Research Article

Stem Cell & Regenerative Medicine

Primitive Stem Cells in Adult Human Peripheral Blood

Henry E. Young[1,2*], Frank Lochner[3], Deborah Lochner[3], Douglas Lochner[3], Gypsy F. Black[1], Julie A. Coleman[1], Valerie E. Young[2], George McCommon[4] and Asa C. Black, Jr.[5]

[1]Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, GA, 31207, USA.

[2]Dragonfly Foundation for Research and Development, Macon, GA 31210, USA.

[3]Cougar Creek Farms, Fort Valley, GA 31030, USA.

[4]Department of Veterinary Sciences, Fort Valley State University, Fort Valley, GA 31030, USA.

[5]Department of Medical Education, Greenville Hospital System, University of South Carolina School of Medicine, Greenville, SC 29605, USA.

***Correspondence:**
Henry E. Young, Chief Science Officer, Dragonfly Foundation for Research and Development, 1515 Bass Rd, Suite E (Corporate Office), Macon, GA 31210, USA, Tel: 478-319-1983; Fax: 478-743-0280; E-mail: young.hey1@yahoo.com.

Received: 12 June 2017; **Accepted:** 29 June 2017

Citation: Young HE, Lochner F, Lochner D, et al. Primitive Stem Cells in Adult Human Peripheral Blood. Stem Cells Regen Med. 2017; 1(1) 005: 1-8.

ABSTRACT

Recent reports demonstrated the presence of primitive endogenous stem cells circulating within adult cat, dog, sheep, goat, pig, cow, and horse peripheral blood. The current study was undertaken to determine whether similar primitive stem cells could be isolated from the peripheral blood of adult humans. Adult humans had their blood withdrawn following the guidelines of Mercer University School of Medicine and the Medical Center of Central Georgia Institutional Review Boards. The blood was obtained by venipuncture and processed to obtain primitive stem cells. Cells were identified and counted using 0.4% Trypan blue inclusion/exclusion analysis and stained with carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) antibody. Totipotent stem cells are both trypan blue and CEA-CAM-1 positive and <2.0 microns in size; transitional-totipotent/pluripotent stem cells are both trypan blue and CEA-CAM-1 positive & negative and >2.0 to <6.0 microns in size; and pluripotent stem cells are both trypan blue and CEA-CAM-1 negative and 6-8 microns in size. The results show that TSCs, Tr-TSC/PSCs, and PSCs are circulating within the peripheral blood of adult humans. Studies are ongoing to address their functional significance during maintenance and healing.

Keywords
Human, Blood, Totipotent Stem Cells, Pluripotent Stem Cells, Transitional-Totipotent/Pluripotent Stem Cells, Healing Cells, Trypan blue staining, CEA-CAM-1,

Introduction
Previous reports by Young and colleagues have noted the presence of endogenous healing cells [1], located within the connective tissues of multiple animal species, including humans [2-16]. Clones of these cells were derived by serial dilution single cell clonogenic analysis [11,13,14] and characterized. Based on multiple criteria, that included size of viable cells by flow cytometry; Trypan blue staining; cell surface markers; cluster of differentiation markers; viability post mortem; growth in serum-free defined medium with and without proliferation agents, progression agents, inductive agents, and inhibitory factors; growth at confluence; optimum cryopreservation conditions; differentiation capabilities both in vitro and in vivo; expressed genes, etc., multiple separate populations of endogenous healing cells were identified. The endogenous healing cells identified included totipotent stem cells (TSCs), transitional-totipotent stem cell/pluripotent stem cells (Tr-TSC/PSCs), pluripotent stem cells (PSCs), transitional-pluripotent stem cell/germ layer lineage stem cells (Tr-PSC/GLSCs), germ layer lineage stem cells (GLSCs), ectodermal stem cells (EctoSCs), mesodermal stem cells (MesoSCs), and endodermal stem cells (EndoSCs) [6,10,11,13-35].

Young et al. [16], Stout et al. [28], McCommon et al. [33], noted the presence of endogenous healing cells in the peripheral blood of multiple species of adult animals, i.e., cats, dogs, sheep, goats, pigs, cows, and horses. The current study was designed to determine if similar types of endogenous healing cells were circulating in the peripheral blood of adult humans.

Materials and Methods

The use of adult humans in this study complied with the guidelines of Mercer University School of Medicine and the Medical Center of Central Georgia Institutional Review Boards. All individuals signed consent forms before participating in this study.

Tissue Harvest

Adult human blood (n=8, four males and four females) was obtained by venipuncture following standard acceptable medical practice. The blood was collected using sterile procedures and placed in 5-ml EDTA hemovac tubes (Beckton-Dickinson), inverted several times to mix and then refrigerated at 4°C for 48 hours until further processing to isolate endogenous stem cells within the peripheral blood plasma fraction [28].

Stem Cell Isolation

After 48 hours of gravity separation, the blood had separated into a floating plasma fraction and a sedimented cellular fraction. The cellular fraction contained hematopoietic stem cells, red blood cells, white blood cells, and most mesodermal stem cells [28,33,36,47]. The plasma fraction was withdrawn using a sterile pipette, placed in a second sterile tube and refrigerated at 4°C.

