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Telomerase-Positive Stem Cells in Adult Porcine and Adult Rat Spleens I. Totipotent Stem Cells

Henry E Young^{1-3*}, Ioannis Jason Limnios⁴, Frank Lochner⁵, George McCommon⁶

¹Dragonfly Foundation for Research & Development, Macon, GA 31210, USA

²Henry E Young PhD Regeneration Technologies LLC, USA

³Mercer University School of Medicine, Macon, GA 31207, USA

⁴Clem Jones Centre for Regenerative Medicine, Bond University, Robina, QLD, Australia

⁵Cougar Creek Veterinary Consultants, Spencer, TN 38585, USA

⁶Department of Veterinary Sciences, Fort Valley State University, Fort Valley, GA 31030, USA

***Corresponding Author:** Henry E Young PhD, Chief Science Officer, Dragonfly Foundation for Research & Development, 101 Preston Ct, Suite 101, (Corporate Office), Macon, GA 31210 USA; Tel: +478-3191983; Email: young.hey1@yahoo.com

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Abstract

Previous studies have reported the presence of endogenous undifferentiated totipotent stem cells within the organs and tissues of various animal species, including the spleen. Since one major function of the spleen is to filter out damaged red blood cells, we wanted to ascertain whether totipotent stem cells existed as a potentially transient circulating population solely within the vasculature and sinusoids of the spleen or whether they existed as an endogenous resident population of primitive stem cells throughout the tissues of the spleen, and potentially involved in the repair of the spleen. The spleens from two separate mammalian species were examined, i.e., adult pigs and adult rats. Adult pigs were euthanized following the guidelines of Fort Valley State University's IACUC. Adult rats were euthanized following the guidelines of Mercer University School of Medicine's IACUC. The spleens were harvested, fixed, frozen, cryosectioned, and stained with an antibody diagnostic for the endogenous totipotent stem cells, i.e., Carcinoembryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1). CEA-CAM-1 positive stem cells were located within the capsule of the spleen, along the splenic trabeculae,

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within the red pulp, within the white pulp, along the central arteries and surrounding the penicillar arteries of the spleen in both the adult pig and in the adult rat. These results suggested that the totipotent stem cells are a resident population of stem cells within the splenic tissues. Studies are ongoing to address their functional significance in repair of the spleen.

Keywords

Adult; Endogenous; Totipotent; Stem Cells; Spleen; Pig; Rat; Regenerative Medicine

Introduction

The spleen is an important organ in the function of the immune system. Although it is the largest secondary lymphatic organ of the body it lacks a cortex and medulla, present in other lymphatic organs. Instead, the parenchyma of the spleen contains two major components with distinct functions, e.g., red pulp and white pulp. The red pulp is composed of an interconnected network of splenic sinusoids lined with splenic cords that separate the sinusoids. The splenic cords (cords of Billroth) contain macrophages, plasma cells, and blood cells, supported by reticular cells and fibers. Cytoplasmic processes of macrophages within the splenic cords project into the lumen of the sinusoids to sample particulate material. The red pulp filters damaged and/or aged red blood cells. It is also involved in recycling hemoglobin and iron from these RBCs. The heme portion of hemoglobin is metabolized to bilirubin, which is removed in the liver. The globin portion of hemoglobin is degraded to its constitutive amino acids. The red pulp also removes microorganisms from the circulating blood. Microorganisms (bacteria and viruses) can be recognized by macrophages and removed from the vascular system either directly or after being coated with immunoglobulins and complement. The immunoglobulins are produced in lymphoid tissues, whereas complement is produced in the liver. The macrophage clearance of bacteria and viruses is rapid and prevents infections in the meninges, lung, and kidneys. The red pulp also serves as a reservoir of erythrocytes, lymphocytes, and platelets, which is useful in the case of emergencies, such as hemorrhagic shock, hypovolemia and hypoxia, where there is insufficient oxygenated blood flow to the body tissues [1-14].

