Research Article

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Location and Characterization of Endogenous Naturally-Occurring Stem Cells in the Skeletal Muscle of the Adult Rat

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ABSTRACT

Endogenous naturally-occurring totipotent stem cells and pluripotent stem cells have been isolated from the skeletal muscle of adult mammals, including humans. This study was undertaken to determine their particular location within adult rat skeletal muscle and to verify the identity of the stained cells. Adult rats were euthanized following the guidelines of Mercer University's IACUC. Skeletal muscle was harvested and processed for immunocytochemistry. Cells were stained with carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) and stage-specific embryonic antigen-4 (SSEA-4). Positive and negative staining controls were run to verify the validity of the immunostaining. CEA-CAM-1+ cells were located preferentially within vascular connective tissues, whereas SSEA-4+ cells were located preferentially within neuronal connective tissues. CEA-CAM-1+ stem cells and SSEA-4+ cells were isolated from adult rat skeletal muscle, segregated, cloned from single cells, and their unique morphologies, differentiation potentials, and attributes in culture were subsequently characterized. Four populations of stem cells were identified that share the CEA-CAM-1 and/or SSEA-4 epitopes. Totipotent stem cells and transitional-totipotent stem cell/pluripotent stem cells share the CEA-CAM-1 epitope, whereas the transitional-totipotent stem cell/pluripotent stem cells, pluripotent stem cells, and transitional- pluripotent stem cell/germ layer lineage stem cells share the SSEA-4 epitope. This report describes native populations of endogenous naturally-occurring totipotent stem cells and pluripotent stem cells in adult rat skeletal muscle. Studies are ongoing to address their functional significance during normal tissue maintenance and repair of body organs.

Keywords

Totipotent stem cells, CEA-CAM-1, Pluripotent stem cells, SSEA-4, Skeletal muscle, Mammals, Immunocytochemistry, Sorting, cloning, Characterization.

Introduction

Four categories of precursor cells have been identified within tissues of postnatal mammals, including humans [1]. Following the nomenclature of Young and Black [2], which is based on the normal embryological development of the zygote, the four categories of precursor cells identified were lineage-uncommitted blastomere-like stem cells (BLSCs) which can form all somatic cells of the body and the gametes, spermatogonia and oogonia (putative totipotent stem cells); lineage un-committed epiblast-like stem cells (ELSCs) which can form all somatic cells of the body, but cannot form the gametes (putative pluripotent stem

cells); cell- and tissue-uncommitted germ layer lineage stem cells (GLSCs, i.e., putative ectodermal stem cells [EctoSCs] which can form all cell types of the ectodermal germ layer linage, putative mesodermal stem cells [MesoSCs] which can form all cell types of the mesodermal germ layer lineage, and putative endodermal stem cells [EndoSCs] which can form all cell types of the endodermal germ layer lineage); and cell- and tissue-committed progenitor cells, such as neuroblasts for nerve cells, glioblasts for glial cells, adipoblasts for fat cells, chondroblasts for cartilage, osteoblasts for bone, and hepatoblasts for liver cells, etc. (Figure 1) [2-18].

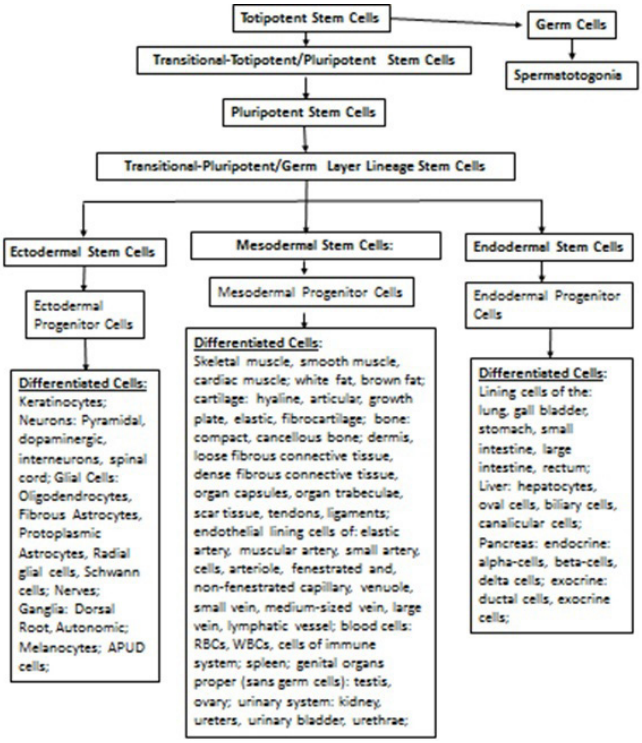

Figure 1: Unidirectional differentiation of precursor cells into adult differentiated cell types. This occurs from the most primitive totipotent stem cell, through transitional totipotent stem cell / pluripotent stem cells, to pluripotent stem cells, to transitional pluripotent stem cell / germ layer lineage stem cells, to germ layer lineage stem cells (e.g., ectodermal stem cells, mesodermal stem cells, and endodermal stem cells) to germ layer lineage progenitor cells (ectodermal progenitor cells, mesodermal progenitor cells, endodermal progenitor cells), to adult differentiated cell types.

The current study was designed to ascertain the location of native populations of carcinoembryonic antigen-1 positive (CEA-CAM-1+) cells and stage-specific embryonic antigen-4 positive (SSEA-4+) cells in selective regions of adult rat skeletal muscle. Further isolation, segregation, cloning, and characterization was included to verify the identities of these precursor cells that expressed epitopes for CEA-CAM-1 and SSEA-4 located within adult rat skeletal muscle.

Methods and Materials

Animal Use

The use of animals in this study complied with the guidelines of Mercer University's Institution Animal Care and Use Committee (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 (National Academy Press, 1996).

Tissue Harvest

Postnatal male Sprague-Dawley rats were euthanized using a lethal dose of a Ketamine/Xylazine mixture [19]. The abdomen and lower extremities were washed with Betadine solution, the skin resected and the underlying muscles, e.g., rectus abdominis, external and internal abdominal obliques, transversus abdominis, and quadriceps femoris (e.g., rectus femoris, vastus lateralis, vastus medialis, and vastus intermedialis), were removed pre- and post-trauma, cut into pieces of appropriate size, weighed, placed into their respective containers for either fixation with cryosectioning, or cell harvest, segregation, and characterization [1].

Tissue Fixation

The excised skeletal muscle was placed into fixative (20), consisting of aqueous 0.4% v/v glutaraldehyde, 2% w/v paraformaldehyde, and 1% w/v glucose, pH 7.4, with an osmolality 1.0. The tissue was fixed for 2 weeks and then rinsed with GIBCO's Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen Corporation, Carlsbad, CA).

Cryosectioning

The tissue was embedded in Tissue Tek OCT Compound 4583 (Miles Laboratory, Ames Division, Elkhart, IN) and then frozen at -20°C. The tissue was then sectioned on a Tissue Tek Cryostat II (GMI, Ramsey, MN) to a thickness of 7 microns. The sections were applied to positively charged slides (Mercedes Medical, Sarasota, FL) and stored at -20°C until stained.

Staining of Sectioned Tissue

The tissue sections were stained using the Enzyme-Linked Immuno-Culture Assay (ELICA) construct (Figure 2) [20] for carcinoembryonic antigen cell adhesion molecule-1 epitope (clone 5.4) (CEA-CAM-1) for totipotent stem cells [8] and stage-specific embryonic antigen-4 cell surface epitope (SSEA-4) (DSHB, Developmental Studies Hybridoma Bank, Iowa City, IA) for pluripotent stem cells [6]. Antibody 1A4 (Sigma, St Louis, MO) was used as a positive procedural control to stain smooth muscle alpha-actin in the tunica media of the vasculature within skeletal muscle. Negative procedural controls consisted of: DPBS buffer only, No 1° Antibody, No 2° Antibody (Rat Adsorbed Biotinylated Anti-Mouse IgG, Vector Laboratories Inc., Burlingame, CA) and No 3° (avidin HRP, Vector). The substrate used was AEC (Sigma). Thirty percent Hydrogen Peroxide (Sigma) and 5% Sodium Azide (Sigma) and were used as blocking agents to inhibit endogenous peroxidases. The slides were removed from -20°C storage, allowed to come to ambient temperature, fixed in situ with 95% Ethanol for 30 minutes, and rinsed with DPBS. Endogenous peroxidases were then blocked for 60 minutes with hydrogen peroxide followed by 30 minutes with sodium azide and then rinsed with DPBS. The tissue sections adherent to the slides were next incubated with the 1° antibodies for 60 minutes and rinsed with DPBS. The slides

were then incubated with the 2° antibody for 60 minutes and rinsed with DPBS. The slides were next incubated with the 3° probe for 60 minutes and rinsed with DPBS several times. The substrate was applied and allowed to incubate for 60 minutes. The substrate was then rinsed off with DPBS and the slide was cover-slipped using VectaMount (Vector) [20].

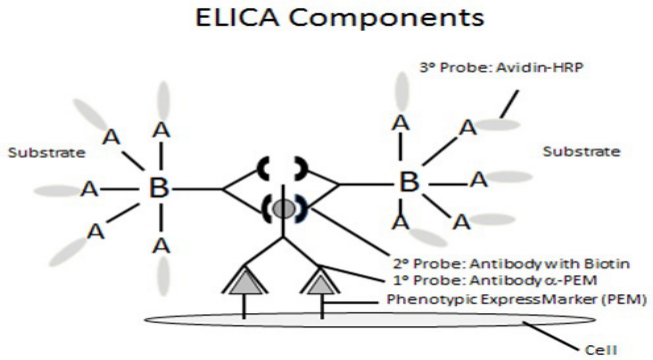

Figure 2: ELICA components consist of a primary probe, an antibody directed against a phenotypic expression marker, such as IA4 for smooth muscle alpha actin; a secondary probe, an anti-species antibody directed against the species that generated the first antibody, to which is attached biotin; a tertiary probe consisting of avidin-HRP (horseradish peroxidase enzyme); and a substrate for the HRP. These ELICA components result in a 10:1 amplification of the signal allowing for quantification of a soluble HRP substrate, followed by visualization of an insoluble HRP substrate at site of primary antibody binding, followed by DNA quantification to standardize the amount of phenotypic expression marker in ng per µg DNA.

Photography and Preparation

The tissue sections were photographed using a COOLPIX 995 digital camera and a Nikon Bright field / Phase Contrast / Fluorescent Fluorophot microscope. The digital photographs were cropped using Adobe Photoshop 7.0.

Isolation of Adult Stem Cells from Skeletal Muscle

Putative stem cells were harvested from skeletal muscle and segregated into separate cell populations following established procedures for differentiated cells, progenitor cells, germ layer lineage stem cells, pluripotent stem cells, and totipotent stem cells by cryopreservation (Figure 3), cell sorting (Figure 4), and single cell serial dilution clonogenic analysis (Figure 5) [4-7,8]. As viewed with phase contrast microscopy, the segregated cell populations demonstrated unique sizes and distinctive morphologies.

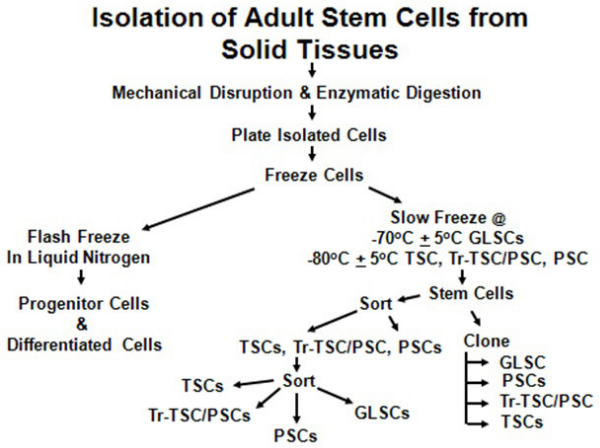

Figure 3: Schematic, Isolation protocol of precursor cells from skeletal muscle: differentiated cells, progenitor cells, germ layer lineage stem cells, pluripotent stem cells, transitional-totipotent stem cell/pluripotent stem cells, and totipotent stem cells. The flow chart is based on cryopreservation, and cloning by repetitive single cell clonogenic analysis or cell sorting using unique cell surface markers to separate GLSCs, PSCs, Tr-TSC/PSCs, and TSCs.