Stem Cell Counting

Totipotent stem cells are Trypan blue positive very small spherical-shaped cells that are <2.0 microns in size [14,15,30]. Transitional-totipotent stem cell/pluripotent stem cells display a peripheral rim that stains with Trypan blue and a central core that does not. They are >2.0 to <6.0 microns in size [15,30]. Pluripotent stem cells do not stain with Trypan blue and are 6.0 to 8.0 microns in size [13,15,30]. Germ layer lineage stem cells do not stain with Trypan blue and are >8.0 to <20.0 microns in size [15,30].

Fifteen microliters of the plasma fraction from each adult human (n=8) was mixed with 15 microliters of sterile-filtered 0.4% w/v Trypan blue (Kodak, Rochester, NY) in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen, GIBCO, Grand Island, NY), pH 7.4. The resulting solution was placed onto a hemocytometer and the isolated cells counted [33].

All cells within the nine large boxes of a standard hemocytometer were counted and then averaged for the number of cells per 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 calculations were based on number of cells per ml for whole blood. Eight separate individuals counted the cells for this study. Figure 3 denotes the average from eight separate counters for each human plasma fraction sample +/- Standard Deviation.

Stem Cell Identification

Cells within the plasma fraction were stained with an antibody to carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) to identify the particular cell types present in the plasma fraction. Totipotent stem cells exhibit complete staining for CEA-CAM-1 antibody. Transitional-totipotent stem cell/pluripotent stem cells exhibit a peripheral rim that stains with CEA-CAM-1 and a central area that does not stain with the antibody. Pluripotent stem cells and germ layer lineage stem cells do not stain with the CEA-CAM-1 antibody [13-15].

In brief, the plasma fraction was placed within 15-ml polypropylene tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and mixed with an equal volume of ELICA fixative. The ELICA fixative was conposed of aqueous 0.4% v/v glutaraldehyde, 2% w/v paraformaldehyde, pH 7.4. D-glucose was used to titrate the fixative to an osmolality of 1.0 [33]. The tissue was fixed for 2 weeks and then rinsed with DPBS [10] at ambient temperature from 5 minutes to 7 days. The plasma / cell / fixative mixture was centrifuged at 2,000 x g. The resultant supernatant was decanted into bleach. The cell pellet was re-suspended and mixed with 14 ml of DPBS pH 7.4 at ambient temperature to wash the fixative from the cells. The cells were centrifuged at 2,000 x g, the resultant supernatant decanted into bleach, and the cells re-suspended. This washing process was repeated a second time to ensure the removal of the fixative from the cells. The cells then underwent the same immunocytochemical staining sequence used for tissue sections [15].

Immunocytochemistry

Fixed cells were stained using the Enzyme-Linked Immuno-Culture Assay (ELICA) construct for carcinoembryonic antigen cell adhesion molecule-1 epitope (clone 5.4) (CEA-CAM-1) [15,38]. The fixed cells located in the plasma fraction were incubated with 95% ethanol and then washed with 14 ml of DPBS. The cells were incubated with 5 ml of 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in DPBS for 60 minutes. They were washed with DPBS and incubated with 5 ml of 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes to irreversibly inhibit endogenous peroxidases [15,26,38]. The cells were rinsed with DPBS, and incubated for 60 minutes with blocking agent (Vecstain ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in DPBS [15,38]. The blocking agent was removed by centrifugation, and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with primary antibody for 60 minutes. The primary antibody consisted of 0.005% (v/v) CEA-CAM-1, clone 5.4 in DPBS [14,15]. The primary antibody was removed by centrifugation and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat adsorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector Laboratories) in DPBS [13,15]. The secondary antibody was removed by centrifugation and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure, Curtain Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vectastain ABC Reagent Kit, Vector Laboratories) in DPBS [13,15]. The avidin-HRP was removed by centrifugation and the supernatant decanted. The cells were re-suspended and washed with DPBS. The cells were incubated with AEC substrate (Sigma) for 60 minutes [13,15]. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed by centrifugation and the supernatant decanted. The cells were re-suspended, and washed with DPBS. Fifteen microliters of re-suspended cells were placed on a hemocytometer and the number of cells counted.

Visual Analysis

Stained cells were visualized using a Nikon TMS phase contrast microscope with bright field microscopy at 40x, 100x, and 200x. Photographs were taken with a Nikon CoolPix 995 digital camera. Photographs were cropped using Photoshop (Adobe).

Results

Three cell types were visualized with Trypan blue staining in the human peripheral blood plasma fraction (Figures 1 and 2A). Numerous, very small spherical structures that stained completely with the Trypan blue dye were designated as TSCs. Slightly larger cells with a stained peripheral rim and a clear central area were designated as Tr-TSC/PSCs. The third category of cells was larger than the other cell types and did not stain with Trypan blue dye. They appeared as white (glowing) spheres on a blue background of Trypan blue dye. These cells were designated as PSCs.