The function of the white pulp is to promote adaptive immune responses to blood-borne antigens. The spleen is the focal point of the mononuclear phagocyte system. The white pulp, which is analogous to a large lymph node, encompasses the immune components of the spleen by synthesizing antibodies and immunoglobulins for the recognition of foreign material, e.g., bacteria, viruses, etc. The white pulp consists of four regions, e.g., follicular arteriole (central artery), Periarteriolar Lymphoid Sheath (PALS), corona formed by B-cells and antigen presenting cells, and germinal center. The central artery gives rise to the radial arterioles, which end in the marginal sinus surrounding the white pulp. The white pulp of the spleen has activities analogous to a lymph nodule, producing B-cell clones in the presence of T-cells derived from the PALS. Functioning white pulp is extremely important during bacteremia

where there is presence of viable bacteria in the blood stream. Macrophages can sequester bacteria, process, and present their antigens to lymphocytes to stimulate an innate immune response. Antigens enter the spleen from the blood stream and reach the white pulp through the trabecular arteriole, to the central artery, to the radial arterioles, and then into the marginal sinus. Antigen-presenting cells in the corona region detect the blood-borne antigens that are sampled by the PALS-derived T-cells. The T-cells then interact with B-cells, causing B-cell proliferation and differentiation into plasma cells that release immunoglobulins. Monocytes, developed in the spleen, move to damaged tissues, and turn into macrophages while promoting tissue healing [1,4,15-22].

The spleen is covered by a capsule, consisting of dense irregular connective tissue interspersed with elastic fibers and smooth muscle cells. Blood enters and exits the spleen through the splenic artery and splenic vein which pierce the capsule at the hilum and divide into progressively smaller branches surrounded by trabeculae. Trabeculae emanating from the splenic capsule, form conduits to carry trabecular arteries and trabecular veins, and nerve branches of the splenic plexus to and from the red pulp. As an artery leaves a trabecula it becomes surrounded by T-cells forming a Periaarteriolar Lymphoid Sheath (PALS) and penetrates a lymphatic nodule of the white pulp. Within the white pulp the artery is designated as the follicular arteriole (central artery). As the central artery leaves the white pulp it becomes the penicillar artery (arteriole) and ends as macrophage-sheathed capillaries. The spleen has both an open and closed circulation system. Terminal capillaries either terminate as open-ended vessels within the red pulp (open circulation system) or drain directly into splenic sinusoids (closed circulation system). The closed circulation route leaving the spleen is splenic sinusoids, to pulp veins, to trabecular veins, to splenic vein [4,8].

Splenic injury can occur by multiple events. Blunt force abdominal trauma from traffic accidents, sports injuries, or falls can cause splenic rupture which requires immediate medical intervention. In severe cases, removal of the spleen (splenectomy) is required for patient survival. Unfortunately, splenectomy causes serious damage to the functions of the immune system. Non-traumatic causes of ruptured spleen include infectious diseases, hematological diseases, medical procedures, medications, and pregnancy. The spleen can be damaged in sickle cell disease, in which the patient gradually loses splenic function due to the trapping of sickled red blood cells in the splenic cords and sinusoids. The termination of this process is the destruction of the spleen. Spleen enlargement can also occur and is known as splenomegaly. Splenomegaly can be caused by obstruction of blood flow, underlying abnormality, infiltration, or antigenic stimulation [23-30].

Fifty percent of the cells in adults consist of differentiated parenchyma and stroma, while the remaining 50% are composed of various types of endogenous stem cells [31,32]. Of those stem cells, approximately 40% are telomerase negative stem cells, approximately 10% are telomerase positive stem cells, and less than 0.1% are totipotent stem cells [31,32]. Both telomerase negative MSCs, VSELs, and MAPCs, and telomerase positive stem cells, have been identified in the spleen [32-41].

Stout et al., reported that telomerase positive stem cells were present within the skeletal musculature and circulating within the vasculature of adult pigs and demonstrated their increased circulation in the bloodstream following trauma [42]. Since one of the major functions of the spleen is to filter out damaged red blood cells, the goal of the study was to ascertain whether the endogenous population of totipotent (CEA-CAM-1⁺) stem cells existed as a potentially transient circulating population solely within the vasculature of the spleen or whether they existed as a resident population of primitive stem cells throughout the tissues of the spleen. The hypothesis to delineate transient versus resident cells was based on the location of the cells within specific regions of the spleen, e.g., splenic blood vessels and sinusoids (transient) versus splenic capsule, trabeculae, red pulp, etc. (resident).

