platelets were lysed during the commercial collection of serum releasing these components into the serum [65].

To do any type of repetitive testing for biological activity, one needs to maintain the cells in their own undifferentiated state. Initially, when single cell repetitive serial dilution cloning was performed in chicken cells, over 400 lots of serum from bovine, calf, horse, pig, and human were screened for absence of biological activities. Only a single lot of (horse) serum (HS7) was found that was void of any biological agents. The entire lot was purchased and stored at -80^{o}C for testing. That was the horse serum lot used for cloning avian cells and initially testing human recombinant proteins for biological activity [60,61]. For cloning mouse stem cells, a heat inactivated serum procedure was devised to negate the potential biological activities of factors inhibiting differentiation, proliferation, progression, and induction [69,70]. The process for heat inactivation was to incubate any serum from bovine, fetal calf, equine, porcine or human origin, at 56^{o}C for 8 hours, high speed centrifugation at 100,000 RCF to remove precipitate, and then filter through a 0.1-micron filter for sterilization [65]. For cloning rat cells and culturing human cells for study, the heat inactivation technique was given

to Atlas Biologicals, in Fort Collins, CO and they generated the heat inactivated serum [13], which was/is commercially available.

5.8. Anerobic Environment

The aTPSCs exist in a dormant, quiescent, hibernating anaerobic state. To mimic that environment, beta-mercaptoethanol was added to the culture medium to reduce the oxygen tension in the medium for all cells for in vitro propagation [84]. For testing, beta-mercaptoethanol was removed from the medium to allow differentiation to occur [65]. However, if human aTPSCs were to be utilized for clinical treatments, they were grown within a BioSpherix unit where gases were regulated, e.g., oxygen 5%, carbon dioxide 5%, and nitrogen 90%, to simulate an anaerobic environment [65].

5.9. Doubling Rate, Propagation, and Potential for Mutations

The majority of the naturally-occurring adult cells used for testing, e.g., maintenance progenitor cells and functional differentiated cells, have a doubling rate from days to weeks to months, depending on the particular cell type examined [56,72,83]. The time period for a cell to double in number is inherent to the length of time it takes the cell to progress through its cell cycle + $\geq$ 2 hours [56,104]. During this time period the newly formed DNA strand is checked and rechecked for aberrant DNA match(es) that can lead to mutations [54,104]. Also, during this time frame is the replication of cellular components prior to mitotic division. Telomerase positive TSCs require 12-14 hours; HLSCs and CLSCS require 13-15 hours; PSCs require 14-16 hours; GLSCs require 16-18 hours; EctoSCs, MesoSCs, and EndoSCs require 18-24 hours; while telomerase negative MSCs require days to double in number (Table 1) [13,65].

5.10. Release from Tissue Culture Vessel Surface

Release of telomerase negative progenitor cells or differentiated cells from a culture vessel is usually performed using scraping cells from the surface of the vessels and/or enzymatic digestion with trypsin [34]. Scraping lyses cell membranes, leaving a majority of the cells either dead and/or dying. Trypsin cleaves proteins associated with paired lysine/arginine amino acids [56]. Plasma cell membranes also contain paired lysine/arginine amino acids and thus trypsin degrades the plasma membranes of cells to get the cells to release from the surface of the vessel, with a less-than-optimal viability after release [65].

Telomerase positive stem cells prefer a type-1 collagen substratum for cell attachment and growth, utilizing two binding sites, a calcium-dependent binding site [79] and an RGD-fibronectin dependent binding site [80] to basement membranes. This knowledge was used to design a releasing solution for the TPSCs from the plate surface. Cells were first incubated in an electrolyte-free buffer containing EDTA to release cells from the substratum [65]. Next, the type-1 collagen substratum was digested with EDTA buffer containing type-1 collagenase and dispase to degrade the RGD-binding sites [79,80] and thus remove the aTPSCs intact, where the majority (>99%) of the cells are still viable after release [13]

6. Conclusion

Since the aTPSCs do not conform to previously published protocols for cell culture, new methodologies had to be developed. During this developmental process, we listened to what the cells wanted rather than forcing them into pre-determined protocols. This begins a series of articles, explaining in step-by-step detail, the newly developed methodologies (e.g., rationale, standardization, reagents, manufacturers, catalog numbers, step-by-step instructions, and examples of expected results via text, tables, and figures) that were developed for in vitro characterization and in vivo use of the aTPSCs. The current report describes the methodologies developed to optimize the viability of the aTPSCs during their isolation, plating, and propagation for in vitro and pre-clinical in vivo analyses.