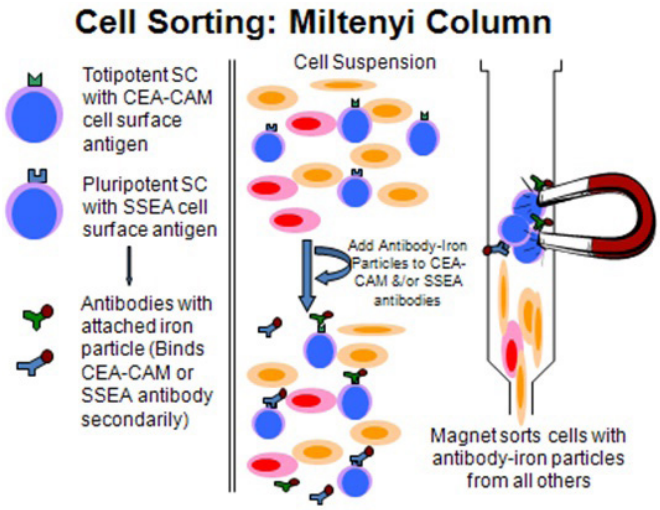

Figure 4: Miltenyi sort of pluripotent stem cells (PSCs are SSEA+) and totipotent stem cells (TSCs are CEA-CAM-1+) isolation from remaining cell types from stem cell harvest. Yields routinely in the 95-98% purity range.

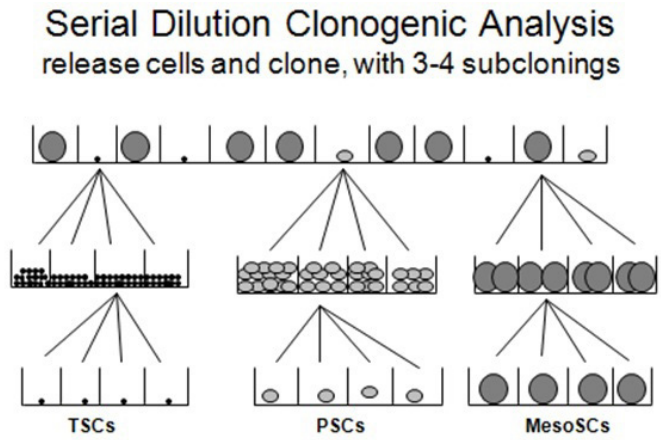

Figure 5: Single cell serial dilution clonogenic analysis for TSCs, PSCs, and MesoSCs. Single cells plated per well of 96-well plates in conditioned medium, grown post confluence, replated as single cells per well, grown post confluence, 3-4 times to insure that population of cells came from a single cell. Yields routinely in the 100% purity range.

Cell Counts

Final volume of each cell suspension was determined and recorded. Fifteen microliters of each cell suspension was removed and placed into separate 2.0 ml polypropylene tubes. Fifteen microliters of sterile 0.4% Trypan Blue solution (0.4% w/v Trypan blue [Kodak, Rochester, NY] in DPBS with Calcium and Magnesium [GIBCO], sterile filtered) was added to each tube. The contents were mixed by trituration 5-6 times and 15 µl of cell suspension-trypan blue mixture was placed on a hemocytometer for cell counting. All trypan blue-positive cells (BLSCs), positive/negative cells (Tr-BLSC/ELSCs), and negative cells (ELSCs, Tr-ELSC/GLSCs, GLSCs, MesoSCs, and MSCs) within the nine large boxes were

counted and then averaged for the number of adult cells per each large box. The formula to determine final cell number per ml was [(((average number)/5)/5) x 0.25) x 2] = cells x 10^6 cells per ml. Final cell number calculations were based on number of cells per gram of skeletal muscle. The isolated cells were counted on a hemocytometer by four separate individuals, the counts averaged and standardized per gram of tissue.

Karyotypic Analysis

Karyotypic analysis of two generated adult rat clones Scl-44β(BLSCs [TSCs]) and Scl-4β(ELSCs [PSCs]) was performed as described [21].

Culture Conditions

The following culture conditions were analyzed to determine similarities and differences between the six categories of cells isolated and the four adult rat clones generated (i.e., Scl-44β, Scl-40β, Scl-4β, and A2A2β). These conditions included cellular characteristics, cryopreservation parameters, culture conditions, requirements for substrates, growth in serum-free defined media, log phase doubling times, growth at confluence, replicative potential, and activities in the presence of a proliferations agents, progression agents, inductive agents, and inhibitory agents [1-8,20-34].

In Vitro Characterization of Differentiation Potentials

Cells were plated at 1.0 x 10^3 cells per well of 96-well plates in stem cell basal medium. Basal medium consisted of Opti-MEM with Glutamax (GIBCO) containing 1% v/v antibiotic – antimycotic (GIBCO), 10% v/v heat inactivated serum (Atlas Biologicals, Fort Collins, CO), pH 7.4. Culture flasks (Falcon) were pre-coated with 1% gelatin (EMSciences, Cherry Hill, NJ) as the type-I collagen-based substratum. The cells were incubated in a humidified incubator containing 95% air/5% carbon dioxide at 37°C for 24 hours, washed with DPBS (GIBCO) and then incubated with various general (Dexamethasone at 10^-10 to 10^-6M, Sigma) and selective bioactive factors singly and in combinatorial cocktails to induce proliferation, progression, differentiation, and inhibition of the cells, i.e., proliferation agents: platelet-derived growth factor-AA (PDGF-AA), platelet-derived growth factor-BB (PDGF-BB), platelet-derived growth factor-AB (PDGF-AB) [7,23]; progression agents: insulin, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) [7,23]; inductive agents: acidic-fibroblast growth factor (a-FGF), vascular endothelial cell growth factor (VEGF), basic-fibroblast growth factor (b-FGF), transforming growth factor-beta (TGF-β), bone morphogenetic protein-2 (BMP-2), epidermal growth factor (EGF), nerve growth factor (NGF), hepatocyte growth factor (HGF), erythropoietin (EPO), keratinocyte growth factor (KGF); inhibitory agents: leukemia inhibitory factor (LIF), anti-differentiation factor (ADF) [7,23]; and conditioned media from adult differentiated cell types, i.e., spermatogonia, skeletal muscle, smooth muscle, cardiac muscle, unilocular fat cells, multilocular fat cells, hyaline cartilage, articular cartilage, elastic cartilage, growth plate cartilage, fibrocartilage, endochondral bone, intramembranous bone, tendon, ligament, endothelial cells, neurons, glial cells, keratinocytes, gastrointestinal epithelial cells, liver cells, and pancreatic cells). Medium conditioned by differentiated cells was processed as follows. Medium at full establishment of differentiated cell phenotype was removed from the cultures, pooled, freeze/thawed (-20°C/37°C) three times, centrifuged at 100,000 x g for 18 hours, the supernatant decanted, and filtered through a 0.1 µm sterile filter. Processed conditioned medium was aliquoted and stored at -80°C until used. Differentiated cell characterization was performed following previously established procedures [1,4,6,7,22,23]. Living cells were assayed for size by flow cytometry, trypan blue inclusion/exclusion, unique cell surface markers, cryopreservation, culture substrates, cultivation with serum-free defined reagents, activity in serum-free defined medium without differentiation inhibitors, response to a proliferation agent, log phase doubling time, growth at confluence, replicative potential, expressed genes and differentiation potentials (Tables 1 and 2). Positive and negative staining controls were included to assure validity of the immunocytochemical staining (see above). Stained cells were visualized using a Nikon TMS inverted phase/bright field microscope with bright field microscopy at 400x and photographed as above.

Identification of Phenotypic Expression Markers

Immunocytochemical and histochemical analyses of pre- and post-induced cells were performed to ascertain the identity and number of cell types present. Pre- and post-induced cells were incubated with CEA-CAM-1 (Hixson), SSEA-1, stage-specific embryonic antigen-1 antibody MC480 (DSHB); SSEA-3, stage-specific embryonic antigen-3, antibody MC631 (DSHB); SSEA-4, stage-specific embryonic antighen-4, antibody MC-813-70 (DHSB); Thy-1, germ layer lineage stem cells (DSHB); and dhTuAg1 for putative spermatogonia [1,6,8,29,35,36].

Ectodermal germ layer lineage cells were identified as follows. Neuroblasts (neuronal progenitor cells) were identified using FORSE-1 (DSHB) for neural precursor cells, RAT-401 (DSHB) for nestin, HNES (Chemicon, Temecula, CA) for nestin, and MAB353 (Chemicon) for nestin [1,6,8,29,37-39]. Neurons were identified using 8A2 (DSHB) for neurons [40], S-100 (Sigma) for neurons [41], T8660 (Sigma) for β-tubulin III [42-44], RT-97 (DSHB) for neurofilaments [45], N-200 (Sigma) for neurofilament-200 [46,47], and SV2 (DSHB) for synaptic vesicles [48]. Ganglia were identified using TuAg1 for ganglion cells [49,50]. Astrocytes were identified using CNPase (Sigma) for astroglia and oligodendrocytes [51-53]. Oligodendrocytes were identified using Rip (DSHB) for oligodendrocytes [54] and CNPase (Sigma) for oligodendrocytes and astroglia [51-53]. Radial glial cells were identified using 40E-C (DSHB) for radial glial cells [55]. Keratinocytes were identified using VM-1(DSHB) to keratinocyte cell surface protein [56,57].

Mesodermal germ layer lineage cells were identified as follows. Skeletal muscle was identified as mononucleated myoblasts staining with OP137 (Calbiochem, San Diego, CA) for MyoD [58], F5D (DSHB) for myogenin [59], and DEU-10 (Sigma) for desmin [60], and as multinucleated spontaneously contracting structures staining with MF-20 (DSHB) for sarcomeric myosin [61], MY-32

Charac	BLSCs	Tr-BLSC/ELSCs (HLSCs & CLSCs)	ELSCs	Tr-ELSC/GLSCs	MesoSCs	MSCs
Clone (Reference)	Scl-44β [8]	Scl-40β [6]	Scl-4β [6]		A2A2β [4]	
Flow Size	0.1 - 2.0 µm	>2.0 to <6.0 µm	6.0-8.0 µm	>8.0 - <10.0 µm	10-20 µm	Variable
Trypan blue	Positive	Positive/Negative	Negative	Negative	Negative	Negative
Cell Surface Markers	CEA-CAM-1	CEA-CAM-1 / SSEA-4	SSEA-4	SSEA-4 / Thy-1	Thy-1	NYD1
Cryoprotectant DMSO[2]	Ultra-pure	Ultra-pure	Ultra-pure	Ultra-pure	Ultra-pure	90%
Concentration	7.5% v/v[3]	7.5% v/v	7.5% v/v	7.5% v/v	7.5% v/v	10%
Temperature	-80°C	-80°C	-80°C	-70°C	-70°C	Liq Nit4
Freezing	Slow	Slow	Slow	Slow	Slow	Flash
Thaw	Flash	Flash	Flash	Flash	Flash	Flash
Recovery	95-98%	95-98%	95-98%	95-98%	95%	90%
Growth on polystyrene plastic	No	No	No	No	No	Yes
Culture Substrate	Collagen, Type-I	Collagen, Type-I	Collagen, Type-I	Collagen, Type-I	Collagen, Type-I	Makes Own Substrate
Suspension Culture	Yes	No	No	No	No	No
Adherent Culture	Yes	Yes	Yes	Yes	Yes	Yes
Calcium Dependent Binding Site	Yes	Yes	Yes	Yes	Yes	Yes
RGD Fibronectin Dependent Binding Site	No	Yes	Yes	Yes	Yes	Yes
Growth in TSC SFDM[5]	Yes	Yes	Yes	Yes	Yes	Yes
Growth in PSC SFDM[6]	No	No	Yes	Yes	Yes	Yes
Growth in GLSC SFDM[7]	No	No	No	No	Yes	Yes
SFDM only[8]	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence	Quiescence
Proliferation Agent in SFDM	Proliferate	Proliferate	Proliferate	Proliferate	Proliferate	Proliferate
Log Phase Doubling Time	12-14 hours	12-14 hours	12-14 hours	18-14 hours	18-24 hours	24+ hours
Growth at Confluence	Multiple Layers	Multiple Layers	Multiple Layers	Contact Inhibited	Contact Inhibited	Contact Inhibited
Replicative Potential	Extensive	Extensive	Extensive	Extensive	Extensive	Limited
Progression Agent in SFDM	None	None	None	None	None	Phenotypic expression
Inductive Agent in SFDM	Phenotypic Expression	Phenotypic Expression	Phenotypic Expression	Phenotypic Expression	Phenotypic Expression	None
Differentiation Potential	All somatic cells, gametes	All somatic cells	All somatic cells	All somatic cells	Mesodermal lineage cells	Fat, Cartilage, Bone
Number of Cell Types Formed	68	66	65	63	31	3
Functional Activity	Totipotent (TSCs[9])	Pluripotent (Tr-TSC/PSCs[10])	Pluripotent (PSCs[11])	Pluripotent (Tr-PSC/GLSC[12])	Mesodermal (MesoSCs[13])	Progenitor (MSCs[14])
Expressed Genes	Telomerase, BCL-2, Nanog, Nanos, CXCR4	Telomerase, Oct-3/4, Sonic Hedgehog	Telomerase, Oct-3/4, Sonic Hedgehog	Telomerase, Pax-6	Telomerase, Pax-6	NYD
Functional Activities in vivo	Parkinson's disease, insulin secretion, diagnostics, trauma	Parkinson's disease, insulin secretion, diagnostics, cardiovascular ischemia, trauma	Parkinson's disease, insulin secretion	Parkinson's disease, insulin secretion,	Repair: skeletal muscle, bone, cartilage; Bone marrow transplant	NYD