Materials and Methods

The use of animals in this study complied with the guide lines of Mercer University Institutional Animal Care and Use Committee, Fort Valley State University Institutional Animal Care and Use Committee, and criteria of the National Research Council for the humane care of laboratory animals as outlined in the “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 outbred Sprague-Dawley rats were euthanized using inhalation of carbon dioxide. The anterior chest wall and abdomen were washed with Betadine solution and incised. Each spleen was removed under aseptic conditions. Adult pigs were anesthetized with tiletamine and zolazepam and prepared for surgery with a Betadine wash. Sterile drapes were placed on the abdomen and a midline laparotomy incision was performed. The spleens were isolated, resected and removed. Porcine spleens were cut into 1 cm³ pieces. All spleens were placed in 50 ml conical polypropylene tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) containing 40 ml of cold ELICA fixative [43]. The spleens remained in the fixative for one to 24 weeks at ambient temperature. After fixation the spleens were transferred and stored in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen, GIBCO, Grand Island, NY) pH 7.4 at ambient temperatures. Pieces of spleen were removed, placed into OTC embedding medium and frozen at -20°C. The frozen spleens were cryostat sectioned to a thickness of seven microns, placed on positively charged microscope slides (Mercedes Medical, Sarasota, FL) and maintained at -20°C. Immunocytochemical staining was performed following established procedures for ELICA analyses [43].

Immunocytochemistry

Tissue sections were incubated with 95% ethanol to remove the OTC embedding medium and then washed under running water for five minutes. The tissue sections were incubated in 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO, Grand Island, NY) for 60 minutes and washed in running water for five minutes. They were incubated in 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes to irreversibly inhibit endogenous peroxidases [43]. Tissue sections were rinsed with running water for five minutes and incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in PBS [43]. The blocking agent was removed. The sections were rinsed with running water for five minutes and incubated with primary antibody for 60 minutes. The primary antibodies consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1, clone 5.4) in DPBS for totipotent stem cells [32] and smooth muscle alpha-actin (IA4, Developmental Studies Hybridoma Bank) in PBS [41,43] for smooth muscle cells, as a positive procedural control. The primary antibody was removed. The sections were rinsed with running water for five minutes and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated anti-mouse IgG (H + L) affinity purified, rat adsorbed (BA-2001, Vector Laboratories, Burlingame, CA) in PBS [43]. The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector Laboratories) in PBS. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with 3, 3'-diaminobenzadine (DAB) substrate (Vector) for 60 minutes. The DAB substrate consisted of 5 ml of distilled water, 4 drops of DAB stock solution, 2 drops of hydrogen peroxide solution, and 2 drops of Nickel solution (SK-4100, DAB Substrate Kit for Peroxidase, Vector). The substrate solution was removed. The sections were rinsed with running water for 10 minutes and then cover-slipped with Aqua-mount (Vector Laboratories) [43].

Positive and negative procedural controls were included to assure validity of the immunocytochemical staining [43]. The positive control consisted of smooth muscle surrounding blood vessels within the tissue (positive for IA4) [44]. The negative controls consisted of the staining protocol with PBS alone (no antibodies or substrate), without primary antibodies (CEA-CAM-1 or IA4), without secondary antibody (biotinylated anti-mouse IgG), without avidin-HRP, and without substrate (DAB) [44].

Visual Analysis

Stained sections 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. Digital photographs were cropped using Adobe Photoshop 7.0.

Tissue Labelling and Cell Counts

The photographs of spleens from each species were divided into discrete regions for counting. The outer most layer of the spleen was designated as “OCap”; the intermediate portion of the splenic capsule was designated as “Cap”; the inner border of the splenic capsule was designated as “ICap”; the red pulp was designated as “RP”; and the penicillar artery was designated as “PA” (Fig. 1).