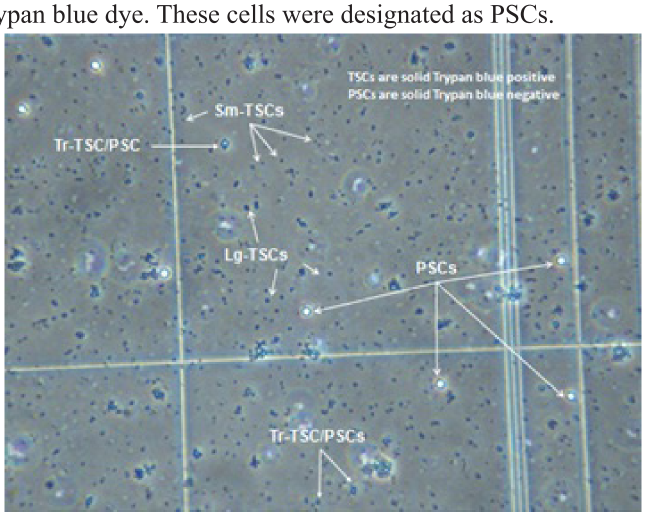

Figure 1: HM001, adult human stem cells isolated, stained 1:1 with 0.4% Trypan blue solution, diluted 1:1000 with sterile saline and mounted onto a hemocytometer, magnification 200X. Note Trypan blue negative cells that are white "glowing" spheres are pluripotent stem cells (PSCs). Spherical cells with a rim of Trypan blue positive staining and centers void of Trypan blue staining are transitional-totipotent stem cell/pluripotent stem cells. Large and small spherical cells that are totally Trypan blue positive are large and small totipotent stem cells.

Staining for CEA-CAM-1 was examined in cells removed from human peripheral blood plasma (Figure 2B). As with the Trypan blue staining, the entities designated as TSCs were very small circular structures that stained with the CEA-CAM-1 antibody. Larger circular structures with a rim of staining for CEA-CAM-1 and a clear center were designated as Tr-TSC/PSCs. PSCs were devoid of CEA-CAM-1 staining and could not be visualized with bright field microscopy.

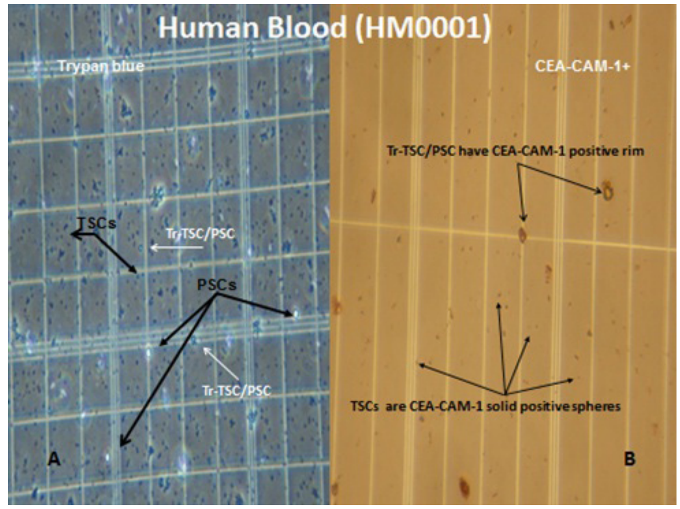

Figure 2: Isolated stem cells from adult human blood (HM0001).
A. HM0001 cells stained 1:1 with 0.4% Trypan blue solution, diluted 1:1000 with sterile saline and mounted onto a hemocytometer, magnification 200X. Note cells that are spherical and solid blue are large-TSCs and small-TSCs (totipotent stem cells). Spherical cells that have a rim of Trypan blue positive staining with centers void of Trypan blue positive staining are transitional-totipotent stem cell/pluripotent stem cells (Tr-TSC/PSCs). Trypan blue negative entities that are white "glowing" spheres are pluripotent stem cells (PSCs), 100x mag.
B. HM001 cells stained with CEA-CAM-1 antibody, diluted 1:1000 with sterile saline and mounted onto a hemocytometer, magnification 200X. Note very small cells that display CEA-CAM-1 throughout are small TSCs, which are 0.2 to 1.0 microns in size on flow cytometry of live cells. Note larger cells with rim of CEA-CAM-1 positive material with relatively "clear" centers are Tr-TSC/PSCs, which are 2 to 6 microns in size, 100x mag.

The results obtained with Trypan blue staining clearly demonstrated the existence of uniquely stained cells, i.e., totipotent stem cells, transitional-totipotent stem cell/pluripotent stem cells, and pluripotent stem cells, in adult human peripheral blood plasma in eight of the eight individuals examined (Figure 3).

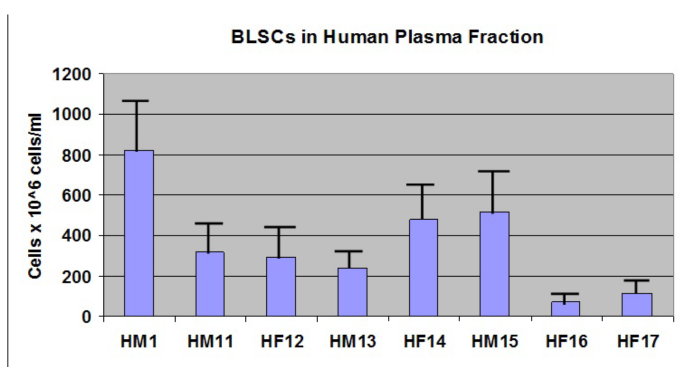

Figure 3: Mean counts (+/- SD) of totipotent stem cells (a.k.a., BLSCs) in human plasma fractions from eight volunteers. HM1 = HM-0001 in figures.