Table 1: BLSCs, Blastomere-Like Stem Cells; Tr-BLSC/ELSC, Transitional-Blastomere-Like Stem Cell/Epiblast-Like Stem Cell (HLSC, Halo-Like Stem Cells and CLSCs, Corona-Like Stem Cells); ELSC, Epiblast-Like Stem Cell; Tr-ELSC/GLSC, Transitional-Epiblast-Like Stem Cell/Germ Layer lineage Stem Cell; MesoSC, Mesodermal Stem Cell; MSC, Mesenchymal Stem Cell; NYD[1], not yet determined; DMSO[2], dimethyl sulfoxide; v/v[3], volume per volume; Liq Nit[4], liquid nitrogen; Growth in TSC SFDM[5], growth in totipotent serum-free defined medium (Moraga Biotechnology Corp, Los Angeles, CA); Growth in PSC SFDM[6], growth in pluripotent serum-free defined medium (Moraga Biotechnology Corp, Los Angeles, CA); Growth in GLSC SFDM[7], growth in germ layer lineage serum-free defined medium (Moraga Biotechnology Corp, Los Angeles, CA); SFDM[8], serum-free defined medium (Moraga Biotechnology Corp, Los Angeles, CA); TSCs[9], totipotent stem cells; Tr-TSC/PSCs[10], transitional-totipotent stem cell/pluripotent stem cells; PSCs[11], pluripotent stem cells; Tr-PSC/GLSC[12], transitional-pluripotent stem cell/germ layer lineage stem cell; MesoSCs[13], mesodermal stem cells; MSCs[14], mesenchymal stem cells.

Cell Types	TSC[1]	Tr-TSC/PSC[2]	PSC[3]	Tr-PSC/GLSC[4]	GLSC[5]	EctoSC[6]	MesoSC[7]	EndoSC[8]	MSC[9]
Spermatogonia[10]	+[11]	-[12]	-	-	-	-	-	-	-
Tr-TSC/PSC	+	-	-	-	-	-	-	-	-
PSC	+	+	-	-	-	-	-	-	-
Tr-PSC/GLSC	+	+	+	-	-	-	-	-	-
GLSC	+	+	+	+	-	-	-	-	-
EctoSCs [11 cell types]	+	+	+	+	+	-	-	-	-
NeuroEctoSCs[13]	+	+	+	+	+	+	-	-	-
Neuronal PCs[14]	+	+	+	+	+	+	-	-	-
Neuroblasts[15]	+	+	+	+	+	+	-	-	-
Pyramidal Ns[16]	+	+	+	+	+	+	-	-	-
Dopaminergic Ns[17]	+	+	+	+	+	+	-	-	-
Neurons[18]	+	+	+	+	+	+	-	-	-
Oligodendroblasts[19]	+	+	+	+	+	+	-	-	-
Oligodendrocytes[20]	+	+	+	+	+	+	-	-	-
Astroblasts[21]	+	+	+	+	+	+	-	-	-
Fibrous astrocytes[22]	+	+	+	+	+	+	-	-	-
Protoplasmic Astrocytes[23]	+	+	+	+	+	+	-	-	-
Synaptic Vesicles[24]	+	+	+	+	+	+	-	-	-
Radial glial Cells[25]	+	+	+	+	+	+	-	-	-
Keratinocytes[26]	+	+	+	+	+	+	-	-	-
MesoSCs [29 cell types]	+	+	+	+	+	-	-	-	-
Myoblasts[27]	+	+	+	+	+	-	+	-	-
Skeletal Muscle[28]	+	+	+	+	+	-	+	-	-
Smooth Muscle[29]	+	+	+	+	+	-	+	-	-
Cardiac Muscle[30]	+	+	+	+	+	-	+	-	-
Adipoblasts[31]	+	+	+	+	+	-	+	-	+
White Fat[32]	+	+	+	+	+	-	+	-	+
Brown Fat[33]	+	+	+	+	+	-	+	-	-
Chondroblasts[34]	+	+	+	+	+	-	+	-	+
Chondrocytes[35]	+	+	+	+	+	-	+	-	+
Hyaline Cartilage[36]	+	+	+	+	+	-	+	-	+
Articular Cartilage[37]	+	+	+	+	+	-	+	-	-
Elastic Cartilage[38]	+	+	+	+	+	-	+	-	-
Growth Plate Cartilage[39]	+	+	+	+	+	-	+	-	-
Fibrocartilage[40]	+	+	+	+	+	-	+	-	-
Perichondrium[41]	+	+	+	+	+	-	+	-	-
Osteoblasts[42]	+	+	+	+	+	-	+	-	+
Endochondral Bone[43]	+	+	+	+	+	-	+	-	+
Intramembranous Bone[44]	+	+	+	+	+	-	+	-	-
Periosteum[45]	+	+	+	+	+	-	+	-	-
Fibroblast[46]	+	+	+	+	+	-	+	-	-
Tendon/Ligament[47]	+	+	+	+	+	-	+	-	-
Loose Fibrous CT[48]	+	+	+	+	+	-	+	-	-
Dense Fibrous CT[49]	+	+	+	+	+	-	+	-	-
Dermis[50]	+	+	+	+	+	-	+	-	-
Scar Tissue[51]	+	+	+	+	+	-	+	-	-
Endothelial Cells[52]	+	+	+	+	+	-	+	-	-
Hematopoietic Cells[53]	+	+	+	+	+	-	+	-	-
Leukocytes[54]	+	+	+	+	+	-	+	-	-
Granulocytes/NK Cells[55]	+	+	+	+	+	-	+	-	-
Monocytes/Macrophages[56]	+	+	+	+	+	-	+	-	-

Megakaryocytes/Platelets[57]	+	+	+	+	+	-	+	-	-
EndoSCs [10 cell types]	+	+	+	+	+	-	-	-	-
Endodermal Progenitor Cells[58]	+	+	+	+	+	-	-	+	-
Gastrointestinal Epithelium[59]	+	+	+	+	+	-	-	+	-
Liver Oval Cells[60]	+	+	+	+	+	-	-	+	-
Hepatocytes[61]	+	+	+	+	+	-	-	+	-
Biliary Cells[62]	+	+	+	+	+	-	-	+	-
Canalicular Cells[63]	+	+	+	+	+	-	-	+	-
Pancreatic Islets[64]	+	+	+	+	+	-	-	+	-
Pancreatic Alpha-cells[65]	+	+	+	+	+	-	-	+	-
Pancreatic Beta-cells[66]	+	+	+	+	+	-	-	+	-
Pancreatic Delta-cells[67]	+	+	+	+	+	-	-	+	-
Pancreatic Ductal Cells[68]	+	+	+	+	+	-	-	+	-

Table 2: Cell Types Formed from Adult Precursor Cells.

TSC[1], totipotent stem cell (BLSC, blastomere-like stem cell); Tr-TSC/PSC[2], transitional-totipotent stem cell / pluripotent stem cell (Tr-BLSC/ELSC [HLSC & CLSC], transitional-blastomere-like stem cell/epiblast-like stem cell (i.e., Halo-Like Stem Cell and Corona-Like Stem Cell]); PSC[3], pluripotent stem cell (ELSC, Epiblast-Like Stem Cell); Tr-PSC/GLSC[4], transitional-pluripotent stem cell / germ layer lineage stem cell (Tr-ELSC/GLSC, transitional-epiblast-like stem cell/germ layer lineage stem cell); GLSC[5], germ layer lineage stem cells; EctoSC[6], ectodermal stem cell; MesoSC[7], mesodermal stem cell; EndoSC[8], endodermal stem cell expresses; MSC[9], mesenchymal stem cell is a tripotent mesenchymal progenitor cell that can differentiate into fat, cartilage, and bone; Spermatogonia[10], precursor to sperm; +[11], presence; -[12], absence. Ectodermal lineage cells: Neuro-EctoSCs[13], neural ectodermal stem cells; Neuronal PCs[14], neuronal progenitor cells; Neuroblasts[15], progenitor cell for neurogenic lineage cells; Pyramidal Ns[16], pyramidal neurons; Dopaminergic Ns[17], dopaminergic neurons; Neurons[18]; Oligodendroblasts[19], were identified by morphology and immuno-staining for MOSP; Oligodendrocytes[20]; Astroblasts[21] glial progenitor cells; Fibrous Astrocytes[22]; Protoplasmic Astrocytes[23]; Synaptic Vesicles[24], contained within pre-synaptic end bulbs; Radial Glial Cells[25]; and Keratinocytes[26], epidermal cells. Mesodermal lineage cells: Myoblasts[27], progenitor cells for muscle; Skeletal Muscle[28]; Smooth Muscle[29]; Cardiac Muscle[30], Adipoblasts[31], adipocyte progenitor cells; White Fat[32]; Brown Fat[33]; Chondroblasts[34], chondrogenic progenitor cells; Chondrocytes[35-40]; Hyaline Cartilage[36]; Articular Cartilage[37]; Elastic; Growth Plate Cart[39]; Fibrocartilage[40]; Perichondrium[41]; Osteoblasts[42], osteogenic progenitor cells; Endochondral Bone[43]; Intramembranous Bone[44]; Periosteum[45]; Fibroblast[46]; Tendon/Ligament[47]; Loose Fibrous CT[48]; Dense Fibrous CT[49]; Dermis[50]; Scar Tissue[51]; Endothelial cells[52]; Hematopoietic Cells[53]; Leukocytes[54]; Granulocytes/NK cells[55]; Monocytes / Macrophages[56]; Megakaryocytes/Platelets[57]; Endodermal Progenitor Cells[58]; Gastrointestinal Epithelium[59]; Liver Oval Cells[60]; Hepatocytes[61]; Biliary Cells[62]; Canalicular Cells[63]; Pancreatic Islets[64]; Pancreatic Alpha-cells[65]; Pancreatic Beta-cells[66]; Pancreatic Delta-cells[67]; and Pancreatic Ductal cells[68].

(Sigma) for skeletal muscle fast myosin [62], ALD-58 (DSHB) for myosin heavy chain [63], and A4.74 (DSHB) for myosin fast chain [64]. Smooth muscle was identified as mononucleated cells staining with antibodies IA4 (Sigma) for smooth muscle α-actin [65] and Calp (Sigma) for calponin [66,67]. Cardiac muscle was identified as binucleated cells co-staining with MF-20 (DSHB) + IA4 (Sigma) for sarcomeric myosin and smooth muscle α- actin [68,69], MAB3252 (Chemicon) for cardiotin [70] and MAB1548 for cardiac muscle (Chemicon). White fat, also denoted as unilocular adipose tissue, was identified as a mononucleated cell with peripherally located nucleus and containing a large central intracellular vacuole filled with refractile lipid and stained histochemically for saturated neutral lipid using Oil Red-O (Sigma) and Sudan Black-B (Chroma-Gesellschaft, Roboz Surgical Co, Washington, DC) [7]. Brown fat, also denoted as multilocular adipose tissue, was identified as a mononucleated cell with a centrally located nucleus containing multiple small intracellular vacuoles filled with refractile lipid and stained histochemically for saturated neutral lipid using Oil Red-O (Sigma) and Sudan Black-B (Chroma-Gesellschaft) [8,9]. Cartilage: structures thought to be cartilage nodules were tentatively identified as aggregates of rounded cells containing pericellular matrix halos.

