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Adult Telomerase Positive Stem Cells: Remain Constant Throughout Life-Span of Individual

Henry E. Young[*1-6]

[1]Dragonfly Foundation for Research & 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

***Corresponding author:** Henry E. Young, Dragonfly Foundation for Research & Development, 101 Preston Ct, Ste 101, Macon, GA 31210 USA.

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Abstract

Previous reports stated (implied) that (all) adult stem cells decrease within increasing age of an individual. Those reports utilized telomerase negative progenitor cells, such as mesenchymal stem cells (MSCs), and other progenitor cells to validate their results. Their manner of testing was to isolate their respective cells from different aged individuals using short term trypsin digests and then counting the released cells. Their results demonstrated a constant number of cells till about age thirty (in humans) and then a slow decrease in number of released cells with increasing age of the individual. The previously reported methods for assessing numbers of telomerase negative progenitor cells were recapitulated in a series of studies with adult telomerase positive cells using biopsy specimens from newborn, adolescent, post-pubescent, sexually-mature, and geriatric-aged individuals, utilizing short term trypsin digests, short term collagenase/dispase digests, and extended term collagenase/dispase digests keyed to the age of the individual. After the subsequent digests the released cells were counted. Both telomerase negative progenitor cells and telomerase positive cells could be released from the connective tissues of newborn, adolescent, post-pubescent, sexually-mature, and geriatric-aged individuals. The results suggest that while progenitor cells decrease with increasing age of the individual, telomerase positive cells remain constant throughout the life-span of an individual.

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I would propose that the decrease in cell numbers of progenitor cells with increasing age of the individual relates to either the absence of the telomerase enzyme, thus limiting their population doublings with increasing age, or an age-based increase in the density of the extracellular matrix surrounding the cells.

Keywords

Stem Cells; Progenitor Cells; Decrease with Age; Constant with Age.

Abbreviations

- 37^0C, thirty-seven degrees centigrade
- aTPSCs, adult telomerase positive stem cells
- CEA-CAM-1, carcinoembryonic antigen-cell adhesion molecule-1
- CD, collagenase-dispase
- CLSC, corona-like stem cell
- DPBS, Dulbecco's Phosphate Buffered Saline
- EctoSC, ectodermal stem cell
- EGTA, ethylene glycol bis (b-aminoethyl ether)-N, N, N', N'- tetraacetic acid, a monospecific calcium chelator
- EndoSC, endodermal stem cell
- GLSC, germ layer lineage stem cell
- Ger, geriatric (85-years-old)
- HLSC, halo-like stem cell
- IRB, Institutional Review Board
- MAPCs, multipotent adult progenitor cells
- MesoSC, mesodermal stem cell
- MIAMIs, marrow-isolated adult multilineage inducible cells
- mm, millimeter
- mM, millimolar
- MSC, mesenchymal stem cell
- MUSEs, multilineage-differentiating stress-enduring cells
- NB, newborn, < 1-year-old
- NIH, National Institutes of Health
- NIRS, National Institute Research Services
- PSC, pluripotent stem cell
- Pub, pubescent (10-12-years of age)
- RCF, relative centrifugation force
- RGD, tripeptide Arginine (R), glycine (G), aspartic acid (D) sequence found in fibronectin, acting as a key recognition site for cell attachment, migration, and tissue development
- SM, sexually mature (30-years of age)
- SMS, small mobile stem cells

- VSELs, very-small embryonic-like stem cells
- T, trypsin
- TSC, totipotent stem cell

Introduction

The post-natal (adult) human is composed of trillions and trillions of cells. These cells can be divided into two categories: differentiated cells and precursor cells [1,2]. The differentiated cells are composed of functional parenchyma and stroma, approximating 40% of all cells of the body. Whereas, precursor cells are composed of cells that self-replicate and repair and/or replace worn out differentiated cells. Examples of these precursor cells are mesenchymal stem cells (MSCs) [3-12], very-small embryonic-like stem cells (VSELs) [13-18], multilineage-differentiating stress-enduring cells (MUSEs) [19-27], marrow-isolated adult multilineage inducible cells (MIAMIs) [28,29], small mobile stem cells (SMS) [30,31], and multipotent adult progenitor cells (MAPCs) [32-36].

