Review Article

ISSN 2639-9512

Stem Cell & Regenerative Medicine

Characterization of Endogenous Telomerase-Positive Stem Cells for Regenerative Medicine, A Review

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Received: 12 May 2020; **Accepted:** June 2020

Citation: Henry E. Young, Mark O. Speight. Characterization of Endogenous Telomerase-Positive Stem Cells for Regenerative Medicine, A Review. Stem Cells Regen Med. 2020; 4(2): 1-14.

ABSTRACT

Numerous studies demonstrated the ability of stem cells in general, and endogenous adult stem cells in particular, to be a positive influence in the field of regenerative medicine. Endogenous naturally-occurring stem cells comprise approximately 50% of all cells in an adult organism. Within this group are two categories of cells based on the absence or presence of the enzyme telomerase. Telomerase-negative stem cells comprise approximately 40% of all cells in a post-natal adult, while the telomerase-positive stem cells comprise approximately 10%. Telomerase-negative stem cells are comprised of multipotent adult progenitor cells, mesenchymal stem cells, medicinal signaling cells, multilineage differentiating stress enduring cells, etc. Telomerase-negative progenitor stem cells have a restricted ability for cell doubling, based on Hayflick's Limit for humans of 50-70 population doublings before programmed senescence and cell death. While there has been a virtual explosion of papers detailing the attributes of various forms of telomerase-negative stem cells in the field of regenerative medicine, relatively few papers have been published with respect to telomerase-positive stem cells. The following is a review of work detailing the basic science characterization of telomerase-positive stem cells as well as their use in regenerative medicine.

Keywords

Stem cells, Enzymes, Regenerative medicine, Cell death.

Introduction

Numerous studies have demonstrated the ability of stem cells in general [1-6], and endogenous naturally-occurring stem cells in particular [7-9], to be a positive influence in the field of regenerative medicine. The endogenous naturally-occurring adult-derived stem cells comprise approximately 50% of all cells in an adult (post-natal) organism [10]. Within this group of endogenous adult-derived stem cells are two categories of cells based on the absence or presence of telomerase, i.e., telomerase-negative progenitor stem cells, approximating 40%, and telomerase-positive stem cells, approximating 10% [11]. Telomerase is responsible for the maintenance of the length of the telomeres, with its activity being present in gametes, tumor cells, and stem cells [12]. Cells that are absent of telomerase have a defined life-span before they are genetically preprogrammed to age and die [13,14]. Examples of telomerase-negative endogenous adult stem cells include hematopoietic stem cells [15,16], multipotent adult progenitor cells [17,18], marrow stromal cells [19], mesenchymal stem cells [20,21], multilineage differentiating stress enduring cells [22], medicinal signaling cells [23], myoblasts [24], etc. In contrast, cells that maintain telomerase have essentially an unlimited proliferation potential as long as they maintain an undifferentiated state [9]. While there has been a virtual explosion of papers detailing the attributes of various forms of endogenous naturally-occurring adult-derived telomerase-negative progenitor stem cells in the field of regenerative medicine, relatively few papers have been published with respect to endogenous naturally-occurring adult-derived telomerase-positive stem cells. The following is a review of work from our group and our collaborators detailing the basic science characterization of adult-derived telomerase-positive stem cells as well as their use in regenerative medicine.

Endogenous adult-derived telomerase-positive stem cells were discovered while examining limb regeneration in adult salamanders. Previous studies noted that only juvenile and/or aquatic amphibians, frogs and salamanders, could regenerate limbs and that adult amphibians had lost that particular ability when they had metamorphosed from an aquatic form to a terrestrial form [25,26]. There were two schools of thought regarding what stimulates regeneration in urodele aquatic amphibians versus higher order terrestrial animals: the threshold quantity of nervous tissue [27,28] and/or the presence of a stimulated epidermis, the apical epidermal cap [29,30].

The goal of the initial study was to determine how an adult terrestrial salamander (amphibian) could completely regenerate a lost appendage. Initially, the regeneration process was examined, including formation of appropriate structures [31-33], abnormal limb development [32,34], environmental conditions [32,35], nerve involvement [32,36], the regenerative extracellular matrix [32,36], involvement of hyaluronic acid and nervous tissue in the process [32,37], a thorough histological analysis of the regenerative process [32,38], a histochemical analysis of the effect of denervations on glycoconjugate composition and tissue morphology [32,39] as well as during the normal regeneration process [32,40]. While we noted that both nervous tissue and a stimulated epidermis were involved in complete regeneration of the terrestrial salamander's limb, we also noted the presence of homogenous very small nondescript cells within the blastema [41] that could regenerate tissues from both the ectodermal (e.g., epidermis, submucous skin glands, nerves) and mesodermal (blood vessels, dermis, skeletal muscle, cartilage, bone, associated connective tissues) embryonic germ layer lineages (Fig. 1) [42]. This suggested a potential third player necessary to regenerate tissues in higher order animals, i.e., stem cells with multiple differentiative capabilities [41].

To more completely understand the tissue repair portion of regenerative medicine, one needs to understand the normal developmental process of tissue differentiation from zygote to differentiated cells and tissues [42], as well as the cellular interaction with expressed extracellular matrix macromolecules. Previous histochemical studies in the adult salamander of normal [40] and regenerating [32,41] tissues noted distinctive "fingerprints" of carbohydrate extracellular matrix (ECM) macromolecules associated with particular cells and tissues.

For example, histochemical staining for hyaluronic acid was associated with nervous tissue [37,40]; keratan sulfate and chondroitin sulfate were noted in the ECM of normal and regenerating cartilage [32,36,40]; dermatan sulfate and chondroitin sulfate were noted in the ECM of normal and regenerating dermis [32,36,40]; and only chondroitin sulfate was present in normal and regenerating ECM of skeletal muscle and its associated connective tissues [32]. These particular fingerprints appear to match proteoglycans isolated from the ECM of cartilage, the keratan sulfate-chondroitin sulfate proteoglycan known as Aggrecan [43]; the pure chondroitin sulfate proteoglycan of skeletal muscle [44,45]; and the chondroitin sulfate, dermatan sulfate, and heparan sulfate proteoglycans of skeletal muscle-associated connective tissues from embryonic chick [46-48] and embryonic to senescent CBF-1 mouse (NIH's mouse model for aging) [49].