Cartilage nodules were confirmed by both histochemical and immunochemical staining. Histochemically, cartilage nodules were visualized by staining the pericellular matrix halos for proteoglycans containing glycosaminoglycan side chains with chondroitin sulfate and keratan sulfate moieties. This was accomplished using Alcian Blue (Alcian Blau 8GS, Chroma-Gesellschaft), Safranin-O (Chroma-Gesellschaft) at pH 1.0, and Perfix/Alcec Blue (Aldrich Chemical Company, Milwaukee, WI). Verification of glycosaminoglycans specific for cartilage was confirmed by loss of extracellular matrix staining following digestion of the material with chondroitinase-AC (ICN Biomedicals, Cleveland, OH) and keratanase (ICN Biomedicals) [7,8,22,72,73] before staining (negative staining control). Immunochemically, the chondrogenic phenotype was confirmed by initial intracellular staining followed by subsequent staining of the pericellular and extracellular matrices with CIIC1 (DSHB) for type II collagen [74], HC-II ((ICN Biomedicals, Aurora, OH) for type II collagen [75,76], D1-9 (DSHB) for type IX collagen [77], 9/30/8A4 (DSHB) for link protein [78], and 12C5 (DSHB) for versican [79,80].

Types of cartilage were segregated based on additional attributes. Hyaline cartilage was identified by a perichondrial-like connective tissue surrounding the previously stained cartilage nodule and histochemical costaining for type I collagen [81]. Articular cartilage was identified as the above stained cartilage nodule without a perichondrial-like connective tissue covering [25]. Elastic cartilage was identified by nodular staining for elastin fibers and a perichondrial-like connective tissue surrounding the above stained cartilage nodule and histochemical co-staining for type I collagen [81,82]. Growth plate cartilage was identified by nodular staining for cartilage phenotypic markers and co-staining for calcium phosphate using the von Kossa procedure [6-8].

Fibrocartilage was identified as three-dimensional nodules

demonstrating extracellular histochemical staining for type I collagen [81,82] and co-staining for pericellular matrices rich in chondroitin sulfates A and C. The latter were assessed by Alcian Blue pH1.0 staining. Negative staining controls were digested prior to staining with chondroitinase-ABC or chondroitinase-AC [7,8,71,72]. Intramembranous bone was identified as a direct transition from stellate-shaped stem cells to three-dimensional nodules displaying only osteogenic phenotypic markers WV1D1(9C5) (DSHB) for bone sialoprotein II [83], MPIII (DSHB) for osteopontine [84], and the von Kossa procedure, (Silber Protein, Chroma-Gesellschaft) for calcium phosphate. In the von Kossa procedure, negative staining controls were preincubated in EGTA, a specific chelator for calcium (Sigma) [6–8,82]. Endochondral bone was identified as the formation of a three-dimensional structure with progressional staining from one displaying chondrogenic phenotypic markers i.e., pericellular type II collagen, type IX collage, chondroitin sulfate/keratan sulfate glycosaminoglycans (see previous for chondrogenic cell types) to three-dimensional nodules displaying osteogenic phenotypic markers; that is, WV1D1(9C5) (DSHB) for bone sialoprotein II [83], MPIII (DSHB) for osteopontine [84], and the von Kossa procedure (Silber Protein, Chroma-Gesellschaft) for calcium phosphate. In the von Kossa procedure, negative staining controls were preincubated in EGTA, a specific chelator for calcium (Sigma) [6–8,25].

Tendons and ligaments were identified as linear structures with cellular staining for fibroblast-specific protein IB10 (Sigma) [85], displaying extracellular matrix rich in chondroitin sulfate glycosaminoglycans assessed by Alcian Blue pH 1.0 staining. In the latter procedure, negative staining controls were digested with chondroitinase-ABC or chondroitinase-AC or keratanase prior to staining [6,7,22,81,82], and displaying extracellular histochemical staining for type I collagen [81]. Dermis was identified by the presence of interwoven type I collagen fibers [81] interspersed with spindle-shaped cells staining for fibroblast-specific protein IB10 (Sigma) [85] with an extracellular matrix rich in chondroitin sulfate and dermatan sulfate glycosaminoglycans as assessed by Alcian Blue pH 1.0 staining. In the latter procedure, negative staining controls were digested with chondroitinase-ABC or chondroitinase-AC prior to staining [6,7,22,81,82]. Scar tissue was identified as interwoven type I collagen fibers [81] interspersed with spindle-shaped cells staining for fibroblast specific protein IB10 (Sigma) [85] with an extracellular matrix rich in chondroitin sulfate glycosaminoglycans as assessed by Alcian Blue pH 1.0 staining. In the latter procedure, negative staining controls were digested with chondroitinase-ABC or chondroitinase-AC prior to staining [6,7,22,81,82].

Endothelial cells were identified by staining with antibodies P2B1 (DSHB) for CD31-PECAM [8], H-Endo (Chemicon) for CD146 [86,87], P8B1 (DSHB) for VCAM [8,88], and P2H3 (DSHB) for CD62e selectin-E [8]. Hematopoietic cells were identified using H-CD34 (Vector) for sialomucin-containing hematopoietic cells [8,13]; Hermes-1 (DSHB) for CD44, the hyaluronate receptor [89-91]; and H5A4 (DSHB) for DC11b-granulocytes, monocytes; and natural killer cells, H5H5 (DSHB) for CD43 leukocytes, H4C4 (DSHB) for CD44 hyaluronate receptor, H5A5 (DSHB) for CD45 all leukocytes, and H5C6 (DSHB) for CD63 macrophages, monocytes, and platelets [90-93].

Endodermal germ layer lineage cells were identified as follows. Endodermal progenitor cells were identified with H-AFP (Vector) and R-AFP (Nordic Immunological Laboratories, Tilburg, The Netherlands) for α-fetoprotein [94]. GI Epithelium was identified with HESA (Sigma) for GI-epithelium [2,3,10]. Liver biliary cells were identified with OC2, OC3, OC4, OC5, OC10, DPP-IV, and OV6 (Hixson) for biliary epithelial cells, liver progenitor cells, oval cells, and canalicular cells [50,95-99]. Liver canalicular cells were identified with antibodies H4Ac19 (DSHB), DPP-IV, OV6, and LAP (Hixson) for bile canalicular cells, liver progenitor cells, biliary epithelial cells, and canalicular cell surface protein [49,50,95-100]. Liver hepatocytes were identified with H-1 and H-4 (Hixson) for hepatocyte cell surface marker and hepatocyte cytoplasm, respectively [97,98], and 151-IgG for liver progenitor cells, and biliary epithelial cells [98,99]. Liver oval cells were identified with OC2 and OV6 (Hixson) for oval cells, liver progenitor cells, and biliary epithelial cells [98,99]. Pancreatic progenitor cells were tentatively identified as three-dimensional structures void of chondrogenic or osteogenic phenotypic markers. This identity was confirmed by the presence phenotypic markers for pancreatic ductal cells, β-cells, α-cells, and δ-cells [1–3,10]. Pancreatic ductal cells were identified with cytokeratin-19 (Chemicon) to pancreatic ductal cells [1–3,10]. Pancreatic α-cells were identified with YM-PS087 (Accurate, Westbury, NY) an antibody to glucagon [1–3,10]. Pancreatic α-cells were identified with YM-PS088 (Accurate) an antibody to insulin [1–3,10]. Pancreatic δ-cells were identified with 11180 (ICN) an antibody to somatostatin [1–3,10].

Gene Expression

Molecular analysis was performed to determine genetic expression in the six categories of cells isolated from adult rat skeletal muscle, including the four clones that were generated [2].

Functional Activities In Vivo

The identified cells were examined in animal model systems and in an IRB-approved human clinical trial to determine their functional activities in vivo [6,7,31,34,101,102].

Results

Stage-specific embryonic antigen-4 (SSEA-4) positive cells were located in the connective tissues surrounding nervous tissue, e.g., epineurium, perineurium, and endoneurium (Figure 6A), while CEA-CAM-1 positive cells were located in the tunica intima and tunica adventitia of blood vessels (Figure 6B). IA4 positive staining for smooth muscle alpha-actin was located within the tunica media of the vasculature (Figure 6C). Negative procedural controls demonstrated absence of staining in the tissue sections (Figure 6D). After tissue harvest and plating, the SSEA-4+ cells and CEA-CAM-1+ cells were present in the initial plating mixture of cells [107]. As germ layer lineage stem cells (EctoSCs,

MesoSCs, and EndoSCs) and progenitor cells reached contact inhibition, the SSEA-4+ cells and CEA-CAM-1+ cells appeared on their cell surfaces as small and very small spherical refractile entities, respectively (Figure 8). With time the SSEA-4+ cells and CEA-CAM-1+ cells formed colonies of cells growing on the surface of the confluent germ layer lineage stem cells and progenitor cells (Figure 9). The SSEA-4+ cells and CEA-CAM-1+ cells were allowed to proliferate past confluence forming initially several layers of cells (Figure 10) and then multiple layers of cells (Figure 11).

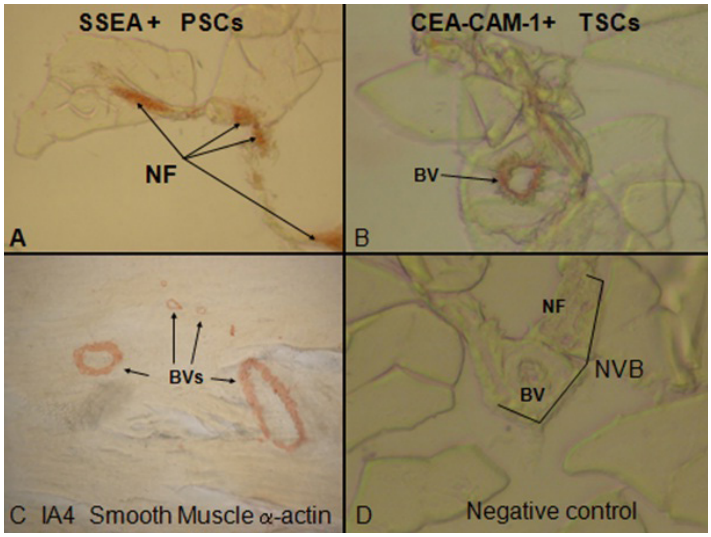

Figure 6: Post-traumatic adult male rat skeletal muscle (RM1025), euthanized, skeletal muscle harvested, fixed in a glutaraldehyde-paraformaldehyde-glucose ELICA fixative, cryosectioned at 7 microns, mounted on positively charged glass slides, and incubated with designated antibodies. A. SSEA positive (PSCs) within nerve fiber connective tissues. B. CEA-CAM-1 positive (TSCs) staining within tunica intima of blood vessels. C. IA4 positive staining for smooth muscle alpha-actin in the tunica muscularis of medium and small blood vessels within the perimysium of skeletal muscle, positive procedural control. D. No primary antibody, negative procedural control.

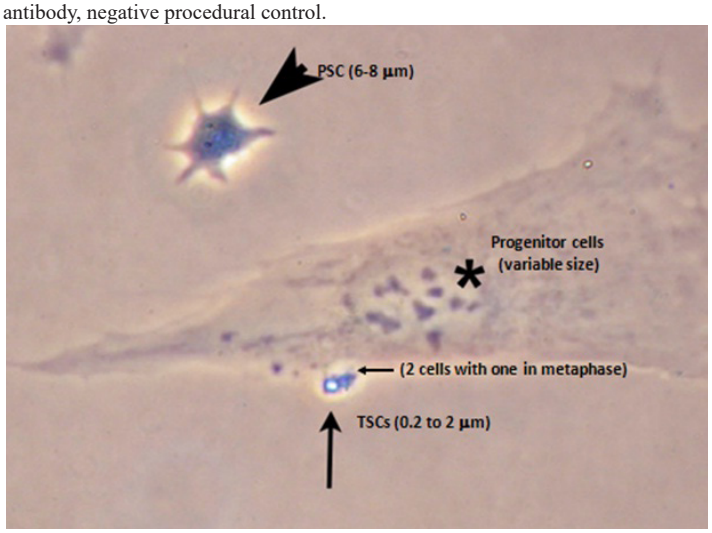

Figure 7: Note presence of single PSC (SSEA-4+) (large arrowhead), a portion of a progenitor cell (asterisk), and two TSCs (CEA-CAM-1+) (small arrow) in photograph. Of the two TSCs in the photograph one is in prophase and the other is in metaphase (see chromosomes along metaphase plate). Magnification of original photograph, 800x, electronically enlarged to 1600x.