After labelling by tissue location, each photograph was divided into a 10 x 10 matrix, vertical labelling A through J, and horizontal labelling 1-10. Therefore, each photograph was divided into 100 individual squares for counting, 1A through 10J. An Excel spreadsheet was constructed for summation of counts for each tissue region within each photograph as each of the 100 squares were counted. Tabulated percentages of cell counts (unstained and stained with CEA-CAM-1 antibody) are in Table 1.

Results

In adult rat spleens and adult pig spleens, there were different configurations of CEA-CAM-1+ stained cells with respect to their presence in the outer capsule, intermediate capsule, inner capsule, red pulp, and penicillar arteries. In the rat spleen (Fig. 1, Table 1), CEA-CAM-1+ cells were located throughout the layers of the capsule. In the outer capsule, the percentages of unstained versus stained cells were almost even (49/51), whereas in the intermediate capsule (37/63) and inner capsule (34/66) the unstained cells approximated one-third while the CEA-CAM-1+ cells approximated two-thirds of the cells present. This differed considerably to that of the pig (Fig. 1B, Table 1) [(unstained/CEA-CAM-1+)], where the preponderance of the CEA-CAM-1+ cells were located in both the outer capsule (30/70) and inner capsule (10/80), with very few CEA-CAM-1+ cells in the intermediate capsule (92/8).

In rat and pig spleens (Fig. 1 and Table 1), CEA-CAM-1+ cells were in both the red pulp and penicillar arteries. However, in the rat spleen, these cells were slightly less abundant than unstained cells in the red pulp (55/45) and penicillar arteries (58/42). Whereas there was a greater disparity between unstained cells versus CEA-CAM-1+ cells in the red pulp (83/17) and the penicillar arteries (79/21) in pig spleens.

Figure 1: CEA-CAM-1+ stained cells in adult rat spleen (A and C) and adult pig spleen (B and D). Legend: OCap, outer capsule; Cap, capsule; ICap, inner capsule; RP, red pulp; S, splenic sinusoid; PA, penicillar artery. A: Capsule of the adult rat spleen with underlying red pulp. Note CEA-CAM-1+ dark-stained cells amongst the connective tissues of the capsule (outer capsule, capsule, and inner capsule) as well as within the adjacent underlying parenchyma of the red pulp. B: Capsule of the adult pig with underlying red pulp. Note CEA-CAM-1+ dark stained cells predominantly along the outer and inner borders of the capsule as well as fewer stained cells within the parenchyma of the red. C: Splenic cords (cords of Billroth) of the adult rat cut in cross section. Note dark stained CEA-CAM-1+ cells within the wall of the penicillar arteriole as well as in the parenchyma of the surrounding red pulp. D: Splenic cords of the adult pig cut in cross section. Note a few CEA-CAM-1+ cells within the wall of the trabecular vessel and the paucity of CEA-CAM-1+ cells within the parenchyma of the red pulp surrounding the trabecular vessel. Positive controls (i.e., smooth muscle alpha-actin staining of smooth muscle) and negative controls (absence of staining in the absence of primary antibody) were appropriate (data not shown).

Tissue Location within the Spleen	Rat	Pig
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Outer Capsule (OCap)		
Unstained Cells	49	30
CEA-CAM-1+ Cells	51	70
Capsule, Intermediate (Cap)		
Unstained Cells	37	92
CEA-CAM-1+ Cells	63	8
Inner Capsule (ICap)		
Unstained Cells	34	20
CEA-CAM-1+ Cells	66	80
Red Pulp (RP)		
Unstained Cells	55	83
CEA-CAM-1+ Cells	45	17
Penicillar Artery		
Unstained Cells	58	79
CEA-CAM-1+ Cells	42	21

Table 1: Cell Count Percentages.