Development of Model Systems

To develop model systems to test for eventual clinical treatments in humans one needs a multi-prong approach. We choose to examine conditions for culturing and freezing cells from different species, e.g., avian [50,51], mouse [52], rat [53], rabbit [54], bovine [9] and human [55,56], from different source tissues [11,52,57-68]; devise appropriate in vitro [9,69-71] and *in vivo* [9,72-75] assay procedures; characterize the effects of various bioactive factors on cell-based phenotypic expression [9,52-54,57-62,68,69,76]; characterization of cell surface markers [9,11,55,64]; determine how telomerase-positive stem cells could be used within in vivo models of repair [9,65-68,76-83]; cloned the telomerase-positive stem cells from single cells, using repetitive single cell clonogenic analysis, for characterization of cell populations derived from a single cell, e.g., mesodermal stem cells [84], pluripotent stem cells [85], and totipotent stem cells [86]; have outside laboratories validate the results [87-91]; karyotype the telomerase-positive stem cells at various stages of proliferation past Hayflick's limit [71,94]; compare the telomerase-positive stem cells to a telomerase-negative stem cell in regenerative medicine and determine where the different types of stem cells would fit during the normal developmental processes [9-11,93,94,95]; determine the extent of proliferation of the telomerase-positive stem cells with respect to the biological clock of telomerase-negative cells [13,14]; develop animal model systems of diseases for neuronal repair [80], pulmonary disease [65,94], myocardial repair [83], diabetes [71,81,82], articular cartilage repair [9,77,78], bone repair [9,77,78], skeletal muscle repair [9,77,78], bone marrow transplant [9]; devise various methods to release the telomerase-positive stem cells from their connective tissue niches [61,62]); and design human trials to test effects of telomerase positive stem cells in regenerative medicine for ectodermal lineage disorders, i.e., central nervous system disorders, e.g., Parkinson disease (PD) [80,96], neurodegenerative diseases, and central nervous system injuries; mesodermal lineage disorders, i.e., myocardial infarction (MI) [83], cartilage and bone repair [9], skeletal muscle repair [9], systemic lupus erythematosus, celiac disease, chronic kidney disease; and endodermal lineage disorders, i.e., chronic obstructive pulmonary disease (COPD) [65,94], interstitial (aka, inflammatory, idiopathic) pulmonary fibrosis [65,94], and create an immuno-protected composite pancreatic organoid for the treatment of type-I diabetes [71,81].

Types of Endogenous Adult-Derived Stem Cells

We utilized three categories of endogenous adult-derived telomerase-positive stem cells for our proposed regenerative medicine therapies, e.g., totipotent stem cells (TSCs), pluripotent stem cells (PSCs), and mesodermal stem cells (MesoSCs).

Our studies revealed that the telomerase-positive totipotent stem cells were equivalent in differentiation potential to the blastomeres of the blastocyst during embryonic development of an individual

(compare Figure 2 with Figure 1), forming all tissues of the individual, including the germ cells (sperm and ova) and the notochord [10,11,86,93]. The telomerase-positive pluripotent stem cells were equivalent in differentiation potential to the embryonic epiblast germ layer lineage, forming all tissues of the individual except the germ cells and notochord [9-11,41,42,85,93]. And the telomerase-positive mesodermal stem cells had the capability to form at least 37 separate and distinct cell types within the mesodermal embryonic germ line lineage and was probably equivalent in differentiation potential to the embryonic mesoderm germ layer lineage, forming all tissues within the mesodermal germ layer lineage [9-11,41,42,84,93].

Two additional populations of telomerase-positive stem cells were identified within the body, i.e., ectodermal stem cells, capable of forming multiple cells within the ectodermal germ layer lineage, and endodermal stem cells, forming multiple cells within the endodermal germ layer lineage (Figures 1 and 2). Those studies also demonstrated that the telomerase-positive ectodermal stem cells, mesodermal stem cells, and endodermal stem cells would NOT cross over (would not transdifferentiate) to form cells of the other respective germ layer lineages (Figure 2) [9,11,41,42,50-61,65,70,71,76,77,79,84-89,93].

Phenotypic Expression Assay

We developed a unique immunocytochemical procedure (ELICA, enzyme-linked immuno-culture assay) (Figure 3) [69] for tissue sections and cultured cells where we could quantify (based on a standard curve) phenotypic expression using soluble substrates with a UV/Visible spectrophotometer, visualize phenotypic expression using insoluble substrates with bright-field microscopy, and quantify DNA content (based on a standard curve) all on a separate tissue section or within a single well of a 96-well plate. Because of the 10:1 amplification procedure, the sensitivity of our probes, the high throughput capabilities, and the sensitivity of the equipment, the lower limit of detectability was nanogram quantities of phenotypic expression markers per nanogram quantity of DNA. That gave us a very powerful biological assay to identify and quantify factors that influenced cells involved in tissue replacement and repair, e.g., inhibitory, stimulate proliferation, gain or loss of expressed genes, acceleration of phenotypic expression, and multiple inductive capabilities. We utilized the ELICA procedure for all of the in-house studies [9,11,41,42,50-68,70,71,76,77,79-86,93,95].