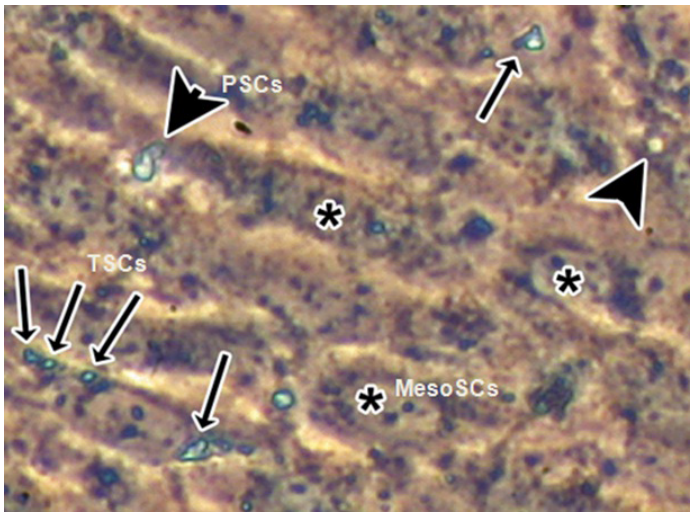

Figure 8: Single contact inhibited layer of mesodermal stem cells (MesoSCs, asterisk) overlain with pluripotent stem cells (PSCs, arrowheads, SSEA-4+) and totipotent stem cells (TSCs, arrows, CEA-CAM-1+). Cells were grown on a substratum of type-I collagen.

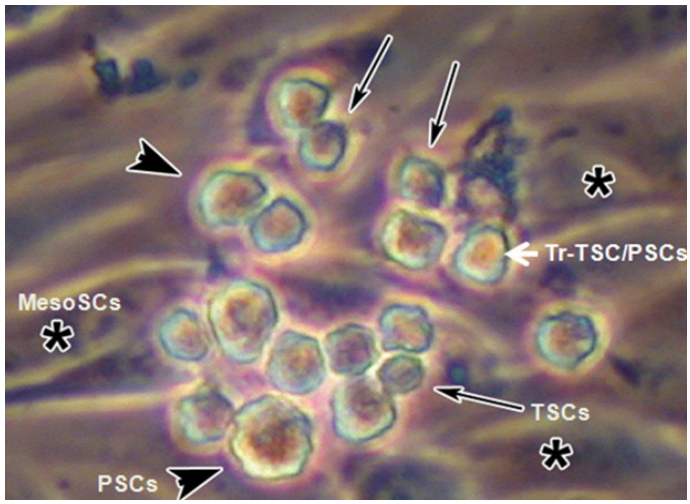

Figure 9: Mixed culture demonstrating mixed colony of larger pluripotent stem cells (PSC, arrowheads, SSEA-4+), intermediate sized transitional-totipotent stem cell/pluripotent stem cells (Tr-TSC/PSCs, white arrow, CEA-CAM-1+/SSEA-4+), and smaller totipotent stem cells (TSCs, arrows, CEA-CAM-1+), overlying an almost contact inhibited single layer of mesodermal stem cells (MesoSCs, asterisk, Thy-1+). Cells were grown on a substratum of type-I collagen.

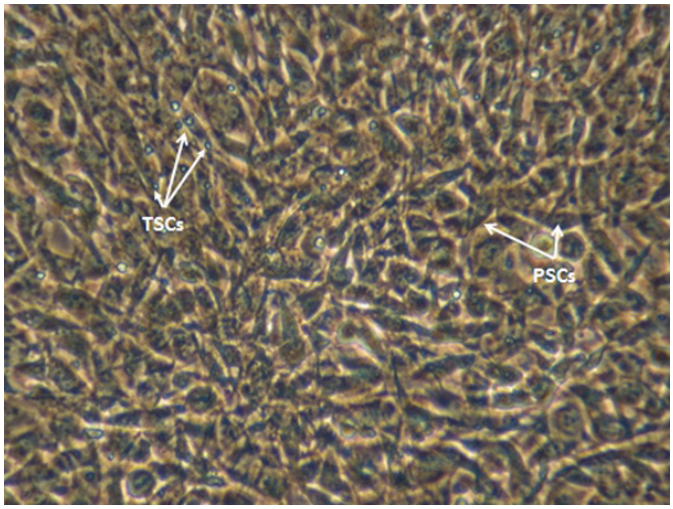

Figure 10: A mixed culture of several confluent layers of post-natal totipotent stem cells (TSCs, CEA-CAM-1+) and post-natal pluripotent stem cells (PSCs, SSEA-4+), grown on a type-I collagen substratum.

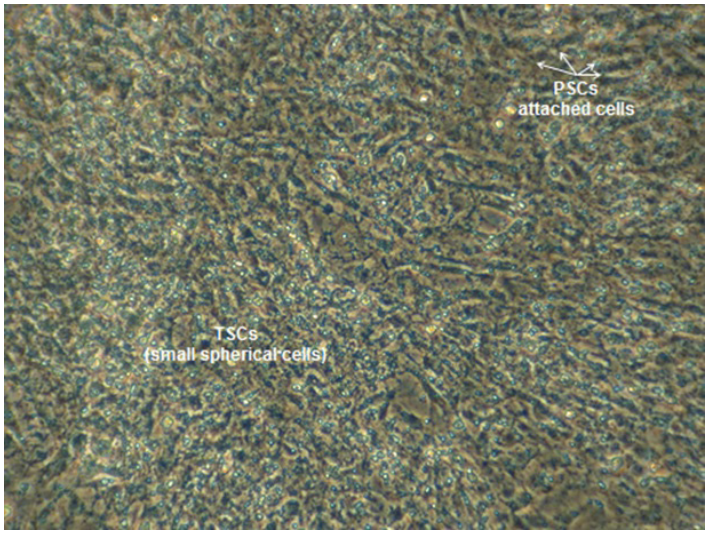

Figure 11: A mixed culture of multiple confluent layers of post-natal totipotent stem cells (TSCs, CEA-CAM-1+) and post-natal pluripotent stem cells (PSCs, SSEA-4+) plated on a decellularized collagen substratum.

The cells were harvested. The mixed population of precursor cells were separated first by cryopreservation [24], and then by either sorting using specific cell surface markers (i.e., CEA-CAM-1 and SSEA-4) or cloning using single cell serial dilution clonogenic analysis [4,6]. The cloning was coupled with log-phase growth stem cell-specific conditioned medium as described previously [4,6,21]. The cloning technique generated 58 clones which were subsequently transfected with Lac-Z using the lipofectin technique so that they could be tracked in vivo or in vitro. Of the 58 clones generated and transfected with Lac-Z, 4 clones were characterized for this study (Scl-44β, Scl-40β, Scl-4β, and A2A2β) (Table 1). Subsequent karyotypic analyses of clone Scl-44β and clone Scl-4β revealed that the adult rat skeletal muscle-derived cell clones, propagated well past Hayflick's limit [25,26], contained a normal compliment of 42 chromosomes [21,31].

Similarities and differences of culture conditions are shown in Table 1.

Six categories of putative precursor cells were isolated from adult rat skeletal muscle, including the four single cell-derived clones (Scl-44β, Scl-40β, Scl-4β, and A2A2β) were then analyzed with our Phenotypic Expression / Progression Medium / General Induction Medium / ELICA Bioassay in combination with general and specific agents singly and in combination, i.e., proliferation agents, progression agents, inductive agents, and inhibitory agents, to determine identity of the cells and their respective differentiation potentials (Table 2) [1,4,6,20-23,25,26,31].

The isolated and cloned cells incubated with proliferation agents demonstrated an increase in log phase cellular proliferation while retaining their initial cell surface markers and did not express any differentiated cell types [21]. The isolated and cloned cells incubated in progression medium retained their initial cell surface markers and did not express any differentiated cell types [21].

Putative totipotent stem cells (BLSCs and Scl-44β) incubated in induction medium containing general or specific inductive growth factors both singly and in combination were induced to form 68 individual cell types (Table 1, Table 2), including a cell displaying a phenotypic expression marker for spermatogonia, when incubated with dispersed testis-conditioned medium. In contrast, putative pluripotent stem cells (Tr-BLSC/ELSCs and Scl-40β; ELSCs and Scl-4β; and Tr-ELSC/GLSCs) formed only 65 individual cell types (Tables 1 and 2), but did not form spermatogonia under the same culture conditions (incubated with dispersed testis-conditioned medium) as the putative totipotent stem cells. Putative mesodermal stem cells (MesoSCs and A2A2β) were induced to form 31 individual cell types within the mesodermal lineage (Tables 1 and 2), but did not form spermatogonia under the same culture conditions (incubated with dispersed testis-conditioned medium) as the putative totipotent stem cells. And putative mesenchymal stem cells (MSCs) were induced to form three cell types (Tables 1 and 2), but did not form spermatogonia under the same culture conditions (incubated with dispersed testis-conditioned medium) as the putative totipotent stem cells.

Genetic expression was analyzed to determine which cell categories/clones expressed which genes. The BLSCs and clone Scl-44β expressed telomerase, Bcl-2, Nanog, Nanos, and CXCR4; Tr-BLSC/ELSCs, clone Scl-40β, ELSCs, and Scl-4β expressed telomerase, Oct-3/4, and Sonic Hedgehog; Tr-ELSC/GLSCs, MesoSCs, and clone A2A2β expressed telomerase and Pax-6 (Table 1) [2].

Stereotactic injection of the Lac-Z-labeled clone Scl-40β incorporated into a neurotoxin-induced model of Parkinson's disease. The labeled cells were seen regenerating dopaminergic neurons within the substantia nigra of the midbrain and pyramidal neurons, interneurons, glial cells, and vasculature within the cerebral cortex [34,101]. Direct injection or systemic delivery of the Lac-Z-labeled clone Scl-40β incorporated into myocardium, vasculature, and connective tissue skeleton in two myocardial infarction animal models undergoing repair [6,7]. Pancreatic islet tissue consisting of glucagon-secreting alpha-cells, insulin-secreting beta-cells, and somatostatin-secreting delta-cells was created in the Lac-Z-labeled clone Scl-40β using exosomes from conditioned media of endodermal stem cells and endodermal progenitor cells and an islet-inducing cocktail of inductive factors [31,103]. The induced islets secreted 25-50% of the amount of insulin secreted by native islets in a glucose challenge assay [104,105] as assessed by a rat-specific radioimmunoassay [31].

Discussion

Previous studies from Young and colleagues have dealt with identifying and characterizing precursor cells, e.g., progenitor cells and stem cells, located in adult post-natal tissues of amphibians,

avians, non-human mammals, and humans [1-8,20-31,34,71-73,81,82,101,102,106-118]. These studies, collectively, identified four categories of precursor cells within the tissues, e.g., lineage-uncommitted blastomere-like (totipotent) stem cells, lineage-uncommitted epiblast-like (pluripotent) stem cells, embryonic germ layer lineage-committed (ectodermal, mesodermal, and endodermal) stem cells, and tissue- and cell-committed progenitor cells. The original intent of the current study was to determine the location of the BLSCs (totipotent stem cells) and ELSCs (pluripotent stem cells) within the skeletal muscle of the adult rat. Immunocytochemical studies, reported herein, demonstrate that pluripotent (SSEA-4+) stem cells preferentially reside within neural-associated connective tissues of skeletal muscle (Figure 6A), while the totipotent (CEA-CAM-1+) stem cells preferentially reside within the connective tissues associated with the vasculature of skeletal muscle (Figure 6B).

Studies of limb regeneration in adult salamanders noted that both an intact vasculature and intact nervous supply were crucial elements for formation of the epidermal ridge with underlying blastema and subsequent outgrowth of the regenerating limb [108,110,111]. Originally, it was thought that chemical trophic factors were released from these two tissues that were instrumental in controlling the regenerative process [118]. While secretion of chemical trophic factors may still be involved, the apparent location of totipotent and pluripotent stem cells in the supporting tissues of vascular and nervous tissues within skeletal muscle suggests an additional if not alternative hypothesis. If these particular stem cells are also present in similar locations in adult amphibians, this might represent at least two categories of stem cells emanating from skeletal muscle that contribute to formation of the blastema with subsequent outgrowth of the limb [7]. To confirm or deny this proposed hypothesis would necessitate immunostaining of sectioned regenerating amphibian limbs with CEA-CAM-1 (TSC marker) and SSEA-4 (PSC marker) to determine location of these cells. Additionally, dual chamber secretory experiments using filters with pore sizes less than 0.1 μm would also need to be performed to exclude the contributions of the totipotent stem cells (0.1 – 2.0 μm) and pluripotent stem cells (>2.0 to 10 μm) to the process.