Compliance with ethical standards

Acknowledgments

This work was supported by grants from Rubye Ryle Smith Charitable Trust, Dragonfly Foundation for Research and Development, MedCen Community Health Foundation, and MorphoGen Pharmaceuticals, Inc. I like to thank my long-time collaborator Dr. Asa C. Black Jr for his mentorship with respect to teaching and research; my clinical collaborators; research associates and assistants J Floyd-Collins-Coleman, GF Long-Black, NL Henson, LW Blake, KC Hawkins, N Walsh, C Alena, MBL Cole, V Krishna, S Ellis, WJ Butler, PE Kross, J Wang, J Wong, D Hixson, and C Duplaa; my multitude of co-authors, clinical attendings, clinical residents, graduate students, medical students, and a high school student, for their

insight, strong work ethic, and for help in conducting 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.

Disclosure of conflict of interest

No conflict of interest was disclosed.

Statement of ethical approval

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

Statement of informed consent

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

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] Baslow MH. The languages of neurons: an analysis of the coding mechanisms by which neurons communicate, learn, and store information. Entropy. 2009; 11(4): 782-797.

[3] Lin S-C, Bergles DE. Synaptic signaling between neurons and glia. Glia. 2004; 47(3):290-298.

[4] Noguchi GM, Huising MO. Integrating the inputs that shape pancreatic islet hormone release. Nature Metabolism. 2019; 1(12): 1189-1201.

[5] Erlandsen SL, Herge OD, Parsons JA, McEvoy RC, Elde RP. Pancreatic islet hormones distribution of cell types in the islet and evidence for the presence of somatostatin and gastrin within the D cell. J Histochem Cytochem. 1976; 24(7): 883-897.

[6] Rorsman P, Braun M. Regulation of insulin secretion in human pancreatic islets. Ann Rev Physiol. 2013; 75(1):155-179.

[7] Scott D, Blizzard L, Fell J, Jones G. Prospective associations between ambulatory activity and muscle function in older adults. Scand J Med Sci Sports. 2011; 21(6): e168-e175.

[8] Barnard AM, Willcocks RJ, Finanger EL, Daniels MJ, Triplett WT. Skeletal muscle magnetic resonance of biomarkers correlate with function and sentinel events in Duchene muscular dystrophy. PLoS One. 2018; 13(3): e0194283.

[9] Montes J, McDermott MP, Mirek E, Mazzone ES, Main M. Ambulatory function in spinal muscular atrophy: age-related patterns of progression. PLoS One. 2018; 13(6): e0199657.

[10] Blondet NM, Messner DJ, Kowdley KV, Murray KF. Mechanisms of hepatocyte detoxification. Physiol Gastrointest Tract. 2018; 981-1001.

[11] Apte U, Krishnamurthy P. Detoxification functions of the liver. Molec Path Liver Diseases. 2010; 147-163.

[12] Grant DM. Detoxification pathways in the liver. J Inherited Metabolic Disease. 1991; 14(4): 421-430.

[13] 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.

[14] Weissman IL, Shizuru JA. The origins and identification and isolation of hematopoietic stem cells, and their capacity to induce donor-specific transplantation tolerance and treat autoimmune disease. Blood. 2008; 112:3543-3553.

[15] Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. Blood, J Amer Soc Hematology. 2015; 125(17): 2605-2613.

[16] Mikkola HKA, Orkin SH. The journey of developing hematopoietic stem cells. Oxford Univ Press, Company Biologists Limited. 2006; 133(19): 3733-3744.