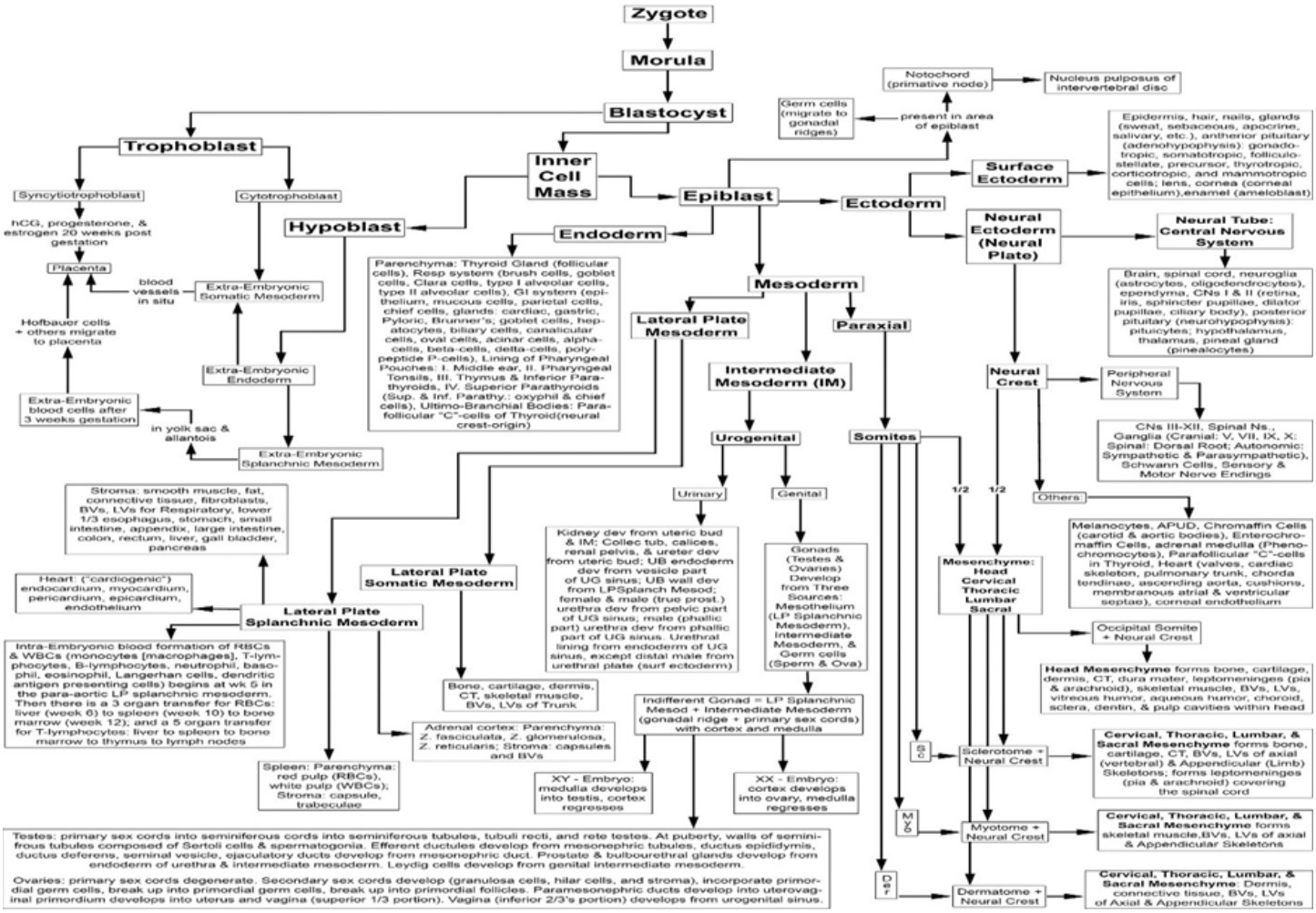

Figure 1: Lineage Map of Embryonic Development. Reprinted with permission from Young HE, Black Jr AC. Adult stem cells. Anat. Rec. 276A:75-102, 2004.

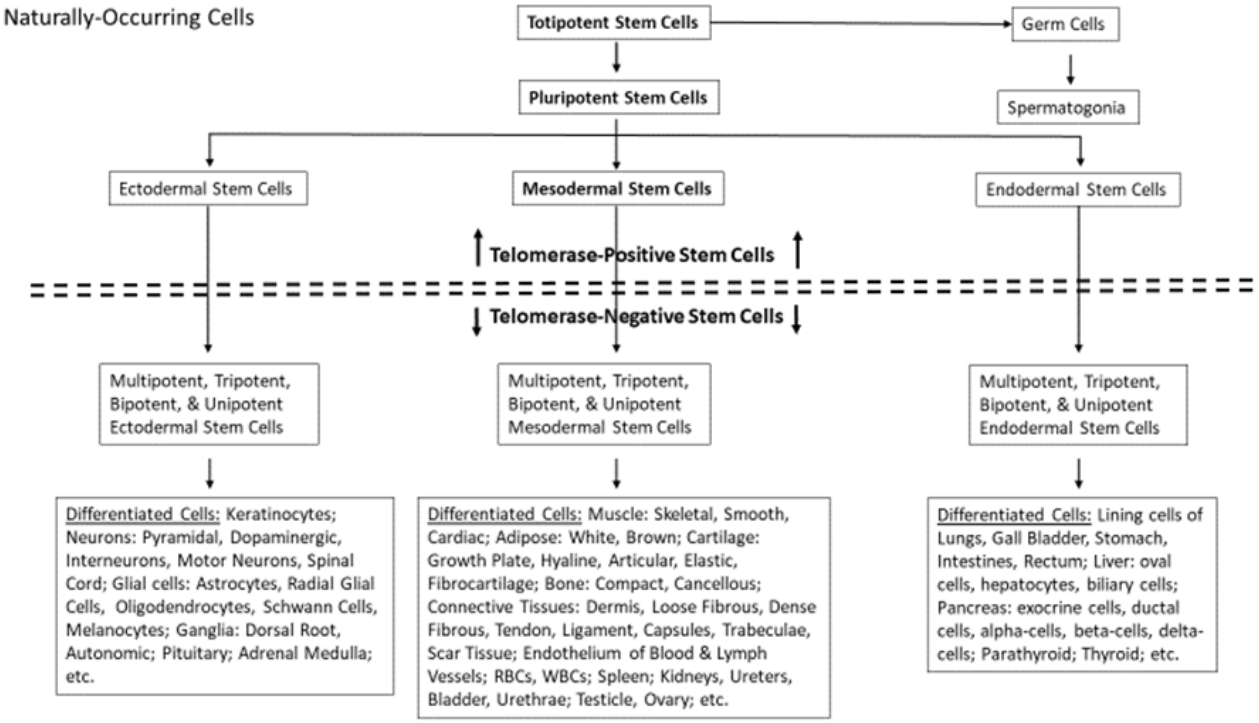

Figure 2: Diagram of telomerase-positive stem cells with essentially unlimited proliferation potential (above dotted line) and telomerase-negative stem cells, which conform to Hayflick's limit of 50-70 population doublings before senescence and cell death (below dotted line) found within the body and their respective downstream differentiation potentials.

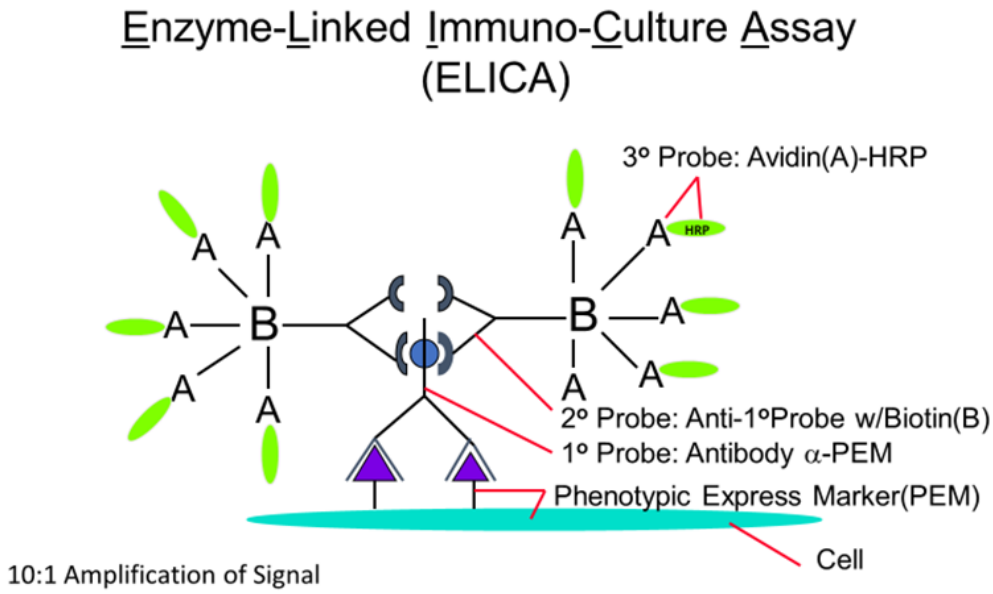

Figure 3: Enzyme-Linked Immuno-Culture Assay (ELICA) immunocytochemical procedure was developed to amplify signal expression to quantify antibody binding using a soluble horseradish peroxidase (HRP) substrate, followed by visualizing antibody binding using an insoluble HRP substrate (AEC or DAB), followed by quantification of DNA, all within the same well of a 96-well plate or on a single tissue section. This procedure utilizes multiple wash and blocking steps to prevent non-specific binding of reagents to cultured cells and/or tissue sections. Reagents used are a primary (1o) probe, which is an antibody to a cell surface or intracellular phenotypic expression marker (PEM); a secondary (2o) probe, which is an antibody directed against the species of the 1o probe with an attached biotin; a tertiary (3o) probe, consisting of an avidin molecule with an attached HRP enzyme; and HRP substrates. Hydrogen peroxide and sodium azide were used in the initial blocking step because cells contain an endogenous peroxidase that would react with the HRP substrate giving a false positive. Reprinted with permission from Young et al. Location and characterization of totipotent stem cells and pluripotent stem cells in the skeletal muscle of the adult rat. J Stem Cell Res 1(1) 002: 1-17, 2017.