Phenotypic expression markers (Table 2) were used to define induced differentiated cell types in the cell categories and clones analyzed in this study [1-8,26,29,31,34]. The ELICA bioassay (Figure 2) was developed to quantify phenotypic expression, visualize phenotypic expression, and standardize to amount of DNA (ng phenotypic expression marker per μg DNA) present within a 96-well format [20]. This allowed us to perform multiple concentration curves and combinatorial experiments to quantify phenotypic expression in our stem cells as well as determine their proliferation potential. The ELICA bioassay was originally developed to quantify the biological activity for the isolation of endogenous bioactive factors isolated from bone, i.e., adipogenic-morphogenetic protein (AMP), skeletal muscle-morphogenetic protein (Sk-MMP), smooth muscle-morphogenetic protein (Sm-MP), cardiac muscle-morphogenetic protein (C-MMP), fibroblastic-morphogenetic protein (F-MP), endochondral bone-morphogenetic protein (EB-MP), intramembranous bone-morphogenetic protein (IB-MP), and scar inhibitory factor (SIF) (7); to quantify the inductive capabilities of exosomes from conditioned medium derived from cultured precursor cells and differentiated cells [31]; and to quantify the activities of various recombinant bioactive factors, such as the proliferation agents: platelet-derived growth factor- AA (PDGF-AA), platelet-derived growth factor-BB (PDGF-BB), platelet-derived growth factor-AB (PDGF-AB) [7,23]; progression agents: insulin, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) [7,23]; inductive agents: acidic-fibroblast growth factor (a-FGF), vascular endothelial cell growth factor (VEGF), basic-fibroblast growth factor (b-FGF), transforming growth factor-beta (TGF-β), bone morphogenetic protein-2 (BMP-2), epidermal growth factor (EGF), nerve growth factor (NGF), hepatocyte growth factor (HGF), erythropoietin (EPO), keratinocyte growth factor (KGF); and inhibitory agents: leukemia inhibitory factor (LIF), anti-differentiation factor (ADF) [7,23]; and to generate serum-free defined medias for our pluripotent and totipotent stem cells.

Serum-free medium containing proliferation agents accelerated expansion of the various cell populations without affecting either the expression of their respective cell surface markers or their respective phenotypic marker expression. There was a definitive hierarchy in the ability of the proliferation agents examined to induce cellular expansion, i.e., PDGF-BB > PDGF-AA > PDGF-AB. Platelet-derived growth factor-BB at 2 ng/ml optimized proliferation in all cells examined [7].

Serum-free medium containing progression agents, i.e., insulin, insulin-like growth factor-I (IGF-I), and insulin-like growth factor-II (IGF-II) accelerate phenotypic expression of tissue- and cell-committed progenitor cells, but have no effect on lineage-uncommitted stem cells (BLSCs [TSCs], Tr-BLSC/ELSCs [Tr-TSC/PSCs], ELSCs [PSCs], Tr-ELSC/GLSC [PSC/GLSCs]) or germ layer lineage-committed stem cells (EctoSCs, MesoSCs, EndoSCs). While there was no discernible difference with respect to the activity of insulin, IGF-I, or IGF-II to accelerate phenotypic expression in lineage-committed progenitor cells, the significant difference in cost between insulin and IGF-I or IGF-II dictated our choice of insulin as the preferred progression agent for all our subsequent studies.

The isolated and cloned stem cells (BLSCs [Scl-44β, TSCs], Tr-BLSC/ELSCs [Scl-40β, Tr-TSC/PSCs] ELSCs [Scl-4β, PSCs], Tr-ELSC/GLSC [PSC/GLSCs], MesoSCs [A2A2β]) incubated in progression medium retained their initial cell surface markers and did not express any differentiated cell types [1,7,31]. For example, clone Scl-44β was incubated in the presence of 2 μg insulin/ml medium to attempt to accelerate phenotypic expression if the clone was committed to a particular cellular phenotype, such as a tissue- or cell-committed progenitor cell. The clone was assayed for CEA-CAM-1 (TSC marker), FORSE-1 (neuroectoderm marker), 8A2 (neuronal marker), S-100 (neuronal marker), CNPase (glial cell marker), 40E-C (ependymal cell marker), SV2 (synaptic vesicle

marker), VM-1 (keratinocyte marker), IA4 (smooth muscle cell marker), CIIC1 (type-II collagen marker for cartilage), MPIII (osteopontine marker for bone), WV1D1 (osteocalcin marker for calcified tissues) and representative markers for endodermal lineage cells. In all phenotypic expression markers examined, the only antibody that stained positive was CEA-CAM-1 [21]. Similar experiments were performed for the remaining eight cell populations identified (Table 2).

In contrast, serum-free defined medium containing general or specific inductive agents both singly and in combination induce stem cells to commit to and express phenotypic markers for differentiated cell types, but it has no effect on tissue- and cell-committed progenitor cells outside the progenitor cell's tissue or cell-commitment. In other words, osteoblasts (the progenitor cells for bone) are not affected by VEGF or a-FGF (endothelial cell lineage inductors), NGF (neuronal cell lineage inductor), or HGF (liver cell lineage inductor), but is only affected by BMP-2 (osteogenic cell lineage inductor). Putative totipotent stem cells (BLSCs, Scl-44β) incubated in induction medium containing general (dexamethasone) or specific inductive growth factors both singly and in combination (a-FGF, VEGF, b-FGF, TGF-β, BMP-2, EGF, NGF, HGF, EPO, KGF, and conditioned medium from differentiated cell types, i.e., spermatogonia, skeletal muscle, smooth muscle, cardiac muscle, unilocular fat cells, multilocular fat cells, hyaline cartilage, articular cartilage, elastic cartilage, growth plate cartilage, fibrocartilage, endochondral bone, intramembranous bone, tendon, ligament, endothelial cells, neurons, glial cells, keratinocytes, gastrointestinal epithelial cells, liver cells, and pancreatic cells) were induced to form 68 individual cell types (Tables 1, Table 2), including cells displaying a phenotypic expression marker for spermatogonia. In contrast, putative pluripotent stem cells (ELSCs, Scl-4β) formed only 65 individual cell types (Table 1 and 2), but did not form spermatogonia under the same culture conditions as the putative totipotent stem cells. Putative mesodermal stem cells (MesoSCs, A2A2β) were induced to form 31 individual cell types within the mesodermal lineage (Tables 1 and 2), but did not form spermatogonia under the same culture conditions as the putative totipotent stem cells. And putative mesenchymal stem cells (MSCs) were induced to form three cell types (Tables 1 and 2), but did not form spermatogonia under the same culture conditions as the putative totipotent stem cells [1,6-8,21,107].

During the isolation and characterization of totipotent stem cells, pluripotent stem cells, germ layer lineage stem cells, and progenitor cells from adult rat skeletal muscle isolates two additional categories of adult-derived stem cells were discovered. These two categories of putative stem cells was based on particular size, trypan blue inclusion-exclusion, cell surface markers, differentiation potentials, and selective culture parameters (Tables 1 and 2). These two categories of stem cells were designated as transitional-totipotent stem cell/pluripotent stem cells (Tr-BLSC/ELSC, Scl-40β), an intermediary cell type between totipotent stem cells (BLSCs) and pluripotent stem cells (ELSCs), and transitional-pluripotent stem cell/germ layer lineage stem cells (Tr-ELSC/GLSCs), an intermediary between pluripotent stem cells (ELSCs) and the germ layer lineage stem cells (GLSCs), e.g., ectodermal stem cells, mesodermal stem cells, and endodermal stem cells. The identity and independent nature of these newly discovered stem cell categories were confirmed with full characterization studies (Table 1).

Our current proposed sequential unidirectional developmental sequence for stem cells within postnatal tissues is as follows (Figure 1). The most primitive stem cells in skeletal muscle appear to be totipotent stem cells (BLSCs). These stem cells are 0.1 to 2 μm in size; they are Trypan blue positive; they express cell surface epitopes for the CEA-CAM-1 antibody; and they are the immediate precursor cells to the transitional-totipotent stem cell/pluripotent stem cells. The totipotent stem cells have the potential to form all somatic cells of the body and the gametes (e.g., spermatogonia) (Tables 1 and 2) [9,38].

The transitional totipotent stem cell/pluripotent stem cells (Tr-BLSC/ELSCs, HLSCs, CLSCs) range in size from >2 to <6 μm; they are Trypan blue positive around their periphery and Trypan blue negative in the center; and they share cell surface epitopes of both CEA-CAM-1 (TSCs) and SSEA-4 (PSCs). They are the immediate precursor cells between the totipotent stem cells and pluripotent stem cells and have the potential to form all somatic cells of the body, but have not shown the capacity to form gametes. Therefore, they are designated as a category of pluripotent stem cells (Tables 1 and 2). Ratajczak and colleagues have described a similarly-sized stem cell, the very small embryonic-like (VSEL) stem cell that is 3-5 μm. It is well within the size range of the Tr-BLSC/ELSCs, >2.0 - <6.0 μm. Ratajczak and colleagues found the VSEL stem cells in the bone marrow of adult animals [14]. The VSEL stem cells stain with stage-specific embryonic antigens, similar to the Tr-BLSC/ELSCs, and have been described as being pluripotent [14]. They have suggested that their VSELs are only located within the bone marrow, whereas the Tr-BLSC/ELSCs are located in at least 37 separate tissues and organs throughout the body, including the bone marrow [1]. Whether the VSELs and the Tr-BLSC/ELSCs are the same pluripotent stem cell or are very similar pluripotent stem cells is unknown at this time. Side by side experiments need to be performed with single-cell derived clonal populations of both VSELs and Tr-BLSC/ELSCs through the various parameters listed in Table 1 to make that determination.

The pluripotent stem cells (ELSCs) range in size from 6-8 μm, they are Trypan blue negative, and express cell surface epitopes for SSEA-4. They are the immediate precursor cell to the transitional-pluripotent stem cell/germ layer lineage stem cells (Tr-ELSC/GLSCs) and have the potential to form all somatic cells of the body, but have not shown the capacity to form gametes utilizing similar culture parameters as the TSCs. Therefore, these cells have been designated as a category of pluripotent stem cells (Tables 1 and 2) [1,6].

The transitional pluripotent stem cell/germ layer lineage stem cells (Tr-ELSC/GLSCs) are 8-10 μm in size, are Trypan blue negative,

and express the cell surface epitopes for both PSCs (SSEA-4) and GLSCs (Thy-1). They are the immediate precursor cell to the germ layer lineage ectodermal stem cells, mesodermal stem cells, and endodermal stem cells and have the potential to form cells from all three primary germ layer lineages, i.e., ectoderm, mesoderm, and endoderm. Therefore, these cells have also been designated as a category of pluripotent stem cells (Tables 1 and 2) [1].

Germ layer lineage ectodermal stem cells (EctoSCs), mesodermal stem cells (MesoSCs), and endodermal stem cells (EndoSCs) stem cells are initially in the size range from 10-20 μm, but will alter their initial size based on subsequent differentiation potentials. They are the precursor cells for tissue- and cell-specific progenitor cells within their respective germ layer lineages. EctoSCs have the potential to form multiple cell types belonging to the ectodermal lineage; MesoSCs have the potential to form multiple cell types within the mesodermal embryological lineage; and EndoSCs have the potential to form multiple cell types within the endodermal embryological lineage. Additional characteristics for each category of stem cell are listed in Tables 1 and 2 [1,4,7].

Studies are ongoing to address the functional significance of endogenous naturally-occurring stem cells, both singly and in combination, during normal tissue maintenance and repair of body organs and tissues. Three animal model systems were developed to address this issue, i.e., Parkinson's disease (ectodermal model) [34], cardiovascular ischemia (mesodermal models) [6,7], and creation of pancreatic islets de novo (endodermal model) [31] from pluripotent stem cells. Young and colleagues examined a Lac-Z transfected clone (i.e., Scl-40β) of transitional-totipotent stem cells/pluripotent stem cell with pluripotent capabilities for its ability to incorporate in vivo into damaged ectodermal, mesodermal, and endodermal tissues and assist in the normal reparative processes within these models. The LAC-Z transfected pluripotent clone, Scl-40β, was used in all three (ectodermal, mesodermal, and endodermal) animal model systems.

A Parkinson's disease model [34] was created by the stereotactic injection of a neurotoxin, 6-hydroxydopamine, into the midbrain (substantia nigra) of adult rat brains. Two weeks after neurotoxin injection the rat brains were stereotactically injected with the Lac-Z transfected clone, Scl-40β or buffer control into the midbrain substantia nigra. The buffer controls demonstrated glial scars along the needle tracks through the cerebral cortex and into the midbrain. Rat brains that were injected with Scl-40β demonstrated regeneration of dopaminergic neurons within the midbrain. An unexpected finding was the regeneration and repair of pyramidal neurons, interneurons, glial cells, and the vasculature along the needle tracks within the cerebral cortex by the Lac-Z labeled Scl-40β cells.