In 1975, an extremely rare population of endogenous adult telomerase positive cells was discovered. These telomerase positive cells were located in multiple postnatal individuals [58-60] as quiescent hibernating cells within the connective tissue interstitium, and only following trauma would become activated, proliferate, migrate to the site of tissue damage and repair the damage [58]. Since 1975, these endogenous adult telomerase positive cells have been extensively characterized [61]. They have been identified in multiple species, isolated, sorted, cloned from single cells, genomically-labeled, expanded exponentially, functions examined with biological agents, long term cryopreservation [62-68], used in pre-clinical animal models of disease [69-73], and fresh isolates of autologous [74-82] and gender-matched ABO blood group-matched allogeneic cells [83-88] used in clinical studies of chronic disease, including from individuals in the ninth decade of their lives [88-90]. The current terminology for these endogenous adult cells is “adult telomerase positive stem cells”, for the presence of the telomerase enzyme, their ability for unlimited self-replication, their ability to form every cell type within the body, and their ability to repair and replace damaged cell, tissues, and organs. Utilizing adult telomerase positive stem cells, autologous and/or gender-matched ABO blood group-matched (or O-negative) allogeneic cells, reverse the signs and symptoms of their respective disease, restoring functionality to the individual [89,90].

Recently, a series of articles have been published detailing comparison and contrast methodologies used to work with these adult telomerase positive stem cells versus telomerase negative progenitor mesenchymal stem cells and other telomerase negative progenitor stem cells [62-68]. Unfortunately, early on it was noted that procedures for isolation and subsequent culturing differed significantly between adult telomerase positive stem cells and the telomerase negative progenitor cells [62-68]. In this study, the adult telomerase positive stem cells were tested to confirm or deny the assertion that ALL adult stem cells, be they telomerase negative progenitor cells or telomerase positive stem cells, decrease in number with increasing age of an individual.

Materials and Methods

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).

Skeletal muscle biopsy specimens from newborn, pubescent (10-12-year-olds), sexually-mature (30-year-old), and geriatric (85-year-old) human [Homo sapiens] individuals were obtained from NIH/NIRS. Five-ml of Dulbecco's Phosphate Buffered Saline, pH 7.4 (DBPS, GIBCO, Grand Island, NY) was added to each of 12 fifty-ml centrifuge tubes (Falcon, Thermo-Fisher Scientific, Waltham, MA), and labeled according, experiment number (#s: 1, 2, 3), age of biopsied individual (newborn < 1-year-old [NB], pubescent 10-12-year-old [Pub], sexually mature 30-year-old [SM], and geriatric 85-year-old [Ger]), and enzyme mixture to be used (trypsin [T], collagenase-dispase [CD]), and tare weights taken.

The approximately 10-g of each biopsy specimen was individually placed into separate sterile 100-mm glass petri dishes in DPBS. Each biopsy specimen was macerated to the consistency of orange marmalade and separated into thirds. Each third was placed into separate pre-tared tubes. Final tube designations were as follows, e.g., #1-NB-T, #2-NB-CD, #3-NB-CD; #1-Pub-T, #2-Pub-CD, #3-Pub-CD; #1-SM-T, #2-SM-CD, #3-SM-CD; #1-Ger-T, #2-Ger-CD, and #3-Ger-CD. All tubes were placed on wet ice or at 4^{0}C until further processing.

Experiment #1: Short term trypsin digest [37,91,92], as 10% (w/v) Trypsin (Sigma) in DPBS for 15 min. Volume in tubes were measured, subtracted from 20, and resultant volume of 37°C pre-warmed enzymatic solution (20% trypsin) added to each tube for a final concentration of 10% trypsin. The tubes were capped, sealed with Parafilm (Diagger, Buffalo Grove, IL), and placed into a weighted holder in a 37°C shaker water bath (Thermo-Fisher Scientific) for 15 min. The tubes were removed from the water bath, Parafilm removed, and 20-ml of ice cold 1% collagen solution [63] added to each tube to stop protease/enzymatic process. The tubes were then centrifuged at 2,000 RCF (Relative Centrifugation Force) (centrifuge, Thermo-Fisher Scientific) to separate cell pellet from enzyme solution.