Char[1]	us-TSC[2]	s-TSC[3]	HLSC[4]	CLSC[5]	ELSC[6]	GLSC[7]	MesoSC[8]	MSC[9]
Size	0.1-1 µm	1-2 µm	2-4 µm	4-6 µm	6-8 µm	8-10 µm	10-12 µm	10-20 µm
Try Blue[10]	Pos[11]	Pos	Pos/Neg[12]	Neg/Pos	Neg	Neg	Neg	Neg
Telomerase	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg
With Aging	Constant	Constant	Constant	Constant	Constant	Constant	Constant	Decrease
Viab PM[13]	30+ days	30+ days	>2 days	>2 days	>7 days	>5 days	3 days	1 day
Viab T[14]	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C
Sol Tiss[15]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
In CTs[16]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
B Mar[17]	Yes	Yes	NYD[18]	NYD	Yes	Yes	Yes	Yes
Adipose	Yes	Yes	NYD	NYD	Yes	Yes	Yes	Yes
Blood	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Species Utilized[19]	Av, KD, M,Rt,Rb,F, Cn,O,Cp, P,B,SpB, E,H	Av, KD, M,Rt,Rb,F, Cn,O,Cp, P,B,SpB, E,H	F, Cn, O, Cp, P, H	F, Cn, O, Cp, P, H	Av, KD, M,Rt,Rb,F, Cn,O,Cp, P,B,SpB, E,H	H	Av, KD, M,Rt,Rb,F, Cn,O,Cp, P,B,SpB,E,H	Rt,
Clone[20]	Rat-Scl-44β, Rat-Scl-4β	Rat-Scl-9β	NYD	NYD	Rat-Scl-40β	NYD	Rat-A_2A_2	Rt-Adip[21], Rt-Chon[22], Rt-Os[23]
Con Hib[24]	No	No	No	No	No	Yes	Yes	Yes
Survive Con Hib	NA	NA	NA	NA	NA	Yes	Yes	No
Growth[25]	Suspension[26]	Adherent[27]	Adherent	Adherent	Adherent	Adherent	Adherent	Adherent
Substrate	None	Collag-I[28]	Collag-I	Collag-I	Collag-I	Collag-I	Collag-I	None
SFM[29]	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
No F[30]	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent	Quiescent
Inhib F[31]	Respond	Respond	Respond	Respond	Respond	Respond	Respond	Respond
Prolif F[32]	Prolif[33]	Prolif	Prolif	Prolif	Prolif	Prolif	Prolif	Prolif
Prog F[34]	No	No	No	No	No	No	No	Yes
Induc F[35]	Yes	Yes	Yes	Yes	Yes	Yes	Only Mesodermal[36]	Only Fat, Cart[37], Bone
Commit[38]	s-TSC	HLSC	CLSC	ELSC	GLSC	EctoSCs[39], MesoSCs, EndoSCs[40]	MesoPCs[41]	Fat, Cartilage, Bone
# Cs ID[42]	68	67	66	65	63	62	37	3
Lineages[43]	3 + Sp44	3 + Sp	3	3	3	3	1	1
Prolif Rt[45]	12-14 hours	12-14 hours	12-14 hours	12-14 hours	12-14 hours	14-18 hours	18-24 hours	Days to weeks
Pop Dbl[46]	>300	>300	>300	>300	>400	>400	>690	50-70
Cryo Ag[47]	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
Con Ag[48]	7.5%	7.5%	7.5%	7.5%	7.5%	7.5%	7.5%	10%
# Cs Cry[49]	1-10 B[50]	1-10 B	1-10 B	1-10 M[51]	1-10 M	1-10 M	1-10 M	1-10 M
Op Fr T[52]	-80°C	-80°C	-80°C	-80°C	-80°C	-80°C	-70°C	-196°C
Fr Prot[53]	Slow	Slow	Slow	Slow	Slow	Slow	Slow	Flash
Op St T[54]	-80°C	-80°C	-80°C	-80°C	-80°C	-80°C	-70°C	-196°C
Thaw T[55]	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C
Recovery	>98%	>98%	>98%	>98%	>98%	>98%	>98%	>95%
Karyotype	Normal	Normal	NYD	NYD	Normal	NYD	Normal	Normal
Genes[56]	Telom[57], Bcl-2, Nanog, Nanos, CXCR4	Telom, Bcl-2, Nanog, Nanos, CXCR4	NYD	NYD	Telom, Oct-4[58], Sonic-hh[59]	NYD	Telom	NYD
Cell Surf Markers[60]	CEA[61] HCEA[62] CD66e[63]	CEA[61] HCEA[62] CD66e[63]	$SSEA^{low}$ $CD10^{low}$ CEA^{high} $CD66e^{high}$	$SSEA^{high}$ $CD10^{high}$ CEA^{low} $CD66e^{low}$	SSEA[64], CD10[65]	$SSEA^{high}$ $CD10^{high}$ $CD90^{low}$ Thy-1^{low}	CD90[66] Thy-1[67] CD13[68] MHC-I[69]	CD105[70] CD117[71] CD166[72] MHC-I