Discussion

Fifty percent of the cells in adult animals, humans included, consist of differentiated parenchymal and stromal cells, while the remaining 50% are composed of various types of endogenous telomerase negative and telomerase positive stem cells [31]. Of that 50% endogenous stem cell percentage, the ratio is approximately 40% telomerase negative stem cells, e.g., MSCs, VSELs, MAPCs, and other progenitor stem cells and approximately 10% telomerase positive stem cells. The telomerase positive stem cells can be further subdivided into Ectodermal Stem Cells (EctoSCs), Mesodermal Stem Cells (MesoSCs), Endodermal Stem Cells (EndoSCs), Pluripotent Stem Cells (PSCs), and Totipotent Stem Cells (TSCs) [32]. The germ layer lineage stem cells, e.g., EctoSCs, MesoSCs, and EndoSCs comprise about 9% of the telomerase positive stem cells within an individual, whereas the PSCs comprise about 0.9% and the TSCs comprise about 0.1% of the telomerase positive stem cells within an individual [32]. As noted from a multitude of studies, within the spleen both telomerase negative and telomerase positive stem cells have been demonstrated [33-41].

The demonstration of endogenous stem cells in the spleen was initially performed by isolating the cells from the spleen and characterizing the resulting cells using multiple methodologies, e.g., flow cytometry for size, cell surface staining, optimal cryopreservation temperature and storage, differential plating, propagation past confluence, proliferation potential past 70 population doublings, differential density gradient centrifugation, karyotyping, differentiation potential, etc. [32,41]. While these studies demonstrated the presence of telomerase positive stem cells in the spleen, they did not address the issue of whether the stem cells might be related to transiently circulating stem cells in the bloodstream or their being a permanent resident population of stem cells within the tissues of the organ. A previous trauma study in pigs noted

that telomerase positive stem cells were present in lower quantities in the bloodstream and in higher quantities in skeletal musculature before trauma, but would increase significantly in the bloodstream and decrease significantly in the skeletal musculature after trauma [42]. The trauma study suggested the potential for the stem cells to be a transient population in the organ by virtue of the organ's vascularization. Since one of the major functions of the spleen is to filter out damaged red blood cells, the goal of the current study was to ascertain whether a particular population of telomerase positive stem cells, i.e., CEA-CAM-1+ totipotent stem cells, existed either as a transient population of stem cells solely within the vasculature of the spleen and/or as a resident population of stem cells throughout the tissues of the spleen. As shown from the results section (Fig. 1-rat and Fig. 1-pig), CEA-CAM-1+ totipotent stem cells appear to be a resident population of endogenous telomerase positive stem cells within the tissues of the spleen, e.g., surrounding and within the splenic capsule, at the capsule/red pulp interface, and within the red pulp cords of Billroth.

The next question is, why are totipotent stem cells present throughout tissues of the adult spleen? The spleen is an important organ in the functioning of the lymphatic system. The red pulp filters damaged and/or aged red blood cells, bacteria, viruses, and recycling hemoglobin and iron from red blood cells for RBCs, WBCs, and platelets, and serves as a reservoir in cases of emergency where there is insufficient blood flow to the body. The white pulp is involved in promoting adaptive immune responses to blood-borne antigens. It synthesizes antibodies and immunoglobulins for the recognition of bacteria and viruses. Macrophages can phagocytose bacteria, process, and present their antigens to T-lymphocytes to stimulate the innate immune response. The T-cells interact with B-cells, causing B-cell proliferation and differentiation into plasma cells that release immunoglobins. Monocytes, developed in the spleen, move to damaged tissues, and differentiate into macrophages that promote the healing process [1,4,15-22]. There are at least two possibilities for their presence in the spleen. Since totipotent stem cells were present as early as the eight-cell blastomere morula, as the blastomeres of the morula continue to divide and differentiate into an individual, the totipotent stem cells divide in parallel to keep pace with the developing embryo result in their presence in every organ and tissue in the body. This can be noted by the differentiation sequence of the zygote into an individual composed of multiple organs and tissues, including the spleen and component cells and tissues. The derivation of the spleen is from the lateral plate splanchnic mesoderm and is as follows. The zygote divides to form two blastomeres, which continue to divide to form a solid ball of cells, termed the morula. The solid morula forms a shell of cells with a hollow center termed the blastocyst. The blastocyst differentiates into the trophoblast (future extra-embryonic membranes) and the inner cell mass. The inner cell mass differentiates into the hypoblast (future extra-embryonic membranes) and epiblast. The epiblast differentiates into ectoderm, mesoderm, and endoderm. The mesoderm further differentiates into lateral plate mesoderm, intermediate mesoderm, and paraxial mesoderm. The lateral plate mesoderm differentiates into lateral plate somatic and lateral plate splanchnic mesoderm. The lateral plate splanchnic mesoderm differentiates into the spleen, e.g., red pulp, white pulp, capsule, trabeculae, and other lateral plate mesodermal structures (Fig. 2 and 3) [45].