Following centrifugation, the supernatant was removed to bleach (Diagger), and cell pellet reconstituted in sterile DPBS. One hundred microliters of cell suspension were placed into 1.5-ml polypropylene uncapped microcentrifuge tubes (Corning, Corning, NY) using a P-200 Ranin micropipettor (Mettler Toledo, Columbus, OH). One hundred microliters of 0.4% Trypan blue (Kodak, Rochester, NY) in DPBS buffer [63] were added to the 1.5-ml polypropylene tubes containing the cell suspensions. The microcentrifuge tubes were capped, vortexed at 1-sec x 10, and returned to their holders. Each microcentrifuge tube was uncapped, 50 microliters of cell suspension/Trypan blue solution was removed, and placed onto each groove of a hemocytometer (Thermo-Fisher, Scientific). Using a NIKON TMS Brightfield/Phase Contrast microscope, samples were viewed at 10x magnification with brightfield optics. Cells were scored according to Figure 1, based on size and Trypan blue staining characteristics.

Experiment #2: Collagenase/Dispase digest for 15 min. Volume in tubes were measured, subtracted from 20, and resultant volume of 37°C pre-warmed enzymatic solution were added to each tube to a final concentration of 250 units/ml type-1 collagenase (Worthington Biochemicals, Lakewood, NJ) and 33.3 units/ml dispase (R&D Laboratories, Minneapolis, MN) in calcium-free, magnesium-free DPBS containing

25 mM ethylene glycol bis (b-aminoethyl ether)-N,N,N',N'- tetraacetic acid (EGTA, Sigma) (25 mM = 0.475 g EGTA/500 ml calcium-/magnesium-free PBS), pH 7.4, was used to separate precursor cells from their extracellular matrices [63]. The tubes were capped, sealed with Parafilm, and placed in a weighted holder in a 37°C shaker water bath for 15 min.

The tubes were removed from the water bath, Parafilm removed, and 20-ml of ice cold 1% collagen solution [63] added to each tube to stop enzymatic process. The tubes were then centrifuged at 2,000 RCF to separate cell pellet from enzyme solution. Following centrifugation, the supernatant was removed to bleach, and cell pellet reconstituted in sterile DPBS. One hundred microliters of cell suspension were placed into 1.5-ml polypropylene microcentrifuge tubes using a P-200 Ranin micropipettor. One hundred microliters of 0.4% Trypan blue in DPBS buffer were added to the 1.5-ml polypropylene capped tubes containing the cell suspensions.

The microcentrifuge tubes were capped, vortexed at 1-sec x 10, and returned to their holders. A microcentrifuge tube was uncapped, 50 microliters of cell suspension/Trypan blue solution was removed, and placed onto each groove of a hemocytometer (Thermo-Fisher, Scientific). Using a NIKON TMS Brightfield/Phase Contrast microscope, samples were viewed at 10x magnification with brightfield optics. Cells were scored according to Figure 1, based on size and Trypan blue staining characteristics.

Experiment #3: Samples were digested with type-1 collagenase/dispase for 15-min (NB, newborn), 1-hr. (Pub, 10-12-year-old), 3-hr. (SM, 30-year-old), and 8.5 hr. (Ger, 85-year-old). Volume in tubes were measured, subtracted from 20, and resultant volume of 37°C pre-warmed enzymatic solution was added to each tube to a final concentration of 250 units/ml type-1 collagenase (Worthington Biochemicals, Lakewood, NJ) and 33.3 units/ml dispase (R&D Laboratories, Minneapolis, MN) in calcium-free, magnesium-free DPBS containing 25 mM ethylene glycol bis (b-aminoethyl ether)-N,N,N',N'- tetraacetic acid (EGTA, Sigma) (25 mM = 0.475 g EGTA/500 ml calcium-/magnesium-free PBS), pH 7.4, was used to separate precursor cells from their extracellular matrices [63].

The tubes were capped, sealed with Parafilm, and placed in a weighted holder in a 37°C shaker water bath for 15 min. The tubes were removed from the water bath, Parafilm removed, and 20-ml of ice cold 1% collagen solution added to each tube to stop enzymatic process. The tubes were then centrifuged at 2,000 RCF to separate cell pellet from enzyme solution. Following centrifugation, the supernatant was removed to bleach, and cell pellet reconstituted in sterile DPBS.