Animal Models	Parkinson disease, Myocardial Infarction, Pulmonary Disease, Skeletal Muscle, Bone, Cartilage, Blood Vessels, Inhibit Scar Formation, Pancreatic Islets	Parkinson disease, Myocardial Infarction, Pulmonary Disease, Skeletal Muscle, Bone, Cartilage, Blood Vessels, Inhibit Scar Formation, Pancreatic Islets	NYD	NYD	Parkinson disease, Myocardial Infarction, Pulmonary Disease, Skeletal Muscle, Bone, Cartilage, Blood Vessels, Inhibit Scar Formation, Pancreatic Islets	Parkinson disease, Myocardial Infarction, Pulmonary Disease, Skeletal Muscle, Bone, Cartilage, Blood Vessels, Inhibit Scar Formation, Pancreatic Islets	Myocardial Infarction, Skeletal Muscle, Bone, Cartilage, Blood Vessels, Inhibit Scar Formation,	Cartilage, Fat, Bone
Human Models	AD[73], ALS[74], B[75], CIDP[76], D[77], MD[78], MS[79], N[80], PD[81], Sc[82], Sk[83], TBI[84], TSCI[85], MI[86], CVD[87], IPF[88], COPD[89], OA[90], RA[91], SLE[92], SkM[93], Sk-MMP[94], SIF[95], Diabetes-I[96], CKD[97]	AD, ALS, B, CIDP, D, MD, MS, N, PD, Sc, Sk, TBI, TSCI, MI, CVD, IPF, COPD, OA, RA, SLE, SkM, Sk-MMP, SIF, Diabetes-I, CKD	AD, CIDP, PD, Sc, IPF, COPD, SLE	AD, CIDP, PD, Sc, IPF, COPD, SLE	AD, ALS, B, CIDP, D, MD, MS, N, PD, Sc, Sk, TBI, TSCI, MI, CVD, IPF, COPD, OA, RA, SLE, SkM, Sk-MMP, SIF, Diabetes-I, CKD	AD, CIDP, PD, Sc, IPF, COPD, SLE	AD, ALS, B, CIDP, D, MD, MS, N, PD, Sc, Sk, TBI, TSCI, MI, CVD, IPF, COPD, OA, RA, SLE, SkM, Sk-MMP, SIF, CKD	NYD

Table 1: Char[1], characteristics tested; us-TSC[2], ultra-small totipotent stem cell; s-TSC[3], small totipotent stem cell; HLSC[4], halo-like stem cell; CLSC[5], corona-like stem cell; ELSC[6], epiblast-like stem cell; GLSC[7], germ layer lineage stem cell; MesoSC[8], mesodermal stem cell – will form all cell types of the mesodermal germ layer lineage; MSC[9], mesenchymal stem cell; Try Blue[10], 0.4% Trypan Blue staining; Pos[11], Positive; Neg[12], Negative; Viab PM[13], viability post mortem (after removal from the animal); Viab T[14], viability temperature; Sol Tiss[15], presence in solid tissues; B Mar[17], bone marrow; NYD[18], not yet determined; Species[19]: Av (chicken, Waddle Crane), KD (Komodo Dragon), M (mouse), Rt (rat), Rb (rabbit), F (feline, cat), Cn (canine, dog), O (ovine, sheep), Cp (caprine, goat), P (porcine, pig), B (bovine, cow), SpB (Speckled Bear), E (equine, horse), H (human); Clone[20], clonal cell populations generated by repetitive single cell clonogenic analysis; Rt-Adip[21], rat-adipogenic progenitor cell; Rt-Chon[22]; rat-chondrogenic progenitor cell; Rt-Os[23], rat-osteogenic progenitor cell; Con Hib[24], contact inhibition of cells at confluence of growth in culture; Growth[25], growth attachment capabilities in culture; Suspension[26], ability to grow in culture suspended in the medium without any attachment to a substratum; Adherent[27], ability to grow in culture when attached to a substratum; Collag-I[28], collagen type-I substratum needed to be provided for cell attachment to occur; SFM[29], serum-free defined medium; No F[30], no growth factors added to the medium; Inhib F[31], inhibitory factors added to the medium to prevent differentiation of the cells, e.g., LIF, leukemia inhibitory factor or ADF, anti-differentiation factor; Prolif F[32], proliferation factor added to the medium, i.e., platelet-derived growth factor BB (PDGF-BB) to stimulate cellular proliferation; Prolif[33], proliferation of cells tested for increased content of DNA per well as assessed by the DNA portion of the ELICA procedure; Prog F[34], progression factor (2 ng/ml insulin) added to the medium to accelerate the phenotypic expression of lineage-committed progenitor cells; Induc F[35], induction factor added to the medium to induce the expression of multiple phenotypes in culture. We routinely used dexamethasone at 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} molar concentrations as a non-specific induction agent for mesodermal lineage cells. We also used specific induction agents to induce specific cellular phenotypes, e.g., SkM-MP for skeletal muscle, Sm-MP for smooth muscle, Cm-MP for cardiac muscle, Adip-MP for adipocytes, Ch-MP for cartilage, Os-MP for bone, BMP-2 for bone, VEGF for endothelial cells, EPO for erythrocytes, HGF for hepatocytes, BDGF for neurons and glial cells, NGF for neurons, and conditioned medium (exosomes) from differentiated cells and tissues to induce differentiation into pancreatic islets, spermatogonia, etc.; Mesodermal[36], mesodermal germ layer lineage cells; Cart[37], cartilage; Commit[38], commitment into a specific cell type as defined by its unique characteristics of cell surface markers, pattern of phenotypic expression markers, and differentiation capabilities; EctoSCs[39], ectodermal stem cells – will form all cell types of the ectodermal germ layer lineage; EndoSCs[40], endodermal stem cells – will form all cell types of the endodermal germ layer lineage; MesoPCs[41], telomerase-negative mesodermal progenitor cells; # Cs ID[42], number of cells identified by unique phenotypic expression markers. The number of cell types identified was not dependent on the differentiation capabilities of the cells, but rather the limited number of objective assays at our disposal to identify different cell types; Lineages[43], refers to embryonic germ layer lineages, e.g., ectoderm, mesoderm, and endoderm; Sp[44], spermatogonia; Prolif Rt[45], proliferation rate of the cells, assessed during log phase growth; Pop Dbl[46], population doublings; Cryo Ag[47], cryogenic agents used to freeze the cells; Con Ag[48], percent concentration of the cryogenic agent used to freeze the cells; # Cs Cry[49], maximum number of cells that could be frozen in a single aliquot of cells; B[50], billion; M[51], million; Op Fr T[52], optimum freezing temperature; Fr Prot[53], particular freezing protocol used; Op St T[54], optimal storage temperature; Thaw T[55], optimal thawing temperature; Genes[56], genes expressed in the naïve undifferentiated state; Telom[57], telomerase; Oct-4[58], genetic expression of the Oct-3/4 gene; Sonic-hh[59], expression of the sonic-hedge-hog gene; Cell Surf Markers[60], cell surface markers used for FACS (fluorescent-activated cell sorting) analysis included CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56, CD59, CD61, CD62e, CD65, CD66e, CD68, CD69, CD71, CD83, CD90, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, MHC-I, HLA-DR-II, FMC-7, Annexin-V, and Lin; CEA[61], carcinoembryonic antigen-cell adhesion molecule-1; HCEA[62], human carcinoembryonic antigen; CD66e[63], carcino-embryonic antigen, human variant "e"; SSEA^{low}, stage specific embryonic antigen-4 at low levels of detection by FACS analysis; CD10^{low}, CLLA antigen at low levels of detection by FACS