Figure 2: Scanning electron micrograph of eight-blastomere morula of male embryo. Note presence of three pairs of very-small TSCs amongst blastomeres. Presence of TSCs suggests that as embryo develops into individual that TSCs become incorporated into all tissues and organs. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. *MOJ Orthop Rheumatol* 2014; 1(4): 00019 [41].

Figure 3: Lineage map of embryonic development. Reprinted with permission from Young HE, Black Jr AC. Adult Stem Cells. Anat Rec. 2004;276A:75-102 [45].

Although not as elegant as the schematic for zygote differentiation to form an intact individual, totipotent stem cells demonstrate a parallel differentiation pattern with respect to cell types generated. Responding to induction factors from either tissue-specific exosomes or commercially available human recombinant proteins, totipotent stem cells will from all the tissues of the body, including gametes and the nucleus pulposus of the intervertebral disc, the only adult derivative of the notochord [32,46]. The blastomeres that form the gametes and primitive node (notochord), respectively, differentiate early during embryogenesis, before the epiblast forms. And while both gametes and nodal (primary organizer) tissue appear within the epiblast during embryogenesis, neither can be replaced by epiblast if removed prematurely, suggesting they are separate and distinct entities from the developing epiblast [47-56].

Totipotent stem cells start as undifferentiated telomerase positive stem cells with essentially unlimited proliferation potential and unlimited differentiation potential. Instead of being pre-programmed to form particular tissue types in a pre-programmed timed sequence, such as embryonic stem cells that can form teratomas both at caudal and cranial ends of the embryo during development or plated into a culture dish in the absence of inhibitors of differentiation, totipotent stem cells are not preprogrammed to form anything and do not form teratomas when

plated in vitro [41,57]. Rather, in vitro totipotent stem cells respond to purified recombinant human proteins or tissue-derived exosomes to form specific cell types [32]. In vivo, totipotent stem cells respond to local environmental cues (exosomes released by surrounding tissues) to form missing or damaged tissues [32,58]. However, the conversion from totipotent stem cell to differentiated cell is not a direct process. Rather the telomerase positive totipotent stem cells progress through a unidirectional downstream pattern from an undifferentiated stem cell transitioning through several different stem cell types to form a differentiated cell as shown in Fig. 4.

Figure 4: Diagram of telomerase-positive stem cells (with an essentially unlimited proliferation potential) {above red dashed line} and telomerase-negative stem cells (which conform to Hayflick's limit of 50-70 population doublings before programmed cell senescence and death {below blue dashed line}, that are located within the body. The differentiation potential with respect to the stem cells is solely unidirectional in a downstream direction. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. *Stem Cell Regen Med* 2020; 4(2):1-14 [32].

For example, the progressional sequence from a totipotent stem cell to splenic cell types is as follows. Telomerase positive totipotent stem cells differentiate into telomerase positive pluripotent stem cells, which differentiate into telomerase positive ectodermal stem cells, telomerase positive mesodermal stem cells, and telomerase positive endodermal stem cells. At this point, each of the three lineages lose the telomerase enzyme. Once the telomerase enzyme is lost the resulting stem cells conform to a biological clock of 70 population doublings before pre-programmed senescence and cell death occurs. Stem cells within each lineage commit to

become multipotent, tripotent, bipotent, and unipotent stem cells and then further differentiate into selected cell types (Fig. 4).

As shown, totipotent stem cells have the capacity to differentiate into all cell types of the individual, including gametes and nucleus pulposus of intervertebral disc. Totipotent stem cell progeny, i.e., pluripotent stem cells, can form all cell types of the individual, except gametes and nucleus pulposus of intervertebral disc. The progeny of pluripotent stem cells, e.g., ectodermal stem cells, mesodermal stem cells, and endodermal stem cells, will differentiate into their respective cell types given the appropriate induction agents (tissue-specific exosomes and/or recombinant proteins) [32,46,58].