Discussion

Young and colleagues [1,10,14,37,39] described five main categories of stem cells involved in the healing of body tissues and organs. These categories are based on the size of viable cells with flow cytometry, their unique Trypan blue staining patterns, the identity of unique cell surface markers, optimal cryopreservation conditions, doubling times, growth at confluence, capabilities for self-renewal, differentiation potentials and unidirectional developmental lineage patterns. These five major categories of adult stem cells are totipotent stem cells, transitional-totipotent stem cell/pluripotent stem cells, pluripotent stem cells, transitional-pluripotent stem cell/germ layer lineage stem cells, and germ layer lineage stem cells consisting of ectodermal stem cells, mesodermal stem cells, and endodermal stem cells. The TSCs are less than 2 microns in size, stain positive for Trypan blue and express carcinoembryonic antigen (CD66e/CEA-CAM-1) on their cell surface. They have the capability to form any somatic cell in the body, as well as forming the germ cells such as spermatogonia. Therefore, TSCs are designated as an endogenous adult-derived totipotent stem cell. The Tr-TSC/PSCs are >2 to <6 microns in size. These transitional stem cells demonstrate a rim of Trypan blue positive staining and a central area void of Trypan blue staining. The Tr-TSC/PSCs express the stem cell surface markers characteristic of both TSCs and PSCs, i.e., carcinoembryonic antigen (CD66e/CEA-CAM-1), stage specific embryonic antigen (SSEA), and neutral endopeptidase (CD10). Transitional-TSC/PSCs can form any somatic cell type in the body, but do not form the germ cells. They are designated as an endogenous adult-derived pluripotent stem cell. The PSCs are 6-8 microns in size and do not stain with Trypan blue. These stem cells express the cell surface markers stage specific embryonic antigen (SSEA) and neutral endopeptidase (CD10). PSCs can form any somatic cell type in the body, but do not form the germ cells. They are designated as an endogenous adult-derived pluripotent stem cell. The Tr-PSC/GLSCs are 8-10 microns in size and do not stain with Trypan Blue. The Tr-PSC/GLSCs express cell surface markers characteristic of both PSCs and GLSCs, i.e., stage specific embryonic antigen (SSEA), neutral endopeptidase (CD10), CD90, and Thy-1. Like the Tr-TSC/PSCs and PSCs, Tr-PSC/GLSCs will form all somatic cells of the body, but will not form the germ cells. Therefore, they are designated as an endogenous adult-derived pluripotent stem cell. Germ layer lineage stem cells are 10-20 microns in size and will not stain with Trypan blue in the viable state. There are three separate categories of GLSCs based on their respective germ layer of origin, i.e., ectodermal stem cells (EctoSCs), mesodermal stem cells (MesoSCs), and endodermal stem cells (EndoSCs). However, all three types of GLSC express the cell surface markers CD90, Thy-1, and MHC-I. The GLSCs will form cells belonging only to the appropriate germ layer from which they originally arose. For example, EctoSCs can form cells of the ectodermal lineage consisting of both neural ectoderm and surface ectoderm origin, i.e., neurons, oligodendrocytes, astrocytes, ganglion cells, Schwann cells, melanocytes, sensory nerve endings, motor nerve endings, and keratinocytes, hair follicles, sweat glands, etc. Mesodermal stem cells can form cell types derived embryologically from somitic mesoderm, intermediate mesoderm, and lateral plate (somatic and splanchnic) mesoderm origin, i.e., three types of muscle, two types of fat, five types of cartilage, two types of bone, multiple types of connective tissue stroma, scar tissue, multiple types of functional endothelial cells, vasculature, all types of hematopoietic cells, tissues within the genitourinary system (except the germ cells, sperm or ova), mesothelium, spleen, adrenal cortex, etc. Whereas EndoSCs can form pulmonary lining cells, gastrointestinal lining cells, cystic lining cells, hepatocytes, canalicular cells, biliary cells, pancreatic exocrine acinar cells and ducts, pancreatic endocrine alpha-cells, beta-cells, delta-cells, thymus, thyroid, parathyroid, etc.

As shown in Figure 3, there was a tremendous variation in the average number of TSCs in millions per ml of blood. Averages ranged from a high of 820 x 10^6 cells per ml of blood (HM1) to a low of 70 x 10^6 cells per ml of blood (HF16). Interestingly, both the HM1 and HF16 samples were taken from participants suffering from autoimmune disorders. Participant HM1 has systemic lupus erythematosus and has refused the traditional methotrexate therapy. In contrast, participant HF16 has rheumatoid arthritis and is being treated with methotrexate for their disorder. In addition, participant HF17 has an avian form of tuberculosis that is currently not being treated. Therefore, whether methotrexate therapy impacts the number of TSCs in the peripheral blood remains to be determined. The remaining individuals in the study classified themselves as relatively healthy. The average numbers of circulating TSCs in the peripheral blood ranged from 514 x 10^6 cells per ml of blood to 225 x 10^6 cells per ml of blood. More research is needed to determine standard values for circulating TSCs. It is possible that the number of circulating TSCs, PSCs, and MesoSCs might have diagnostic and/or prognostic significance.