analysis; CEA^{high}, carcinoembryonic antigen at high levels of detection; CD66e^{high}, CD66e at high levels of detection by FACS analysis; SSEA[64], stage-specific embryonic antigen-4; CD10[65], cell surface enzyme with neutral metalloendopeptidase activity; CD90[66] (or Thy-1), heavily N-glycosylated, glycophosphatidylinositol anchored conserved cell-surface protein with a single V-like immunoglobulin domain present in adult stem cells, cancer cells, and MSCs; Thy-1[67](or CD90), heavily N-glycosylated, glycophosphatidylinositol anchored conserved cell-surface protein with a single V-like immunoglobulin domain; CD13[68], aminopeptidase; MHC-I[69], major histocompatibility marker-class-I; CD105[70], transmembrane glycoprotein, endoglin; CD117[71], receptor tyrosine kinase protein; CD166[72], a transmembrane glycoprotein that is a member of the immunoglobulin superfamily of proteins; AD[73], Alzheimer's Disease; ALS[74], Amyotrophic Lateral Sclerosis; B[75], Blindness; CIDP[76], Chronic Inflammatory Demyelinating Polyneuropathy; D[77], Dementia; MD[78], Macular Degeneration; MS[79], Multiple Sclerosis; N[80], Neuropathies; PD81, Parkinson Disease; Sc[82], Sciatica; Sk[83], Stroke; TBI[84], Traumatic Brain Injury; TSCI[85], Traumatic Spinal Cord Injury; MI[86], Myocardial Infarction; CVD[87], Cardiovascular Disease; IPF[88], Interstitial Pulmonary Fibrosis; COPD[89], Chronic Obstructive Pulmonary Disease; OA[90], Osteoarthritis; RA[91], Rheumatoid Arthritis; SLE[92], Systemic Lupus Erythematosus; SkM[93], skeletal muscle repair; Sk-MMP[94], Skeletal Muscle Morphogenetic Protein; SIF[95], Scar Inhibitory Factor; Diabetes-I[96], Diabetes type-I; CKD[97], Chronic Kidney Disease.

Attributes	Telom-Positive Totipotent Stem Cells	Telom-Negative Progenitor Stem Cells	Telom-Positive induced Pluripotent Stem Cells (iPSCs)	Telom-Positive Embryonic Stem Cells (ESCs)
% in Adults[1]	10%	40%	50%	NA
Telomerase[2]	Positive	Negative	Positive	Positive
Native Location in the Body[3]	Connective Tissue Matrices Throughout	Organ-Associated	Throughout the Body	2-Cell Stage to Blastocyst
Age Range[4]	Newborn to Geriatric	Newborn to geriatric	Newborn to geriatric	2-Cell Stage to Blastocyst stage
Numbers With Aging[5]	Remain Constant	Decline with age	Remain Constant	End at Blastocyst stage
Native Naïve State	Quiescent	Quiescent	Spontaneous Differentiation	Spontaneous Differentiation
Teratoma Formation In Vivo[6]	Absent	Absent	Present	Present
Responsive to Inhibitory Factors[7]	Yes	Yes	Yes	Yes
Increase in Cell Numbers[8]	In Situ & Ex Vivo	Ex Vivo	Ex Vivo	Ex Vivo
Growth in Culture[9]	Suspension & Adherent	Adherent	Adherent	Adherent
Responsive to Proliferation Factors[10]	Yes	Yes	Yes	Yes
Proliferation Potential[11]	Unlimited	Hayflick's Limit 50-70 Doublings	Unlimited	Unlimited
Responsive to Inductive Factors[12]	Yes	Only in Committed Lineage	No	No
Responsive to Local Cues[13]	Yes	Only in Committed Lineage	No	No
Cell Types Formed[14]	All Cells Types	Lineage Committed Cell Types	All Cell Types	All Cell Types
Time Period Fresh Isolate to In Vivo Use[15]	4 hours	4 hours	1-2 years	1-2 years
Time Period Isolation to Ex Vivo Use[16]	5-10 days	10-20 days	1-2 years	1-2 years
Treatment Number Potential[17]	Billions to Trillions	Millions	Millions	Millions
Ability to migrate to tissue damage[18]	Yes	Yes	Unknown	Unknown
Express MHC Class-I markers[19]	TSCs & PSCs No MesoSCs Yes	Yes	NA	No
Immuno-Protected[22]	Yes	No	Yes	No
Autologous Treatments[20]	Yes	Yes	Yes	No
Allogeneic Treatments[21]	TSCs & PSCs Yes MesoSCs No	Yes	Yes	Yes

Table 2: Comparison of attributes of telomerase-positive stem cells (TSCs, PSCs, and MesoSCs) to telomerase-negative stem cell (MSC), induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs), i.e., 1, percentage of stem cells present in adult individual; 2, presence or absence of the enzyme telomerase; 3, their native location within the body; 4, age range of the individual from which the cells can be removed; 5, their numbers with respect to aging of the individual; 6, native naïve state in vitro; 7, presence or absence of teratoma formation in vivo; 8, responsiveness to inhibitory agents such as leukemia inhibitory factor or anti-differentiation factor; 9, location of where induced increase in cell numbers can occur: in situ (in the body) or ex vivo (in culture); 10, responsiveness to proliferation factors; 11, proliferation potential; 12, responsive to inductive factors; 13, responsive to local environmental cues; 14, cell types formed; 15, time period required from isolation to use in vivo; 16, time period required from isolation to finished ex vivo expansion; 17, treatment number potential; 18, ability to migrate to tissue damage; 19, Express MHC Class-I markers; 20, Immuno-protected from the recipient's; 21, Autologous treatment; 22, Allogeneic treatment.

Characterization of Adult-Derived Stem Cells

As a prerequisite before utilizing the telomerase-positive stem cells for regenerative medicine therapies, we chose to extensively characterize totipotent stem cells [86], pluripotent stem cells [85], and mesodermal stem cells [84], and compared them to mesenchymal stem cells [20,21], a population of telomerase-negative stem cells. While most of our characterization studies occurred with clones of rat telomerase-positive stem cells, derived by repetitive single cell clonogenic analysis, and sorted human telomerase-positive stem cells, based on unique cell surface markers, we also examined these same stem cell populations from avians, reptile, mice, rabbits, cats, dogs, sheep, goats, pigs, cows, bears, and horses (Table 1) in a side by side comparison with a telomerase-negative stem cell, the mesenchymal stem cell derived from rats, as originally defined by Caplan [20] and characterized by Pittenger et al [21].

During the course of these studies we noted that the totipotent stem cells actually consisted of two subsets that could be separated both by size and growth in culture. Size was determined both by flow cytometry and the ability to pass through filters of decreasing pore size. We noted that a small percentage of the TSC population would pass through a 0.2 µm filter, but be retained in a 0.1 µm filter. We examined growth in culture analyzing growth in suspension cultures versus growth on a type-I collagen substratum versus growth on plastic. We noted that the 0.1 to <1.0 µm TSCs would grow in suspension, but not on a substratum; whereas the 1.0 to <2.0 µm TSCs grew on a provided type-I collagen substratum, but not in suspension. Neither would grow on plastic as a substratum. We designated these populations of stem cells as ultra-small totipotent stem cells (us-TSCs) and small totipotent stem cells (s-TSCs) (Table 1).