This scenario, i.e., transplanting naïve totipotent stem cells into damaged tissues, has been utilized for the treatment of individuals with autoimmune, cardiovascular neurodegenerative, orthopedic, pulmonary, and/or systemic disorders, as an attempt to restore function in the damaged tissue(s) and organ(s). Results in toto demonstrate increases in organ function from 5 to 45% per stem cell transplant, dependent on the extent of disease progression, the health status of the individual, and the individual's compliance to guidelines for successful treatment outcomes (Fig. 5 and 6) [44,58-60].

Figure 5: Systemic Lupus Erythematosus (SLE) patient's cardiac output dropped precipitously, 90% to 30%, during the ingestion of hydroxychloroquine to slow progression of SLE. At time of first stem cell transplant, cardiac output was below 25%. First stem cell transplant (autologous) raised cardiac output to 25%. Second stem cell transplant from allogeneic 42-year-old A+ male raised cardiac output to approximately 40%. Third stem cell transplant from allogeneic 73-year-old O-negative male raise cardiac output to approximately 70%. A total of 29 adult-derived autologous and/or allogeneic telomerase-

positive stem cell transplants thus far have maintained his cardiac output at approximately 70% for over nine years and counting. Reprinted with permission from Young HE, Speight MO. Cardiovascular disease treated with telomerase-positive stem cells. *Stem Cells Regen Med.* 2020; 4(2):1-8 [59].

Figure 6: Endogenous telomerase-positive stem cell treatment of two individuals with idiopathic pulmonary fibrosis (IPF), with baseline FEV₁ values of less than 30% (Gold-4). The female, age 80 with a baseline FEV₁ of 14%, was transplanted with a single treatment of autologous telomerase-positive stem cells (TSCs and PSCs by nebulization and MesoSCs by intravenous infusion). Within one month after treatment her FEV₁ rose to 27%, and then stabilized at 25% for eight years. The male, age 61 with a baseline FEV₁ of 25% was transplanted with a single autologous and three autologous/allogeneic (auto/allo) telomerase-positive stem cell treatments throughout a seven-year time frame. The autologous/allogeneic treatments consisted of pooled auto/allo-TSCs and auto/allo-PSCs by nebulization and autologous MesoSCs by intravenous infusion. His FEV₁ has stabilized at approximately 70% for the past nine years and counting. Reprinted with permission from Young HE, Speight MO. Telomerase-positive stem cells as a potential treatment for idiopathic pulmonary fibrosis. *Stem Cells Regen Med.* 2020; 4(2):1-11 [60].

An alternate scenario was developed for individuals too fragile to undergo harvesting for the derivation of telomerase positive stem cells for treatment. In this scenario, a combinatorial nutraceutical was developed for daily ingestion to selectively stimulate proliferation of telomerase positive stem cell, e.g., TSCs, PSCs, and MesoSCs, within the body in situ, to mobilize the telomerase positive stem cells into the bloodstream (reverse diapedesis), to increase circulation to all organs and tissues into the body, and to support the immune system

for healing of tissues and organs. This scenario has had a successful outcome in a post-myocardial infarction patient with less than 10% cardiac output (Fig. 7) [59].

Figure 7: Sixty-three-year-old male with five previous coronary arterial bypass graft surgeries and 15 stents had a massive acute myocardial infarction leaving him with a 10% cardiac output at discharge from hospital and placed on heart transplant list (March 2019). By four months on CN-SP, his cardiac output had risen to 35% and his name was removed from heart transplant list. Eight additional months on CN-SP and his cardiac output rose an additional 15% (March 2020) (Fig. 2). He is continuing to ingest CN-SP daily. Reprinted with permission from Young HE, Speight MO. Cardiovascular disease treated with telomerase-positive stem cells. *Stem Cells Regen Med.* 2020; 4(2):1-8 [59].