[17] Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991; 9:641-650.

[18] Pittenger MF, Mackay AM, Beck SC, et al. Multilineages potential of adult human mesenchymal stem cells. Science. 1999; 284:143-147.

[19] Caplan AI. Review: Mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005; 11:1198-1211.

[20] Vierck JL, McNamara JP, Dodson MV. Proliferation and differentiation of progeny of ovine unilocular fat cells (adipofibroblasts). In Vitro Cell Dev Biol Anim. 1966; 32:564-572.

[21] Vierk JL, McNamara JP, Dodson MV. Two alternative procedures for isolating adipofibroblasts from sheep skeletal muscle. Meth Cell Sci. 1996; 18(4): 309-314.

[22] 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.

[23] 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.

[24] Young HE. Adult telomerase positive stem cells: introduction and location. GSC Advanced Research and Reviews. 2025; 25(02): 296-331.

[25] Young HE. Limb Regeneration in the Adult Salamander, Ambystoma annulatum Cope 1889 (Amphibia: Ambystomatidae). University of Arkansas Library Press, copyright -1977.

[26] Young HE. A Temporal Examination of Glycoconjugates During the Initiation Phase of Limb Regeneration in Adult Ambystoma. Texas Tech University Library Press, copyright – 1983.

[27] Young HE. Epidermal ridge formation during limb regeneration in the adult salamander, Ambystoma annulatum. Proceedings of the Arkansas Academy of Science, 31:107-109, 1977.

[28] Young HE, Bailey CF, Markwald RR, Dalley BK. Histological analysis of limb regeneration in postmetamorphic adult Ambystoma. Anatomical Record, 212:183-194, 1985.

[29] Young HE, Dalley BK, Markwald RR. Effect of selected denervations on glycoconjugate composition and tissue morphology during the initiation phase of limb regeneration in adult Ambystoma. Anatomical Record, 223:223-230, 1989.

[30] Young HE, Dalley BK, Markwald RR. Glycoconjugates in normal wound tissue matrices during the initiation phase of limb regeneration in adult Ambystoma. Anatomical Record, 223:231-241, 1989.

[31] Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol. 2014; 1(4): 00019.

[32] http://google.com/images/embryonic stem cells

[33] 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.

[34] ATCC Animal Cell Culture Guide, The Essentials of Life Sciences Research, American Type Culture Collection, Gaithersburg, Maryland.

[35] Young HE, Duplaa C, Katz R, Thompson T, Hawkins KC, Boev AN, Henson NL, Heaton M, Sood R, Ashley D, Stout C, Morgan JH, Uchakin PN, Rimando M, Long GF, Thomas C, Yoon JI, Park JE, Hunt DJ, Walsh NM, Davis JC, Lightner JE, Hutchings AM, Murphy ML, Boswell E, McAbee JA, Gray BM, Piskurich J, Blake L, Collins JA, Moreau C, Hixson D, Bowyer FP, Black AC Jr. Adult-derived stem cells and their potential for tissue repair and molecular medicine. J Cell Molec Med 9:753-769, 2005.

[36] Young HE, Limnios IJ, Lochner F, McCommon G, Black GF, Coleman JA, Hawkins KC, Black Jr AC. Cardiovascular disease and adult healing cells: From bench top to bedside. J Stem Cell Res 2017; 1(3) 002:1-8.

[37] Young HE, Black GF, Coleman JA, Hawkins KC, Black Jr AC. Pulmonary diseases and adult healing cells: from bench top to bedside. J Stem Cell Res 2017; 1(2) 003:1-9.

[38] Young HE, Black AC Jr. Differentiation potential of adult stem cells. In: Contemporary Endocrinology: Stem Cells in Endocrinology, L.B. Lester, ed., The Humana Press Inc., Totowa, NJ. Chap. 4, p. 67-92, 2005.

[39] Young HE, Limnios JI, Lochner F, McCommon G, Cope LA, Black AC Jr. Pancreatic islet composites secrete insulin in response to a glucose challenge. J Stem Cell Res. 2017; 1(1) 001: 1-12.