Two models of cardiovascular ischemia [6,7] were utilized, i.e., freezing of the apex of the heart with liquid nitrogen and transiently ligating the left anterior descending coronary artery. Scl-40β was delivered by direct injection into the damaged tissue or infused systemically through a tail vein injection. In either mode of delivery the Scl-40β clone incorporated into regenerating cardiac muscle, vasculature, and the connective tissue skeleton of the heart.

Pancreatic islets that were receptive to a glucose challenge were created de novo in the Scl-40β pluripotent stem cell clone [31]. Exosomes from endodermal stem cell-conditioned medium and endodermal progenitor cell-conditioned medium were used to convert Scl-40β sequentially into those particular cell types, i.e., pluripotent stem cells to endodermal stem cells and then to endodermal progenitor cells. The final induction into pancreatic islets was accomplished using the pancreatic islet inductive cocktail of Bonner-Weir [103]. The islets that were generated demonstrated staining for glucagon-secreting alpha-cells, insulin-secreting beta-cells, and somatostatin-secreting delta-cells. Next the induced pancreatic islets were compared to native islets for insulin secretion in response a glucose challenge [104,105]. Insulin secretion was measured using a rat-specific insulin-radioimmunoassay. As noted, the induced islets secreted 25% to 50% of the insulin secreted by the native islets [31].

Based on the results from the animal model for Parkinson's disease, an IRB-approved human clinical trial was performed in individuals with Parkinson's disease to determine the safety and efficacy of a single treatment of autologous stem cells. Isolated and segregated endogenous naturally-occurring autologous stem cells were used for the trial. TSCs and Tr-TSC/PSCs were delivered intra-nasally, while PSCs, Tr-PSC/GLSCs, and MesoSCs were delivered by intravenous infusion. At one month post infusion all individuals treated were either stable or improving. At the 7th month and 14th month follow-ups the same individuals demonstrated the following results. Twenty-five percent began to decline but at a slower rate than before treatment began; 50% remained stable; and 25% continued to improve (Figure 12) [101,102]. This trial suggested that autologous stem cells were safe to use for treatment and that 75% of the individuals treated demonstrated positive results for at least 14 months after treatment.

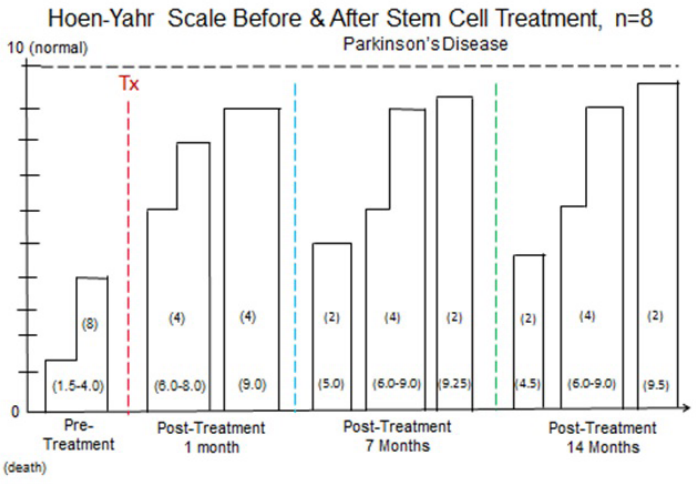

Figure 12: An IRB-approved human clinical trial was performed for individuals with Parkinson's disease. The trial used a single treatment of their naturally-occurring autologous stem cells. One month after a single treatment all patients showed an increase in their quality of life (increased Hoen-Yahr score). At the

seventh month follow-up 25% reverted to a slower decline in their quality of life, 50% remained stable, and 25% continued to improve. At the 14th month follow-up the same results were noted for the same individuals that occurred at the seventh month time point, 25% continued to decline at a slow rate, 50% remained stable, and 25% continued to improve.

Future studies, in both animal model systems and IRB-approved clinical trials, are planned to test the utility of using naturally-occurring endogenous stem cells for the replacement and repair of diseased and/or damaged tissues.

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References

1. Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol. 2014; 1: 00019.
2. Young HE, Black AC Jr. Adult stem cells. Anat Rec A Discov Mol Cell Evol Biol. 2004; 276: 75-102.
3. Young HE, Steele T, Bray RA, et al. Human progenitor and pluripotent cells display cell surface cluster differentiation markers CD10, CD13, CD56, and MHC Class-I. Proc Soc Exp Biol Med. 1999; 221: 63-71.
4. Young HE, Duplaa C, Young TM, et al. Clonogenic analysis reveals reserve stem cells in postnatal mammals. I. Pluripotent mesenchymal stem cells. Anat Rec. 2001; 263: 350-360.
5. Young HE, Steele TA, Bray RA, et al. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec. 2001; 264: 51-62.
6. Young HE, Duplaa C, Yost MJ, et al. Clonogenic analysis reveals reserve stem cells in postnatal mammals. II. Pluripotent epiblastic-like stem cells. Anat Rec. 2004; 277A: 178-203.
7. Young HE, Duplaa C, Romero-Ramos M, et al. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys. 2004; 40: 1-80.
8. Young HE, Black AC Jr. Adult-derived stem cells. Minerva Biotechnologica. 2005; 17: 55-63.
9. Vierck JL, McNamara JP, Dodson MV. Proliferation and differentiation of progeny of ovine unilocular fat cells (adipofibroblasts). In Vitro Cell dev Biol Anim. 1996; 32: 564-572.
10. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284: 143-147.
11. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005; 11: 1198-1211.
12. Jahagirdar BN, Verfaillie CM. Multipotent adult progenitor cell and stem cell plasticity. Stem Cell Rev. 2005; 1: 53-59.
13. Munoz JR, Stoutenger BR, Robinson AP, et al. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. Proc Natl Acad Sci USA. 2005; 102: 18171-18176.
14. Kucia M, Zuba-Surma E, Wysoczynski M, et al. Physiological and pathological consequences of identification of very small embryonic like (VSEL) stem cells in adult bone marrow. J Physiol Pharmacol. 2006; 57 Suppl 5: 5-18.
15. Parker AM, Katz AJ. Adipose-derived stem cells for the regeneration of damaged tissues. Expert Opin Biol Ther. 2006; 6: 567-578.
16. Serafini M, Verfaillie CM. Pluripotency in adult stem cells: state of the art. Semin Reprod Med. 2006; 24: 379-388.
17. Schultz SS, Abraham S, Lucas PA. Stem cells isolated from adult rat muscle differentiate across all three dermal lineages. Wound Repair Regen. 2006; 14: 224-231.
18. De Coppi P, Bartsch G Jr, Siddiqui MM, et al. Isolation of amniotic stem cell lines with potential for therapy. Nature Biotechnol. 2007; 25: 100-106.
19. Stout CL, Ashley DW, Morgan III JH, et al. Primitive stem cells reside in adult swine skeletal muscle and are mobilized into the peripheral blood following trauma. American Surgeon. 2007; 73: 1106-1110.
20. Young HE, Sippel J, Putnam LS, et al. Enzyme-linked immuno-culture assay. J Tiss Cult Meth. 1992; 14: 31-36.

21. Henson NL, Heaton ML, Holland BH, et al. Karyotypic analysis of adult pluripotent stem cells. Histol Histopath. 2005; 20: 769-784.
22. Young HE, Dalley BK, Markwald RR. Glycoconjugates in normal wound tissue matrices during the initiation phase of limb regeneration in adult Ambystoma. Anat Rec. 1989; 223: 231-241.
23. Young HE, Wright RP, Mancini ML, et al. Bioactive factors affect proliferation and phenotypic expression in pluripotent and progenitor mesenchymal stem cells. Wound Rep Regen. 1998; 6: 65-75.
24. Young HE, Morrison DC, Martin JD, et al. Cryopreservation of embryonic chick myogenic lineage-committed stem cells. J Tiss Cult Meth. 1991; 13: 275-284.
25. Young HE, Ceballos EM, Smith JC, et al. Pluripotent mesenchymal stem cells reside within avian connective tissue matrices. In Vitro Cell Devel Biol. 1993; 29A: 723-736.
26. Romero-Ramos M, Vourc'h P, Young HE, et al. Chesselet M-F Neuronal differentiation of stem cells isolated from adult muscle. J Neurosci Res. 2002; 69: 894-907.
27. Young HE, Mancini ML, Wright RP, et al. Mesenchymal stem cells reside within the connective tissues of many organs. Devel Dynam. 1995; 202: 137-144.
28. Seruya M, Shah A, Pedrotty D, et al. Clonal Population of adult stem cells: life span and differentiation potential. Cell Transplant. 2004; 13: 93-101.
29. Vourc'h P, Romero-Ramos M, Chivatakarn O, et al. Isolation and characterization with neurogenic potential from adult skeletal muscle. Biochemical and Biophysical Research Communications. 2004; 317: 893-901.
30. Romero-Ramos M, Vourc'h P, Young HE, et al. Neuronal differentiation of stem cells isolated from adult muscle. J Neurosci Res. 2002; 69: 894-907.
31. 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. 2005; 67-92.
32. Seruya M, Shah A, Pedrotty D, et al. Clonal population of adult stem cells: life span and differentiation potential. Cell Transplant. 2004; 13: 93-101.
33. Vourc'h P, Romero-Ramos M, Chivatakarn O, et al. Isolation and characterization of cells with neurogenic potential from adult skeletal muscle. Biochem Biophys Res Commun. 2004; 317: 893-901.
34. Young HE, Duplaa C, Katz R, et al. Adult-derived stem cells and their potential for tissue repair and molecular medicine. J Cell Molec Med. 2005; 9: 753-769.
35. Estrera VT, Luo W, Phan D, et al. The cytoplasmic domain of CCAM1 tumor suppressor is necessary and sufficient for suppressing the tumorigenicity of prostate cancer cells. Biochem Biophys Res Commun. 1999; 263: 797-803.
36. Röhme 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.
37. HAYFLICK L, MOORHEAD PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25: 585-621.
38. Tole S, Patterson PH. Regionalization of the developing forebrain: a comparison of FORSE-1, Dlx-2, and BF-1. J Neurosci. 1995; 15: 970-980.
39. Hockfield S, McKay RD. Identification of major cell classes in the developing mammalian nervous system. J Neurosci. 1985; 5: 3310–3328.
40. Drazba J, Pierce M, Lemmon V. Studies of the developing chick retina using monoclonal antibody 8A2 that recognizes a novel set of gangliosides. Dev Biol. 1991; 145: 154–163.
41. Baudier J, Glasser N, Gerard D. Ions binding to S100 proteins. I. Calcium- and zinc-binding properties of bovine brain S100 alpha, S100a (alpha beta), and S100b (beta beta) protein: Zn2+ regulates Ca2+ binding on S100b protein. J Biol Chem. 1986; 261: 8192–8203.
42. Tole S, Patterson PH. Regionalization of the developing forebrain: a comparison of FORSE-1, Dlx-2, and BF-1. J Neurosci. 1995; 15: 970-980.
43. Hockfield S, McKay RD. Identification of major cell classes in the developing mammalian nervous system. J Neurosci. 1985; 5: 3310-3328.
44. Joshi HC, Cleveland DW. Diversity among tubulin subunits: toward what functional end? Cell Motil Cytoskeleton. 1990; 16: 159–163.
45. Wood JN, Anderton BH. Monoclonal antibodies to mammalian neurofilaments. Biosci Rep. 1981; 1: 263–268.
46. Debus E, Weber K, Osborn M. Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet polypeptides. Differentiation. 1983; 25: 193–203.
47. Franke FE, Schachenmayr W, Osborn M, et al. Unexpected immunoreactivities of intermediate filament antibodies in human brain and brain tumors. Am J Path. 1991; 139: 67–79.
48. Feany MB, Lee S, Edwards RH, et al. The synaptic vesicle protein SV2 is a novel type of transmembrane transporter. Cell. 1992; 70: 861–867.
49. Faris RA, McEntire KD, Thompson NL, et al. Identification and characterization of a rat hepatic oncofetal membrane glycoprotein. Cancer Res. 1990; 50: 4755–4763.
50. Debus E, Weber K, Osborn M. Monoclonal antibodies specific for glial fibrillary acidic (GFA) protein and for each of the neurofilament triplet polypeptides. Differentiation. 1983; 25: 193-203.
51. Franke FE, Schachenmayr W, Osborn M, et al. Unexpected immunoreactivities of intermediate filament antibodies in human brain and brain tumors. Am J Pathol. 1991; 139: 67-79.
52. Sprinkle TJ. 2':3'-cyclic nucleotide 3'-phosphodiesterase, an oligodendrocyte-Schwann cell and myelin-associated enzyme of the nervous system. Crit Rev Neurobiol. 1989; 4: 235–301.
53. Reynolds R, Carey EM, Herschkowitz N. Immunohistochemical localization of myelin basic protein and 2':3'-cyclic nucleotide 3'-phosphohydrolase in flattened membrane expansions produced by cultured oligodendrocytes. Neuroscience. 1989; 28: 181-188.
54. Hixson DC, Faris RA, Thompson NL An antigenic portrait of the liver during carcinogenesis. Pathobiology 1990; 58: 65-77.
55. Alvarez-Buylla A, Buskirk DR, Nottebohm F. Monoclonal antibody reveals radial glia in adult avian brain. J Comp Neurol. 1987; 264: 159-170.
56. Sprinkle TJ. 2',3'-cyclic nucleotide 3'-phosphodiesterase, an