In addition, we noted that there were four subsets of pluripotent stem cells that could be segregated based on size, Trypan blue staining pattern, animal cell surface markers, and expressed cluster of differentiation (CD) (human) cell surface markers. These subsets of pluripotent stem cells were designated as halo-like stem cells (HLSCs), corona-like stem cells (CLSCs), epiblast-like stem cells (ELSCs), and germ layer lineage stem cells (GLSCs), as described below. Size was determined by flow cytometry. Staining was with 0.4% Trypan blue. Animal cell surface markers used were carcinoembryonic cell adhesion marker-1 (CEA-CAM-1), stage-specific embryonic antigen-4 (SSEA-4), and Thy-1. And CD markers used with human stem cells were CD66e, CD10, and CD90.

The pluripotent cell population with a of size from 2-4 µm had a complete halo of Trypan blue positive staining around its periphery with the center area of the cell absent of stain, hence the designated name 'Halo-Like Stem Cells'. The cell surface staining was high for CEA-CAM-1 & CD66e and low for SSEA-4 & CD10. The pluripotent cell population with a of size from 4-6 µm had a 'crown' of Trypan blue staining along one side of its periphery with the majority of the cell absent of stain, hence the designated name 'Corona-Like Stem Cells'. It's cell surface staining was low for CEA-CAM-1 & CD66e and high for SSEA-4 & CD10. The cell population with a size from 6-8 µm was Trypan blue negative, and stained with both SSEA-4 and CD10, and designated epiblast-like stem cells (ELSCs). The cell population with a size from 8-10 µm was Trypan blue negative, and stained high with SSEA-4 & CD10 and low for Thy-1 and CD90. This population was designated as germ layer lineage stem cells (Table 1). All four populations of pluripotent stem cells, HLSCs, CLSCs, ELSCs, and GLSCs would grow if a type-I collagen substratum was provided, but none would grow either in suspension or on bare plastic as a substratum.

The cell population that would only form cells of the embryonic mesodermal linage was 10-12 µm in size. It was absent of Trypan blue staining and stained for Thy-1, CD13, CD90, and MHC Class-I. This telomerase-positive stem cell population was originally designated as "pluripotent mesenchymal stem cells" [50,52]. However, with continued confusion with respect to Caplan's mesenchymal stem cell that could form three cell types [20,21] versus our "pluripotent mesenchymal stem cell" that could form at least 37 different cell types [9,84], we re-designated the population as "mesodermal stem cells". The telomerase-positive mesodermal stem cells (MesoSCs) differed from telomerase-negative mesenchymal stem cells (MSCs) with respect to presence or absence of telomerase, continued presence versus decline in numbers with respect to aging of the individual, days of survivability at 4°C after removal from the individual, survival when the cells reached contact inhibition, whether the cells would grow on bare plastic due to their ability to produce their own substratum for cell growth, whether the two populations were responsive to progression factors to accelerate phenotypic expression, the differentiative capabilities of the cells, how many different cell types of the populations would form, cell doubling time, number of population doublings, etc., and henceforth designated as a separate population of stem cells (Table 1).

Animal and Human Models for Regenerative Medicine

To determine the capabilities of adult-derived telomerase-positive stem cells to effect repair in ectodermal, mesodermal, and endodermal associated diseases, pre-clinical animal studies utilized a rat-derived PSC clone (Scl-40), derived from a single cell using repetitive single cell clonogenic analysis [85] and then labeled with the gene Lac-Z using lipofectin (Scl-40b). This was done so that the beta-galactosidase-genomically labeled pluripotent stem cell clone could be tracked in situ after implantation into various IACUC-approved animal model systems of germ layer lineage-associated diseases: Parkinson disease (ectodermal) [79,80,95], myocardial infarction (mesodermal) [9,83,85], and pulmonary diseases (COPD, IPF) [65]. These pre-clinical studies were then followed with IRB-approved protocols for the treatment of these same conditions in humans using adult-derived autologous and/or allogeneic telomerase-positive stem cells.

Neurodegenerative Disease Repair

Previous to our initial study [79], the preferred method to treat Parkinson disease, other than medicating with L-DOPA, was either surgical implantation of embryonic stem cells [96], surgical

implantation of fetal brain tissues [96] or surgical implantation of electrodes for deep brain stimulation [97]. All of these techniques required serious invasive surgeries for placement of either embryonic tissue, fetal tissue or electrodes in or near the substantia nigra of the midbrain.

Previous reports suggested that pharmacological drugs could be delivered by infusion through the olfactory epithelium, the nasal mucosa in the roof of the superior meatus of the nose, to bypass the blood-brain barrier and treat problems within the central nervous system (brain and spinal cord) [98-100]. Other investigators tried a similar intranasal route with significantly larger MSCs (10-20 μm), but noted that they needed a hyperosmolarity substance, such as mannitol, to shrink the cells of the olfactory epithelium to such an extent that their MSCs could migrate between the olfactory cells [101,102]. Their technique worked fine for individuals younger than puberty and for individuals older than puberty for a one-time only application. Following treatment, the olfactory epithelium would swell to their normal shape and size and the normal histoarchitecture of the olfactory epithelium was restored. However, for individuals older than puberty, a second treatment with the hyperosmolar mannitol solution created permanent channels between the olfactory epithelial cells that formed direct conduits to the meninges, enabling the potential for an increased propensity for bacterial meningitis.

The results from our IRB-approved trial for Parkinson disease demonstrated that application of autologous telomerase-positive stem cells was both safe and effective at reducing the symptoms of their disease and gave them a better quality of life in at least 75% of the population completing the trial at 14 months post treatment [80,95].

Cardiovascular Repair

Various treatment modalities for cardiovascular diseases have involved the restoration of blood flow to the ischemic heart muscle, either by physically opening the blocked vessels [103], replacing the blocked vessels with unblocked vessels [104], and/or trying to create collateral circulation by various methods, including the application decellularized bioscaffolds to the outside of the heart to act as a reservoir for growth factors, cytokines, and matricellular proteins to direct cardiac remodeling [105]. These techniques include re-opening the blocked arteries using balloon angioplasty followed by stents to keep the vessels open [103]; coronary arterial bypass graft (CABG) surgery [106,107]; and/or placement of a bolus of stem cells (ESCs, iPSCs, CSCs, MSCs, etc.) in the blocked or re-opened coronary vessels [108-111].