[40] Young HE, Hyer L, Black AC Jr, Robinson Jr JS. Adult stem cells: from bench-top to bedside. In: Tissue Regeneration: Where Nanostructure Meets Biology, 3DBiotech, North Brunswick, NJ Chap 1, pp 1-60, 2013a.

[41] Young HE, Hyer L, Black AC Jr, Robinson Jr JS. Treating Parkinson disease with adult stem cells. J Neurological Disorders, 2:107-109, 2013b.

[42] Young HE, Speight MO. Treating Parkinson Disease with Autologous Telomerase-Positive Stem Cells, Update 2021. Stem Cells & Regenerative Medicine. 2021; 5(1): 1-13.

[43] Young HE, Speight MO. Cardiovascular disease treated with telomerase-positive stem cells. Stem Cells Regen Med. 2020; 4(2):1-8.

[44] Young HE. Combinatorial Nutraceutical Supplement Pill (CNSP) Stimulates Naïve Adult Telomerase Positive Stem Cells In-Situ to Heal Cardiomyopathies. GSC Advanced Research and Reviews. 2024; 20(02), 047-056.

[45] Young HE, Speight MO. Potential treatment of chronic obstructive pulmonary disease with allogeneic and autologous telomerase-positive stem cells. Stem Cells Regen Med. 2020; 4(3):1-11.

[46] Young HE, Speight MO. Telomerase-positive stem cells as a potential treatment for idiopathic pulmonary fibrosis. Stem Cells Regen Med. 2020; 4(2):1-11.

[47] 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.

[48] 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

[49] Young HE, Speight MO. Allogeneic telomerase-positive stem cells as a treatment for celiac disease. Stem Cells Regen Med. 2020; 4(2):1-7

[50] Young HE, Speight MO. Age-related macular degeneration treated with autologous telomerase-positive totipotent stem cells. Stem Cells Regen Med. 2020; 4(3):1-9. JSCR-20-055

[51] Young HE, Speight MO. Alzheimer's disease treated with autologous and allogeneic telomerase-positive stem cells. Stem Cells & Regen Med. 2021; 5(1):1-17

[52] Young HE, Speight MO. Blunt force trauma-induced total bilateral vision impairment of 13 years duration treated with autologous telomerase positive stem cells. Stem Cells Regen Med. 2021; 5(1):1-22

[53] Young HE, Speight MO. Traumatic spinal cord injury treated with autologous telomerase-positive stem cells. Stem Cells Regen Med. 2021; 5(1):1-13

[54] Young HE, Speight MO. Chronic inflammatory demyelinating polyneuropathy treated with autologous telomerase-positive stem cells. Stem Cells Regen Med. 2021; 5(2):1-16

[55] Young HE. Proposed treatment plan for restoration of histoarchitecture and function of severed nerve embedded in scar tissue. GSC Advanced Research and Reviews. 2025; 24(01): 323-354.

[56] Lieberman M, Marks AD. Mark's Basic Medical Biochemistry, A Clinical Approach, 4th Edition. Lippincott Williams & Wilkins.

[57] Young HE, Carrino DA, Caplan AI. Histochemical analysis of newly synthesized and resident sulfated glycosaminoglycans during musculogenesis in the embryonic chick leg. Journal of Morphology, 201:85-103, 1989.

[58] Young HE, Carrino DA, Caplan AI. Changes in synthesis of sulfated glycoconjugates during muscle development, maturation, and aging in embryonic to senescent CBF-1 mouse. Mechanisms of Ageing and Development, 53:179-193, 1990.

[59] Yue B. Biology of the extracellular matrix. J Glaucoma. 2014; (Oct-Nov): S20-S23.

[60] Young HE, Ceballos EM, Smith JC, Lucas PA, Morrison DC. Isolation of embryonic chick myosatellite and pluripotent stem cells. Journal of Tissue Culture Methods, 14:85-92, 1992.