One hundred microliters of cell suspension were placed into 1.5-ml polypropylene microcentrifuge tubes using a P-200 Ranin micropipettor. One hundred microliters of 0.4% Trypan blue in DPBS buffer were added to the 1.5-ml polypropylene tubes containing the cell suspensions. The microcentrifuge tubes were capped, vortexed at 1-sec x 10, and returned to their holders. A microcentrifuge tube was uncapped, 50 microliters of cell suspension/Trypan blue solution was removed, and placed onto each groove of a hemocytometer (Thermo-Fisher, Scientific). Using a NIKON TMS Brightfield/Phase Contrast microscope, samples were viewed at 10x magnification with brightfield optics. Cells were scored according to Figure 1, based on size and Trypan blue staining characteristics.

Results

- **Experiment #1:** 15-min digestion with Trypsin: Trypan blue negative cells approximating 15 microns or larger were present in similar numbers (based on per gram tissue starting material) in newborn, pubescent, and sexually mature individuals, but declined in number in the geriatric-aged tissue samples. However, the remaining material in all the samples consisted of cell debris (bits and pieces Trypan blue-positive material with sharp edges and no discernible Trypan blue negative spherical cells).
- **Experiment #2:** 15-min digestion with collagenase/dispase: Trypan blue negative cells approximating 15 microns or larger were present in similar numbers (based on per gram tissue starting material) in newborn, pubescent, and sexually mature individuals, but had declined in number in the geriatric-aged tissue samples. Trypan blue negative, negative/positive, and positive cells approximating 12 microns or smaller were present at similar levels to themselves in all four populations examined, e.g., newborn, pubescent, sexually mature, and geriatric-aged individuals, but were considerably fewer in number than the Trypan blue negative cells that were 15-microns or larger.
- **Experiment #3:** Digestion times increased dependent on age of the individual, e.g., 15-min (newborn), 1-hr. (pubescent), 3-hr. (sexually-mature), and 8.5-hr. (geriatric), with collagenase/dispase: All cells, both Trypan blue negative cells approximating 15 microns or larger, and cells approximating 12-mincrons or less that were Trypan blue negative, negative/positive, and positive, showed about a two-fold increase in the numbers of released cells at all four digestion times examined. However, cells approximating 15-microns or larger still showed a decrease in number of cells in geriatric-aged individuals compared to newborn, pubescent, and sexually mature, while cells approximating 12-microns or less that were Trypan blue negative, negative/positive, and positive, remained constant with respect to themselves at all time points analyzed, but were still considerably fewer in number than the Trypan blue negative cells that were 15-microns or larger.

Discussion & Conclusions

In this study, the precursor cells were examined to confirm or deny the assertion that ALL adult stem cells, be they telomerase negative progenitor cells or telomerase positive stem cells, decrease in number with increasing age of an individual. Multiple fresh skeletal muscle human [Homo sapiens] biopsy specimens (newborn [<1-year-old], puberty [10-12-yer-old], sexually mature [30-32-year-old], and geriatric aged [85-year-old] individuals) were obtained from NIH/NIRS. In the first experiment, the muscle biopsy specimens were digested for 15-minutes with trypsin at 37^{o}C [37,91,92], followed by processing for cell counting using Trypan blue exclusion. Using Figure 1 to distinguish telomerase negative progenitor cells from adult telomerase positive stem cells [62] was based both on Trypan blue staining patterns and size approximations. Telomerase negative progenitor cells were Trypan blue negative cells approximating 15 microns or larger and present in similar numbers (based on per gram tissue starting material) in newborn, pubescent, and sexually mature individuals, but declined in number in the geriatric-aged tissue samples. However, the remaining material in all the samples consisted of cell debris (bits and pieces Trypan blue-positive material with sharp edges and no discernible Trypan blue negative spherical cells).

The theory for using trypsin to release cells from their extracellular matrices is that trypsin cleaves between adjacent lysine and arginine residues [93]. Thus, trypsin will disrupt the core proteins of glycoproteins and proteoglycans within the extracellular matrices, releasing the cells. Apparently, the cell membranes of adult telomerase positive cells also contain an abundance of adjacent lysine and arginine residues [93,94], whereby the trypsin digest caused loss of cell membrane integrity with resultant formation of cell debris.