Adult stem cells are being used experimentally in multiple animal species to treat a variety of conditions [40-45]. One category of maintenance cells, first described as “mesenchymal stem cells” [46], have been the focus of many studies. These cells have been defined as somatic cell populations found most commonly in bone marrow [46-48]. However, they are also found in various organs, such as periosteum [49], adipose tissue [50], and the connective tissue stroma associated with skeletal muscle [15]. Questions have persisted regarding whether these mesenchymal stem cells (MSC’s) are legitimate multipotent stem cells or whether they are simply collections of cell- and tissue-committed progenitor cells derived from connective tissue [51]. Recently, Caplan [52], suggested a name change for “mesenchymal stem cells” to “medicinally secreting cells” thereby retaining the “MSCs” designation. His suggested name change was based on the activity of the cells during various regenerative medicine scenarios [52].

The ability to utilize embryonic stem cells (ESCs) in human medicine is hampered by the complexity of the isolation process, and moral and ethical issues. Alternatively, induced pluripotent stem cells (iPSCs) have now come into vogue as a potential replacement for the ESCs [34] in regenerative therapies. The most widely accepted method to generate induced pluripotent stem cells is the retroviral vector introduction of four genes, a.k.a., the Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc,) into more differentiated cell types, such as fibroblasts [34,53]. Following the initial landmark work Yamanaka, iPSCs have since been generated from differentiated fetal and adult fibroblasts [53-60]; hepatocytes [61]; stomach cells [62]; keratinocytes [63]; cord blood [63-65]; peripheral blood [66,67]; fully differentiated B- and T- lymphocytes [68-73]; dental pulp cells [74-76]; and kidney cells [77].

However, it has also been recognized that the less differentiated the cell type, the fewer the number of Yamanaka factors (genes) are required to induce pluripotency. More efficient reprogramming has been shown to occur in maintenance (progenitor) cells rather than terminally differentiated functional cells [78]. For example, umbilical cord cells that already express Klf4 and c-Myc were found to form iPSCs when challenged with Oct4 and Sox2 [65]. Alternatively, neural progenitor cells, expressing the Sox2 gene could be induced to form IPSCs with the insertion of Oct4 and Klf4 [79-81]. Recent reports demonstrate that iPSCs can be generated from the granulosa cells of ovarian follicles by Oct4 and Sox2 [78]. Indeed, iPSCs demonstrate enhanced expression of the pluripotency-associated genes, Oct4, Telomerase [34], and SSEA [82,83], which are similar to the genes expressed in endogenous pluripotent stem cells [21,13,30,84-86].

Unfortunately, there are problems with ESCs and iPSCs in their ability to maintain lineage and genetic stability, control tumor development, and prevent rejection [87]. The use of endogenous adult healing cells obtained from the host would avoid many of these issues. Young and colleagues identified three major categories of primitive stem cells located in solid tissues and organs of adult mammals. Commencing with the most undifferentiated cell, these categories are totipotent stem cells [14], pluripotent stem cells [13], and germ layer lineage stem cells [11]. Any or all of these adult stem cells have the potential for use for replacement therapies in clinical medicine. However, their isolation from solid tissues, such as adipose tissue [47], for autologous stem cell therapy, is painful for the individual and may create wounds that are slow to heal. The use of endogenous adult stem cells obtained by venipuncture from the peripheral blood may avoid these difficulties. Koerner et al. [36] obtained fibroblastoid cells from the peripheral blood of equines, but reported that these cells exhibited limited multi-lineage differentiation potential. Recently, Stout et al. [28] reported the presence of endogenous TSCs circulating within adult porcine peripheral blood; McCommon et al. [33] reported the presence of TSCs circulating within adult equine peripheral blood; and Young et al. [16] reported the presence of TSCs, Tr-TSC/PSCs, and PSCs circulating in the peripheral blood of multiple mammalian species, i.e., cats, dogs, sheep, goats, cows, and horses. The current study was undertaken to determine whether similar primitive stem cells could be isolated from adult human peripheral blood.

The results of this study confirm that identifiable adult totipotent stem cells and pluripotent stem cells (Tr-TSC/PSCs, and PSCs) can be isolated from adult human peripheral blood. The use of peripheral blood provides a much simpler extraction method than current methods of obtaining adult stem cells from adipose tissue or bone marrow. The presence of TSCs and PSCs in all species makes possible the development of treatments with species-specific autologous totipotent stem cells, pluripotent stem cells, and mesodermal stem cells.

The implications for usage of these cells to repair damaged tissue are enormous. Cells used in autologous transplants for the repair of damaged tissue are frequently isolated from another tissue. Indeed, TSCs, PSCs, and MesoSCs isolated from alternative solid organs and tissues have been utilized in adult transplantation model systems to affect the repair of articular cartilage, bone, skeletal muscle, and myocardium. They have also been used to repopulate the subventricular zone, and neurons of the midbrain and cortex, as well as to generate three-dimensional constructs of pancreatic islets [21,24,27,35, 88].

As a result of this study, TSCs, Tr-TSC/PSCs, and PSCs have now been isolated from the peripheral blood of human test subjects. Current studies are being undertaken to determine whether circulating primitive healing cells could form the basis for autologous stem cell therapy. If so, this therapeutic approach would be less traumatic to the patient than isolating stem cells from fat or bone marrow. Patients are currently receiving peripheral blood stem cells in the treatment for acute myeloid leukemia, acute lymphoblastic leukemia, and multiple myeloma [89,90]. Studies have shown that peripheral blood cells are as effective as bone marrow transplants in the in repopulating of bone marrow. However, stem cells from peripheral blood display an increase in the risk of graft versus host disease (GVHD) [89,90]. Autologous transplants of TSCs, Tr-TSC/PSCs, PSCs, and MesoSCs would significantly decrease the risk of GVHD. Further studies are required to demonstrate the efficacy of autologous peripheral healing cell transplantation in the treatment of damaged or diseased tissues. Studies are in progress to address the functional significance of these circulating adult stem cells during disease states, and during the process of injury and repair.