[61] Young HE, Ceballos EM, Smith JC, Mancini ML, Wright RP, Ragan BL, Bushell I, Lucas PA. Pluripotent mesenchymal stem cells reside within avian connective tissue matrices. In Vitro Cellular & Developmental Biology. 1993; 29A:723-736.

[62] Pate DW, Southerland SS, Grande DA, Young HE, Lucas PA. Isolation and differentiation of mesenchymal stem cells from rabbit muscle. Surgical Forum. 1993; XLIV:587-589.

[63] Young HE, Steele T, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr. 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.

[64] Stout CL, Ashley DW, Morgan III JH, Long GF; Collins JA, Limnios JI, Lochner F, McCommon G, Hixson D, Black Jr AC, Young HE. Primitive stem cells reside in adult swine skeletal muscle and are mobilized into the peripheral blood following trauma. American Surgeon. 2007; 73 (11):1106-1110.

[65] Young HE. Endogenous Adult Telomerase Positive Stem Cells and/or Combinatorial Nutraceuticals, 50 Years in the Making, 50 Years of Discovery; In preparation

[66] Lucke' B, McCutcheon M. The living cells as an osmotic system and its permeability to water. Physiol Rev. 1932; 12(1): 68-139.

[67] Young HE, Duplaa C, Romero-Ramos M, Chesselet M-F, Vourc'h P, Yost MJ, Ericson K, Terracio L, Asahara T, Masuda H, Tamura-Ninomiya S, Detmer K, Bray RA, Steele TA, Hixson D, El-Kalay M, Tobin BW, Russ RD, Horst MN, Floyd JA, Henson NL, Hawkins KC, Groom J, Parikh A, Blake L, Bland LJ, Thompson AJ, Kirincich A, Moreau C, Hudson J, Bowyer III FP, Lin TJ, Black Jr AC. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys. 2004; 40(1):1-80.

[68] www.BioSpherix.com

[69] Rogers JJ, Adkison LR, Black AC Jr, Lucas PA, Young HE. 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.

[70] Young HE, Wright RP, Mancini ML, Lucas PA, Reagan CR, Black AC Jr. Bioactive factors affect proliferation and phenotypic expression in pluripotent and progenitor mesenchymal stem cells. Wound Repair and Regeneration. 1998; 6(1):65-75.

[71] Fowler JF. Potential for increasing the differential response between tumors and normal tissues: can proliferation rate be used? Intl J Radiation Oncology. 1986; 12(4): 641-645.

[72] Daukste L, Basse B, Baguley BC, Wall DJN. Mathematical determination of cell population doubling times for multiple cell lines. Bull Mathematical Biology. 2021; 74(10): 2510-2534.

[73] Lovett ST. Encoded errors: mutations and rearrangements mediated by misalignment at repetitive DNA sequences. Molec Microbiol. 2004; 52(5): 1243-1253.

[74] Iyer RR, Pluciennik A, Burdett V, Modrich PL. DNA mismatch repair: functions and mechanisms. Chem Rev. 2006: 106(2): 302-323.

[75] Karran P, Bignami M. DNA damage tolerance, mismatch repair and genome instability. Bioessays. 1994; 16(11): 833-839.

[76] Young HE. Adult telomerase positive stem cells: isolation, plating, and propagation. GSC Advanced Research and Reviews. 2025; (Submitted)

[77] Abbas AK, Litchman AH. Pillai S. In: Cellular and Molecular Immunology, 7th Ed., ELSEVIER, Saunders, pg. 509, 2012.

[78] www.worthington-biochem.com Introduction to Enzymes.

[79] Tam EM, Wu YI, Butler GS, et al. Collagen binding properties of the membrane type-I matrix metalloproteinase (MT1-MMP) Hemopexin C domain. J Biol Chem. 2002; 277(41):39005-39014.

[80] Benoit YD, Groulx J-F, Gagne D, et al. RGD-Dependent epithelial cell-matrix interactions in the human intestinal crypt. J Signal Transduct. 2012; 2012: 248759.

[81] Conlee KM, Stephens ML, Rowan AN, King LA. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. Laboratory Animals. 2005; 39(2): 137-161.