In the second experiment, instead of using short term trypsin digest, a combination of enzymes was used that would disrupt the cells attachments to their connective tissue collagen substrata, e.g., type-1 collagenase and dispase, rather than to disrupt the cell plasma membranes themselves [63]. Multiple fresh skeletal muscle human [Homo sapiens] biopsy specimens (newborn, puberty, sexually mature, and geriatric aged individuals) were obtained from NIH/NIRS. The muscle biopsy specimens were macerated to the consistency of orange marmalade and then digested for 15-minutes with 250 Units of type-1 collagenase and 33.3 units of dispase at 37^{o}C in DPBS containing EGTA [63], followed by processing for cell counting using 0.4% Trypan blue exclusion.

Instead of using trypsin, 25 mM EGTA in calcium/magnesium-free buffer and two enzymes, collagenase and dispase, were used to release viable cells from their substratum. Cells within an individual are attached to their extracellular matrices through glycoprotein moieties in their basement membranes [95-97]. The basement membrane is a non-cellular structure which contains type-4 collagen, heparan sulfate proteoglycan, laminin, fibronectin, etc. [95,97]. 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 [95,97]. The telomerase positive cells are attached to their collagen-based connective tissue substratum by two binding sites, a calcium-dependent binding site and an RGD-fibronectin-collagen dependent binding site [97].

EGTA is a specific calcium chelator, that has a higher binding coefficient than the calcium-dependent cell binding site [93], thus releasing the cells at this binding site. Collagenase and dispase disrupt the RGD-fibronectin-collagen binding site, releasing the cells from this binding site. By disrupting the extracellular matrix binding sites, rather than the cell membranes, intact viable cells are released from the connective tissues [98-102]. This was noted in the ability to recognize Trypan blue negative, negative/positive, and positive spherical entities of 12-microns or less in size [62].

Using Figure 1 to distinguish telomerase negative progenitor cells from adult telomerase positive stem cells based on Trypan blue staining patterns and size approximations, Trypan blue negative cells approximating 15 microns or larger were present in similar numbers (based on per gram tissue starting material) in newborn, pubescent, and sexually mature individuals, but had declined in number in the geriatric-aged tissue samples. Trypan blue negative, negative/positive, and positive cells approximating 12 microns of less were present at similar levels in all four populations examined, e.g., newborn, pubescent, sexually mature, and geriatric-aged individuals. However, the numbers of adult telomerase positive stem cells were significantly less than those of the progenitor cells at each time point examined.

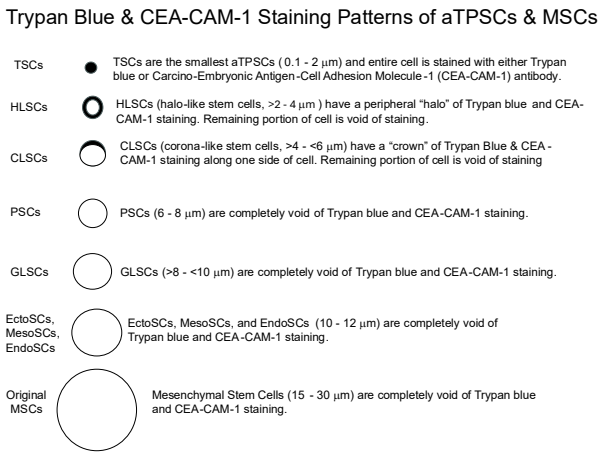

Figure 1: Trypan Blue and CEA-CAM-1 Staining Patterns of a TPSCs and MSCs.

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Competing and conflicting interests

Endogenous adult telomerase positive stem cells (aTPSCs) have competing interests with those laboratories studying mixed populations of telomerase negative progenitor cells termed stem cells, e.g., mesenchymal stem cells (MSCs), very small-embryonic-like stem cells (VSELs), multilineage-differentiating stress-enduring cells (MUSEs), marrow-isolated adult multilineage inducible cells (MIAMIs), small mobile stem cells (SMSs), and multipotent adult progenitor cells (MAPCs). The conflicting interest relates to comparing innate cellular characteristics, putative differentiation potentials and ability to affect a positive regenerative response when transplanted in vivo of single cell-derived clones of aTPSCs versus mixed cell populations of MSCs, VSELs, MUSEs, MIAMIs, SMSs, and MAPCs.

NOMENCLATURE

Humans, *Homo sapiens"*

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