Indeed, we utilized the isolation scheme discussed herein to derive autologous TSCs, PSCs, and MesoSCs for an IRB-approved clinical trial for Parkinson disease [31,32] (Figure 4).

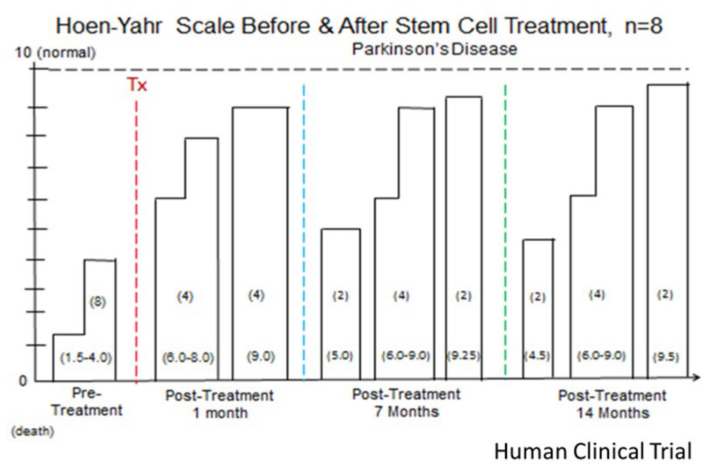

Figure 4: IRB-approved clinical trial for Parkinson disease with eight

participants completing a 14-month trial. Hoen-Yahr scores (quality of life) were averaged across ten separate parameters before treatment began, and at one, seven and 14 months post-treatment. A single treatment with autologous stem cells resulted in 100% of participants demonstrating a better quality of life (H-Y score) than before autologous stem cell treatment at one month following treatment. The seven-month follow-up showed that 25% reverted and began to decline, but at a H-Y score higher than before their stem cell transplant; 50% remained stable; and 25% continued to improve (slight rise in H-Y score). The 14th month follow-up demonstrated the same results as 7-month follow-up in the same participants: 25% continued to decline but with a H-Y score still higher than before stem cell treatment, 50% remained stable, and 25% continued in their improvement with a similar rise in H-Y score.

In this trial, a single autologous stem cell transplant was utilized, which consisted of an intra-nasal infusion of TSCs accompanied by an IV-infusion of PSCs and MesoSCs. While 25% of the participants did revert, and begin to show a decline in symptoms (decreasing H-Y scores), clearly 75% of the participants benefited from the treatment, at least to the end of the trial. Fifty percent of the individuals remained stable throughout the 14-month time period following treatment, whereas 25% demonstrated continued improvement in their disease status (increasing H-Y scores).

The ability to harvest TSCs, PSCs, and MesoSCs by the collection of peripheral blood rather than the use of more invasive protocols that include enzymatic digestion and/or sonication to separate cells from their adherent extracellular matrices greatly simplifies the acquisition of these endogenous healing cells for clinical use. The procedure has the potential for making therapeutic advances possible in tissue engineering in all aspects of medicine. We are currently pursuing IRB-approved clinical trials to ascertain the quality and quantity of endogenous healing cells necessary for positive long-term treatment and increased quality of life outcomes.

Acknowledgements
The authors would like thank M Carriero, O Samples, P Jones, S Fortson, C Alena, V Krishna, L McGill, and FP Boyer III for their technical assistance. The authors would like to thank D Hixson, Department of Internal Medicine, Brown University, Providence, RI, for the kind gift of the CEA-CAM-1 antibody. These studies were funded in part by MedCen Foundation, Rubye Ryle Smith Charitable Trust, and L.M. & H.O. Young Estate Trust.

References
1. Young HE, Speight MO, Black AC Jr. Functional Cells, Maintenance Cells, and Healing Cells. Stem Cells Regen Med. 2017; 1: 003: 1-4.
2. Young HE. Epidermal ridge formation during limb regeneration in the adult salamander, Ambystoma annulatum. Proc Ark Acad Sci. 1977; 31: 107-109.
3. Young HE, Bailey CF, Markwald RR, et al. Histological analysis of limb regeneration in postmetamorphic adult Ambystoma. Anat Rec. 1985; 212: 183-194.
4. Young HE, Ceballos EM, Smith JC, et al. Pluripotent mesenchymal stem cells reside within avian connective tissue matrices. In Vitro Cell Dev Biol. 1993; 29A: 723-736.
5. Pate DW, Southerland SS, Grande DA, et al. Isolation and differentiation of mesenchymal stem cells from rabbit muscle. Surg Forum, 1993; XLIV: 587-589.
6. 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: 231-236.
7. 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.
8. Lucas PA, Calcutt AF, Southerland SS, et al. A population of cells resident within embryonic and newborn rat skeletal muscle is capable of differentiating into multiple mesodermal phenotypes. Wound Rep Regen. 1995; 3: 449-460.
9. 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.
10. Young HE, Steele T, Bray RA, et al. Human progenitor and pluripotent cells display cell surface cluster differentiation markers CD10, CD13, CD56, CD90 and MHC Class-I. Proc Soc Exp Biol Med. 1999; 221: 63-71.
11. 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.
12. Young HE. Existence of reserve quiescent stem cells in adults, from amphibians to humans. Curr Top Microbiol Immunol. 2004; 280: 71-109.
13. 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.
14. Young HE, Black AC Jr. Adult-derived stem cells. Minerva Biotechnologica Cancer Gene Mechanisms and Gene Therapy Reviews. 2005; 17: 55-63.
15. Young HE, Henson NL, Black GF, et al. Location and characterization of totipotent stem cells and pluripotent stem cells in the skeletal muscle of the adult rat. Stem Cells Regen Med. 2017; 1: 002: 1-17.
16. Young HE, Lochner F, Lochner D, et al. Primitive Stem Cells in Adult Feline, Canine, Ovine, Caprine, Bovine, and Equine Peripheral Blood. Stem Cells Regen Med. 2017; 1: 004: 1-6.
17. 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.
18. 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.
19. 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.
20. 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.
21. 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.