[82] Turner PV, Hickman DL, Van Luiik JV, Rotskes-Hoitinga M, Sargent JM, et al. Welfare impact of carbon dioxide on laboratory mice and rats: a systematic review. Front Vet Sci. 2020; 7: 411.

[83] Animal Cell Culture Guide, The Essentials of Life Sciences Research, American Type Culture Collection, Gaithersburg, Maryland.

[84] Humason G. Animal Tissue Techniques. WH Freeman and Co., San Francisco, CA.

[85] Young HE, Dalley BK, Markwald RR. Effect of selected denervations on glycoconjugate composition and tissue morphology during the initiation phase of limb regeneration in adult Ambystoma. Anatomical Record. 1989; 223:223-230.

[86] www.SigmaAldrich.com EGTA specific calcium chelator in reduced electrolyte buffer.

[87] Paulsen DF, Solursh M. Microtiter micromass cultures of limb-bud mesenchymal cells. In Vitro Cell & Develop Biol. 1988; 24(2):138-147.

[88] Schramm CA, Reiter RS, Solursh M. Role of short-range interactions in the formation of cartillage and muscle masses in transfilter-micromass cultures. Develop Biol. 1994; 163(2): 467-479.

[89] Solursh M, Ahrens PB, Reiter RS. A tissue culture analysis of steps in limb chondrogenesis. In Vitro. 1978; 14(1): 51-61.

[90] Swalla BJ, Solursh M. The independence of myogenesis and chondrogenesis in micromass cultures of chick wing buds. Develop Biol. 1986; 116(1): 31-38.

[91] NIH Stem cell information page. In: Stem cell Information, Bethesda, MD, NIH, US DHHS, 2016. Cited July 28, 2020.

[92] Hayflick L, Moorehead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25:585-621.

[93] 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: 5009-5013.

[94] Zvereva MI, Shcherbakova DM, Dontsova OA. Telomerase: structure, function, and activity regulation. Biochemistry (Mosc.). 2010; 75:1563-1583.

[95] Young HE, Lochner F. Telomerase positive totipotent stem cells in the adult brain. I. cerebral cortex. Regen Med Biol Res 2021; 2(1):1-16.

[96] 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.

[97] Young HE. Combinatorial nutraceutical supplement pill (CNSP) stimulates naïve adult telomerase positive stem cells In Situ to reverse signs and symptoms in multiple human health concerns. GSC Advanced Research and Reviews. 2004; 20(02): 047-056. https://doi.org/10.30574/gscarr.2024.20.2.0290

[98] Bennett LD, Fox JM, Signoret N. Mechanisms regulating chemokine receptor activity. Immunology. 2011; 134 (3): 246-256.

[99] Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018: 285(16): 2944-2971.

[100] Costanzo RM, Perrino LA. Peak in matrix metalloproteinases-2 levels observed during recovery from olfactory nerve injury. Neuroreport. 2008; 19(3): 327-331.

[101] Moore KL, Persaud TVN, Torchia MG. The Developing Human, Clinically Oriented Embryology, 9th Edition, 2013. ELSEVIER, Saunders.

[102] 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.

[103] Henson NL, Heaton ML, Holland BH, Hawkins KC, Rawlings B, Eanes E, Bozof R, Powell S, Grau R, Fortney J, Peebles B, Kumar D, Yoon JI, Godby K, Collins JA, Sood R, Bowyer 3rd FP, Black Jr AC, Young HE. Karyotypic analysis of adult pluripotent stem cells. Histology and Histopathology, 20: 769-784, 2005.

[104] 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

[105] Young HE, Black Jr AC. Adult stem cells. Anat. Rec. 276A:75-102, 2004.

[106] 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, pp. 63-93, 2013. [Labeling differences between original paper and above, original paper Figure 4. ELSCs = PSCs and BLSCs = TSCs].

[107] Kumar V, Abbas AK, Fausto M, et al. In: Robbins and Cotran Pathologic Basis of Disease. Elsevier, Saunders, pgs. 226-230, 2010.

[108] 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.

[109] 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).