oligodendrocyte-Schwann cell and myelin-associated enzyme of the nervous system. Crit Rev Neurobiol. 1989; 4: 235-301.
57. Morhenn VB. VM-1 for keratinocyte, basal, cell attachment antigen (human). In: Developmental Studies Hybridoma Bank Product Catalog. The University of Iowa, Iowa City. 2002; 24.
58. Thulasi R, Dias P, Houghton PJ, et al. Alpha 2a-interferon-induced differentiation of human alveolar rhabdomyosarcoma cells: correlation with down-regulation of the insulinlike growth factor type I receptor. Cell Growth Differ. 1996; 7: 531–541.
59. Wright WE, Binder M, Funk W. Cyclic amplification and selection of targets (CASTing) for the myogenin consensus binding site. Mol Cell Biol. 1991; 11: 4104–4110.
60. Debus E, Weber K, Osborn M. Monoclonal antibodies to desmin, the muscle-specific intermediate filament protein. EMBO J. 1983; 2: 2305-2312.
61. Bader D, Masaki T, Fischman DA. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J Cell Biol. 1982; 95: 763–770.
62. Naumann K, Pette D. Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation. 1994; 55: 203–211.
63. Shafiq SA, Shimizu T, Fischman DA. Heterogeneity of type 1 skeletal muscle fibers revealed by monoclonal antibody to slow myosin. Muscle Nerve. 1984; 7: 380-387.
64. Webster C, Silberstein L, Hays AP, et al. Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. Cell. 1988; 52: 503-513.
65. Skalli O, Ropraz P, Trzeciak A, et al. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. J Cell Biol. 1986; 103: 2787–2796.
66. Frid MG, Shekhonin BV, Koteliansky VE, et al. Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. Dev Biol. 1992; 153: 185–193.
67. Lazard D, Sastre X, Frid MG, et al. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. Proc Natl Acad Sci USA. 1993; 90: 999–1003.
68. Eisenberg CA, Markwald RR. Mixed cultures of avian blastoderm cells and the quail mesoderm cell line QCE-6 provide evidence for the pluripotentiality of early mesoderm. Dev Biol. 1997; 191: 167–181.
69. Eisenberg CA, Gourdie RG, Eisenberg LM. Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6. Development. 1997; 124: 525–536.
70. Schaart G, Moens L, Endert JM. Ramaekers FC. Biochemical characterization of cardiotin, a sarcoplasmic reticulum associated protein. FEBS Lett. 1997; 403: 168–172.
71. Young HE, Dalley BK, Markwald RR. Glycoconjugates in normal wound tissue matrices during the initiation phase of limb regeneration in adult Ambystoma. Anatomical Record. 1989; 223: 231-241.
72. Lazard D, Sastre X, Frid MG, et al. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. Proc Natl Acad Sci USA. 1993; 90: 999-1003.
73. Dixon K, Murphy RW, Southerland SS, et al. Recombinant human bone morphogenetic proteins-2 and 4 (rhBMP-2 and rhBMP-4) induce several mesenchymal phenotypes in culture. Wound Rep Regen. 1996; 4: 374-380.
74. Holmdahl R, Rubin K, Klareskog L, et al. Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. Arthritis Rheum. 1986; 29: 400–410.
75. Burgeson RE, Hollister DW. Collagen heterogeneity in human cartilage: identification of several new collagen chains. Biochem Biophys Res Commun. 1979; 87: 1124-1131.
76. Kumagai J, Sarkar K, Uhthoff HK, et al. Immunohistochemical distribution of type I, II and III collagens in the rabbit supraspinatus tendon insertion. J Anat. 1994; 185: 279–284.
77. Ye XJ, Terato K, Nakatani H, et al. Monoclonal antibodies against bovine type IX collagen (LMW fragment): production, characterization, and use for immunohistochemical localization studies. J Histochem Cytochem. 1991; 39: 265–271.
78. Caterson B. 9/30/8-A-4 for link protein, 12/21/1-C-6 for proteoglycan hyaluronic acid binding region. In: Developmental Studies Hybridoma Bank Product Catalog. NICHHD. University of Iowa, Iowa City. 2001; 12.
79. Caterson B, Baker JR, Christner JE, et al. Monoclonal antibodies as probes for determining the microheterogeneity of the link proteins of cartilage proteoglycan. J Biol Chem. 1985; 260: 11348–11356.
80. Asher RA, Scheibe RJ, Keiser HD, et al. On the existence of a cartilage-like proteoglycan and link proteins in the central nervous system. Glia, 1995; 13: 294–308.
81. 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. 1989; 201: 85-103.
82. Young HE, Ceballos EM, Smith JC, et al. Pluripotent mesenchymal stem cells reside within avian connective tissue matrices. In Vitro Cellular & Developmental Biology. 1993; 29A: 723-736.
83. Kasugai S, Nagata T, Sodek J. Temporal studies on the tissue compartmentalization of bone sialoprotein (BSP), osteopontine (OPN), and SPARC protein during bone formation in vitro. J Cell Physiol. 1992; 152: 467–477.
84. Asher RA, Scheibe RJ, Keiser HD, et al. On the existence of a cartilage-like proteoglycan and link proteins in the central nervous system. Glia. 1995; 13: 294-308.
85. Ronnov-Jessen L, Celis JE, Van Deurs B, et al. A fibroblast-associated antigen: characterization in fibroblasts and immunoreactivity in smooth muscle differentiated stromal cells. J Histochem Cytochem. 1992; 40: 475–486.
86. Solovey A, Lin Y, Browne P, et al. Circulating activated endothelial cells in sickle cell anemia. N Eng J Med. 1997; 337: 1584–1590.
87. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science. 2000; 289: 1197-1202.
88. Gorski JP, Griffin D, Dudley G, et al. Bone acidic glycoprotein-75 is a major synthetic product of osteoblastic cells and localized as 75- and/or 50-kDa forms in mineralized phases of bone and growth plate and in serum. J Biol Chem. 1990; 265: 14956-14963.
89. Picker LJ, Nakache M, Butcher EC. Monoclonal antibodies to

human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. J Cell Biol. 1989; 109: 927–937.
90. Lewinsohn DM, Nagler A, Ginzton N, et al. Hematopoietic progenitor cell expression of the H-CAM (CD44) homing-associated adhesion molecule. Blood. 1990; 75: 589–595.
91. Butcher EC. Hermes-1 for CD44. In: Developmental Studies Hybridoma Bank Product Catalog. The University of Iowa. 2002; 12.
92. Hildreth JE, August JT. The human lymphocyte function-associated (HLFA) antigen and a related macrophage differentiation antigen (HMac-1): functional effects of subunit-specific monoclonal antibodies. J Immunol. 1985; 134: 3272–3280.
93. August JT, Hildreth JEK. H5A4 for CD11b (Mac-1, CR3), H5H5 for CD43 (human) sialophorin, H4C4 for CD44 human hyaluronate receptor, H5A5 for CD45 (lymphocyte common antigen) (human), H5C6 for CD63 (human) LIMP. In: Developmental Studies Hybridoma Bank Product Catalog. The University of Iowa, Iowa City. 2002; 12.
94. Mujoo K, Ali M, Sahib MK. Isolation, characterization, and synthesis of alpha-fetoprotein from neonatal rat brain. J Neurochem. 1983; 41: 1223–1228.
95. Hixson DC, De Lourdes Ponce M, Allison JP, et al. Cell surface expression by adult rat hepatocytes of a non-collagen glycoprotein present in rat liver biomatrix. Exp Cell Res. 1984; 152: 402–414.
96. Hixson DC, Brown J, McBride AC, et al. Differentiation status of rat ductal cells and ethionine-induced hepatic carcinomas defined with surface-reactive monoclonal antibodies. Exp Mol Pathol. 2000; 68: 152–169.
97. Walborg EF Jr, Tsuchida S, Weeden DS, et al. Identification of dipeptidyl peptidase IV as a protein shared by the plasma membrane of hepatocytes and liver biomatrix. Exp Cell Res. 1985; 158: 509–518.
98. Faris RA, Monfils BA, Dunsford HA, et al. Antigenic relationship between oval cells and a subpopulation of hepatic foci, nodules, and carcinomas induced by the "resistant hepatocyte" model system. Cancer Res. 1991; 51: 1308–1317.
99. Gordon GJ, Coleman WB, Hixson DC, et al. Liver regeneration in rats with retrorsine-induced hepatocellular injury proceeds through a novel cellular response. Am J Pathol. 2000; 156: 607–619.
100. Hubbard AL, Bartles JR, Braiterman LT. Identification of rat hepatocyte plasma membrane proteins using monoclonal antibodies. J Cell Biol. 1985; 100: 1115–1125.
101. Young HE, Hyer L, Black AC Jr, et al. Adult stem cells: from bench-top to bedside. In: Tissue Regeneration: Where Nanostructure Meets Biology, 3DBiotech, North Brunswick. 2013a; 1-60.
102. Young HE, Hyer L, Black AC Jr, et al. Treating Parkinson disease with adult stem cells. J Neurological Disorders. 2013b; 2: 1.
103. Bonner-Weir S, Taneja M, Weir GC, et al. In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA. 2000; 97: 7999–8004.
104. Lumelsky N, Blondel O, Laeng P, et al. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science. 2001; 292: 1389-1393.
105. Rajagopal J, Anderson WJ, Kume S, et al. Insulin staining of ES cell progeny from insulin uptake. Science. 2003; 299: 363.
106. Mignon L, Vourc'h P, Romero-Ramos M, et al. Transplantation of multipotent cells extracted from adult skeletal muscles into the adult subventricular zone. J Cell Neurol. 2005; 491: 96-108.
107. 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. 2013; 63-93.
108. 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. Anat Rec. 1989; 223: 223-230.
109. Rajagopal J, Anderson WJ, Kume S, et al. Insulin staining of ES cell progeny from insulin uptake. Science. 2003; 299: 363.
110. Mignon L, Vourc'h P, Romero-Ramos M, et al. Transplantation of multipotent cells extracted from adult skeletal muscles into the subventricular zone of adult rats. J Comp Neurol. 2005; 491: 96-108.
111. Young HE. Existence of reserve quiescent stem cells in adults, from amphibians to humans. Curr Top Microbiol Immunol. 2004; 280: 71-109.
112. Young HE, Dalley BK, Markwald RR. Identification of hyaluronate within peripheral nervous tissue matrices during limb regeneration. Edited by Coates PW, Markwald RR, Kenny AD. Alan R. Liss, Inc., New York. In: Developing and Regenerating Vertebrate Nervous Systems, Neurology and Neurobiology. 1983; 6: 175-183.
113. Young HE, Bailey CF, Markwald RR, et al. Histological analysis of limb regeneration in postmetamorphic adult Ambystoma. Anat Rec. 1985; 212:183-194.
114. Young HE, Rogers JJ, Adkison LR, et al. Muscle morphogenetic protein induces myogenic gene expression in Swiss-3T3 cells. Wound Rep Reg. 1998; 6: 543-554.
115. Young HE. Existence of reserve quiescent stem cells in adults, from amphibians to humans. Curr Top Microbiol Immunol. 2004; 280: 71-109.
116. Rogers JJ, Adkison LR, Black AC Jr, et al. Differentiation factors induce expression of muscle, fat, cartilage, and bone in a clone of mouse pluripotent mesenchymal stem cells. Amer Surg. 1995; 61: 1-6.
117. Warejcka DJ, Harvey R, Taylor BJ, et al. A population of cells isolated from rat heart capable of differentiating into several mesodermal phenotypes. J Surg Res. 1996; 62: 233-242.
118. Young HE. Limb Regeneration in the Adult Salamander, Ambystoma annulatum Cope 1889 (Amphibia: Ambystomatidae). Univ Ark Lib Press, ©1977.