22. 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 Com. 2004; 317: 893-901.
23. 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.
24. 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; 4: 67-92.
25. Vourc'h P, Lacar B, Mignon L, et al. Effect of neurturin on mulitpotent cells isolated from the adult skeletal muscle. Biochem Biophys Res Com. 2005; 332: 215-223.
26. Henson NL, Heaton ML, Holland BH, et al. Karyotypic analysis of adult pluripotent stem cells. Histo Histopath. 2005; 20: 769-784.
27. 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. 9: 753-769.
28. 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. Amer Surg. 2007; 73: 1106-1110.
29. Stout CL, McKenzie J, Long G, et al. Discovery of pluripotent and totipotent stem cells in the heart of the adult rat. Amer Surg. 2007; 73: S63.
30. 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; 3: 63-93.
31. 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 NJ. 2013; 1: 1-60.
32. Young HE, Hyer L, Black AC Jr, et al. Treating Parkinson disease with adult stem cells. J Neurol Dis. 2013; 2: 1.
33. McCommon GW, Lochner F, Black Jr AC, et al. Primitive adult-derived stem cells are present in the blood of adult equines and can be increased in number with moderate exercise or ingestion of a cyanobacter, Aphanizomenon flos-aquae. Autocoids. 2013; 2: 103.
34. Young HE, Black Jr AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol. 1: 00019.
35. Young HE, Limnios JI, Lochner F, et al. Pancreatic islet composites secrete insulin in response to a glucose challenge. Stem Cells Regen Med. 2017; 1: 001: 1-12.
36. Koerner J, et al. Stem Cells. 2006; 24: 1613-1619.
37. Young HE, Black Jr AC. Adult stem cells. Anat Rec. 2004; 276A: 75-102.
38. Young HE, Sippel J, Putnam LS, et al. Enzyme-linked immuno-culture assay. J of Tiss Cult Meth. 1992; 14: 31-36.
39. Young HE, Steele T, Bray RA, et al. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264:51-62, 2001.
40. Urbanek K, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 102(24):8692-8697, 2005.
41. Young RG, et al. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res 16:406-413, 1998.
42. Herthel DJ. Enhanced suspensory ligament healing in 100 horses by stem cell and other bone marrow components. AAEP Proceedings 47:319-21, 2001.
43. Smith RK, et al. The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. Matrix Biol. 16: 255-271, 1997.
44. Carstanjen B, et al. Successful engraftment of cultured autologous mesenchymal stem cells in a surgically repaired soft palate defect in an adult horse. Can J Vet Res. 2006; 70:143–147.
45. Arriero M, et al. Adult skeletal muscle stem cells differentiate into endothelial lineage and ameliorate renal dysfunction after acute ischemia Am J Physiol Renal Physiol. 2004; 287: 621-627.
46. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991; 9: 641-650.
47. Haynesworth SE, Goshima J, Goldberg VM, et al. Characterization of cells with osteogenic potential from human marrow. Bone. 1992; 13: 81–88,.
48. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005; 11: 1198-1211.
49. Nakahara H, Bruder SP, Goldberg VM, et al. In vivo osteochondrogenic potential of cultured cells derived from the periosteum. Clin Orthop Relat Res. 1990; 259: 223–232.
50. Parker AM, Katz AJ. Adipose-derived stem cells for the regeneration of damaged tissues. Expert Opin Biol Ther. 2006; 6: 567-578.
51. Phinney, DG. Building a consensus regarding the nature and origin of mesenchymal stem cells. J Cell Biochem. 2002; 38: 7-12.
52. Caplan AI. Mesenchymal stem cells: time to change the name!. Stem Cells Transl Med. 2017; 6: 1445-1451.
53. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126: 663-676.
54. Maherali N, Sridharan R, Xie W, et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell. 2007; 1: 55-70.
55. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol. 2007; 25: 1177-1181.
56. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861-872.
57. Huangfu D, Osafune K, Maehr R, et al. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol. 2008; 26: 1269-1275.
58. Nakagawa M, Koyanagi M, Tanabe K, et al. Generation of induced pluripotent stem cells without Myc from mouse and