While opening the vessels with balloon angioplasty intuitively sounds like a good idea, subsequent use of stents, even drug eluting stents, have led to a tendency to form fibrous connective tissue in about 3-6 months that causes blockage of these vessels. To prevent fibrosis due to foreign material-induced inflammation, coronary arterial bypass graft (CABG) surgery has been utilized and has found to be superior over percutaneous coronary intervention (PCI) with drug eluting stents [104]. This technique uses either a segment of the large vein in the thigh, the great saphenous vein, with a reversal in direction to minimize the effects of the valves inside the vein or diverting the distal ends of the internal thoracic arteries that vascularize the front portion of the rib cage. It was noted that the use of venous conduits over arterial conduits had no significant superiority regarding long-term graft patency, rate of MI, overall mortality, and the rate of revascularization following the CABG procedure [112]. These non-heart vessels are attached to patent vessels to bypass the blocked segments of the coronary vessels allowing blood flow to continue into ischemic areas of the heart.

Regeneration of the patient's own blood vessels could permit the replacement of the patient's own stenotic coronary artery with an autologous clone of the artery. Such advanced regenerative therapy could eliminate some of the risks associated with current therapeutic approaches to the treatment of coronary artery disease and myocardial infarction. The use of stem cells provides promise in the treatment of patients who have suffered a myocardial infarction, as well as those in the earlier stages of CAD. However, the use of stem cells for repair of heart muscle has presented problems. The use of undifferentiated stem cells obtained from the human embryo is very controversial, for moral and ethical reasons. Moreover, the use of allogeneic embryonic stem cells requires immunosuppressive therapy to avoid problems associated with HLA-mismatch and tissue rejection, since immunosuppressive therapy can itself be a source of increased morbidity and mortality [113]. Teratoma formation occurred when using undifferentiated ESCs or iPSCs infusion for cardiac repair [114,115].

Some reports concerning the use of stem cells to attempt to repair the myocardium have appeared [116-118]. This work is in its infancy, and often has revolved around the use of mesenchymal stem cells. There is data indicating that more primitive cells such as very small embryonic-like (VSEL) stem cells are more effective in the treatment of lesions than the more differentiated mesenchymal stem cells, at least in the lung [119]. Subsequent studies with more differentiated cells noted inconsistencies in improvement in the clinical outcomes of heart function and cardiac remodeling and were found to be quite limited [109-111] [. The issue of which cells are most effective in healing the tissues of the body such as the myocardium, will certainly require further study. Unfortunately, if none of the above techniques (PCI, CABG, bioscaffolds, ESCs, iPSCs, MSCs, other stem cells), are successful at restoring blood flow to the ischemic heart and/or cardiac output remains below 15%, the patient is put on the list to receive a heart transplant.

Rather than placing a bolus of the stem cells at the apex of the coronary vessels as had been done for other stem cells, we took an alternative approach to revascularizing the ischemic heart muscle. We used the unique size of the TSCs (0.1-2.0 μm) (Table 1) along with the fact that the heart actually has two vascular systems, not just the coronary arterial/venous system. The heart also contains the vena communicantes minimae (small communicating veins), also known as the Thebesian veins. The Thebesian veins are small vascular channels without valves of less than 5 μm in diameter,

which are too small for blood cells, either RBCs (7 μm) or WBCs (10+ μm), or mesenchymal stem cells (10-20+μm) or MesoSCs (10-12 μm) or even PSCs (6-8 μm) to traverse. The Thebesian veins are found in all four chambers of the heart and run from inside the chambers, through the myocardium, to the pericardium lining the outside of the heart. When the heart undergoes systole (contraction) fluid is pushed from the inside chambers through the thebesian veins in the myocardium to the outside layer of the heart. During diastole (relaxation) fluid returns to the inside chambers through Thebesian veins through the myocardium from the outside layer of the heart. We hypothesized that by giving the heart TSCs (0.1-2 μm), these very small stem cells could repair the damaged myocardium and revascularize the heart as they traversed back and forth through the Thebesian system of vessels.

Utilizing the Thebesian veins approach, TSCs were given by slow intravenous (IV) infusion followed by PSCs and MesoSCs be regular IV infusion to cardiac patients with heart problems that had resulted from decreased cardiac outputs. The results demonstrated increases in cardiac function as noted by increases in their cardiac output by about 20% [83].

Pulmonary Diseases

New therapeutic strategies continue to be developed for the treatment of pulmonary diseases. Presently, much of the promise in treating chronic and incurable diseases of any organ system is centered on stem cell biology and the "holy grail" of regenerative medicine [9,10]. Despite the optimism and explosion of interest surrounding this new field, knowledge of the scientific basis for stem therapy for chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) is still in its infancy. The use of cell-based therapies to treat IPF is still at the experimental phase. Problems include bioethical issues, safety of cell transplantation, routes of delivery, dose, timing of administration, and outcome versus placebo control [120]. And while there have been promising results from early phase-I safety trials, there remain multiple reasons that "stem cells" are not ready for clinical application, starting from a gap in understanding at the bench research level, all the way to optimal clinical application in order to provide effective therapy [121]. This has been proved especially daunting when mesenchymal stem cells have been utilized as a potential treatment modality [122-135].

Autologous telomerase-positive stem cells, i.e., TSCs and PSCs by nebulization and MesoSCs by regular IV infusion, were given to patients exhibiting COPD and IPF. In both diseases, there was an increase in lung function, as expressed by a 13-18% increase in FEV1 in these individuals treated [65].

Comparison of Stem Cells for Regenerative Medicine

Endogenous adult-derived telomerase-positive stem cells comprise approximately 10% of all stem cells in the body. In comparison, telomerase-negative stem cells comprise approximately 40% of all cells in the body. Induced pluripotent stem cells are generated from differentiated cells, which account for the remaining 50% of cells in the body [10,11]. Embryonic stem cells are only present from the two-cell blastomere stage to the end of the blastocyst stage. However, what the telomerase-positive stem cells lack in sheer numbers in their native quiescent state, they make up for in some rather unique attributes (Table 2). Telomerase-positive stem cells display some rather unique attributes when compared to telomerase-negative stem cells such as the mesenchymal stem cell, to induced pluripotent stem cells, and to embryonic stem cells. These attributes include their unlimited proliferation potential, presence throughout the body, no decrease in numbers from newborn to geriatric-aged individuals, absence of teratoma formation from the naïve state, migration to areas of tissue damage, responsiveness to local environmental cues, ease of isolation, expansion of cell numbers in situ, expansion of cell numbers ex vivo by suspension culturing, relatively short time frame for cell number expansion, ease of use for directed treatments, and ability to repair/regenerate cell types from all three embryonic germ layer lineages in the appropriate histoarchitectural framework in the tissue in which they differentiate. From their inherent attributes (Table 2), we hypothesize that telomerase-positive stem cells would make excellent stem cell candidates for regenerative medicine. Preliminary studies with limited numbers of individuals demonstrated their safe application as well as demonstrating efficacious treatment for Parkinson's disease [80,94], Myocardial Infarction [83], and Pulmonary diseases [65]. Future studies will address their involvement as an autologous and/or allogeneic stem cell treatment modality in neurodegenerative diseases, cardiovascular disease, orthopedic/joint injuries, autoimmune diseases, renal disease, pulmonary diseases, and as an immuno-protective mechanism for pancreatic islet transplant to treat type-I diabetes.

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