human fibroblasts. Nat Biotechnol. 2008; 26: 101-106.
59. Li Y, Zhang Q, Yin X, et al. Generation of iPSCs from mouse fibroblasts with a single gene, Oct4, and small molecules. Cell Res. 2011; 21: 196-204.
60. Chen J, Liu J, Yang J, et al. BMPs functionally replace Klf4 and support efficient reprogramming of mouse fibroblasts by Oct4 alone. Cell Res. 2011; 21: 205-212.
61. Aoi T, Yae K, Nakagawa M, et al. Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science. 2008; 321: 699-702.
62. Aasen T, Raya A, Barrero MJ, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol. 2008; 26: 1276-1284.
63. Haase A, Olmer R, Schwanke K, et al. Generation of induced pluripotent stem cells from human cord blood. Cell Stem Cell. 2009; 5: 434-441.
64. Giorgetti A, Montserrat N, Aasen T, et al. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell Stem Cell. 2009; 5: 353-357.
65. Giorgetti A, Montserrat N, Rodriguez-Piza I, et al. Generation of induced pluripotent stem cells from human cord blood cells with only two factors: Oct4 and Sox2. Nat Protoc. 2010l; 5: 811-820.
66. Loh YH, Agarwal A, Park IH, et al. Generation of induced pluripotent stem cells from human blood. Blood. 2009; 113: 5476-5479.
67. Eminli S, Foudi A, Stadtfeld M, et al. Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat Genet. 2009; 41: 968-976.
68. Hanna J, Markoulaki S, Schorderet P, et al. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell. 2008; 133: 250-264.
69. Pereira CF, Terranova R, Ryan NK, et al. Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. PLoS Genet. 2008; 4: e1000170.
70. Brown ME, Rondon E, Rajesh D, et al. Derivation of induced pluripotent stem cells from human peripheral blood T lymphocytes. PLoS One. 2010; 5: e11373.
71. Loh YH, Hartung O, Li H, et al. Reprogramming of T cells from human peripheral blood. Cell Stem Cell. 2010; 7: 15-19.
72. Seki T, Yuasa S, Oda M, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell. 2010; 7: 11-14.
73. Staerk J, Dawlaty MM, Gao Q, et al. Reprogramming of human peripheral blood cells to induced pluripotent stem cells. Cell Stem Cell. 2010; 7: 20-24.
74. Tamaoki N, Takahashi K, Tanaka T, et al. Dental pulp cells for induced pluripotent stem cell banking. J Dent Res. 2010; 89: 773-778.
75. Yan X, Qin H, Qu C, et al. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 2010; 19: 469-480.
76. Oda Y, Yoshimura Y, Ohnishi H, et al. (2010) Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. J Biol Chem. 2010; 285: 29270-29278.
77. Montserrat N, Ramirez Bajo MJ, Xia Y, et al. Generation of induced pluripotent stem cells from human renal proximal tubular cells with only two transcription factors, OCT4 and SOX2. J Biol Chem. 2012; 287: 24131-24138.
78. Mao J, Zhang Q, Xiaoying Y, et al. Efficient induction of pluripotent stem cells from granulosa cells by Oct4 and Sox2. Stem Cells Dev. 2014; 23: 779-789.
79. Eminli S, Utikal J, Arnold K, et al. Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells 2008; 26: 2467-2474.
80. Kim JB, Zaehres H, Wu G, et al. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature. 2008; 454: 646-650.
81. Kim JB, Sebastiano V, Wu G, et al. Oct4-induced pluripotency in adult neural stem cells. Cell. 2009; 136: 411-419.
82. Huang J, F Wang F, Okuka M, et al. Association of telomere length with authentic pluripotency of ES/iPS cells. Cell Res. 2011; 21: 779-792.
83. Wang F, Yin Y, Ye X, et al. Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells. Cell Res. 2012; 22: 757-768.
84. Kucia M, Reca R, Campbell FR, et al. A population of very small embryonic-like (VSEL) CXCR4+SSEA-1+Oct-4+ stem cells identified in adult bone marrow. Leukemia. 2006; 20: 857-869.
85. KuciaM, Halasa M, Wysoczynski M, et al. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood - preliminary report. Leukemia. 2006; 21: 297-303.
86. Wojakowski W, Tendera M, Kucia M, et al. (2009) Mobilization of bone marrow-derived oct-4+ ssea-4+ very small embryonic-like stem cells in patients with acute myocardial infarction. J Am Coll Cardiol. 2009; 53: 1-9.
87. Hayes B, et al. Derivation, characterization, and in vitro differentiation of canine embryonic stem cells. Stem Cells. 2007.
88. Mignon L, Vourc'h P, Romero-Ramos M. Transplantation of multipotent cells extracted from adult skeletal muscles into the adult subventricular zone. J Cell Neurol. 2005; 491: 96-108.
89. Gahrton G, Iacobelli S, Bandini G, et al. Peripheral blood or bone marrow cells in reduced-intensity or myeloablative conditioning allogeneic HLA identical sibling donor transplantation for multiple myeloma. Haematologica/The Hematology Journal. 2007; 92: 1513-1519.
90. Ringden O, Labopin M, Bacigalupo A, et al. Transplantation of peripheral blood stem cells as compared with bone marrow from HLA-identical siblings in adult patients with acute myeloid leukemia and acute lymphoblastic leukemia. J Clin Oncol. 2002; 20: 4655-4665.