As shown (Fig. 4), totipotent stem cells have the differentiative capability to form cell types that compose the spleen, e.g., dense irregular connective tissue capsule and trabeculae, endothelial-lined vasculature (inside of blood vessels and sinusoids), smooth muscle cells (outside of endothelial-lined blood vessels), red pulp, white pulp, monocytes, macrophages, plasma cells, erythrocytes, lymphocytes, etc., given the appropriate inductive agents. The results (Fig. 1) suggest that the CEA-CAM-1+ telomerase positive totipotent stem cells are a resident population of stem cells within the spleen in both rats and pigs. Since these stem cells are present in two different mammalian species, their presence might not be serendipitous, but rather important for splenic function.

The spleen is an important organ in the functioning of the lymphatic system. The red pulp filters damaged and/or aged red blood cells, bacteria, viruses, and recycling hemoglobin and iron from red blood cells for RBCs, WBCs, and platelets, and serves as a reservoir in cases of emergency where there is insufficient blood flow to the body. The white pulp is involved in

promoting adaptive immune responses to blood-borne antigens. It synthesizes antibodies and immunoglobulins for the recognition of bacteria and viruses. Macrophages can phagocytose bacteria, process, and present their antigens to T-lymphocytes to stimulate the innate immune response. The T-cells interact with B-cells, causing B-cell proliferation and differentiation into plasma cells that release immunoglobins. Monocytes, developed in the spleen, move to damaged tissues, and differentiate into macrophages that promote the healing process [1,4,15-22].

The spleen can be damaged in several ways, e.g., abdominal blunt force trauma, infectious diseases, medications, medical procedures, pregnancy, and hematological diseases, such as sickle cell disease, which depending on its severity, can induce scarring, atrophy, and infarction, resulting autosplenectomy [24]. The totipotent stem cells might be present to prevent such an occurrence. Harvested telomerase positive stem cells have been utilized to repair damaged tissues in individuals with autoimmune, cardiovascular neurodegenerative, orthopedic, pulmonary, and/or systemic disorders, as an attempt to restore function in the damaged tissue(s) and organ(s) [58-60]. *In-situ* induced telomerase positive stem cells have stimulated functional recovery in a post-myocardial infarction patient with a cardiac output of less than 10%, and too fragile for standard telomerase positive stem cell transplant procedures [59]. Results in toto from these treatments demonstrated increases in organ function from 5 to 45% per stem cell transplant, dependent on the extent of disease progression, the health status of the individual, and the individual's compliance to guidelines for successful treatment outcomes (Fig. 5-7) [44,58-60]. It is conceivable that the presence of telomerase positive totipotent stem cells in the spleen is necessary for the repair of the various cellular components to forestall irreparable damage that would encourage autosplenectomy.

One potential outcome of this research is the regeneration of splenic tissue as a treatment for splenectomy following blunt force trauma or autosplenectomy following sickle cell disease. Telomerase positive totipotent stem cells and splenic tissues from a patient undergoing splenectomy following blunt force trauma or autosplenectomy following sickle cell disease could be sequestered and administered to the patient at a subsequent date to regenerate splenic tissue, avoiding compromise to immune function. A patient suffering from sickle cell disease could ingest the combinatorial nutraceutical daily to prevent autosplenectomy by continual regeneration of damaged splenic tissues. Additionally, matched donor telomerase positive stem cells and exosomes from allogeneic splenic tissues might be used as opposed to autologous stem cells and exosomes for treatment of splenectomy or autosplenectomy to restore splenic function [44,57]. All are possibilities to prevent loss of splenic function and compromise of the immune system.

Conclusion

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Previous studies reported the presence of undifferentiated endogenous totipotent stem cells within the organs and tissues of various species of mammals, including the spleen. Since one major function of the spleen is to filter out damaged red blood cells, the goal of this study was to ascertain whether totipotent stem cells existed as a potentially transient circulating population solely within the vasculature and sinusoids of the spleen or whether they existed as an endogenous resident population of primitive stem cells throughout the tissues of the spleen, and potentially involved in the maintenance and repair of the spleen. The spleens from two separate mammalian species were examined, i.e., adult pigs and adult rats. The spleens were harvested, fixed, frozen, cryosectioned, and stained with an antibody diagnostic for the telomerase positive totipotent stem cells, i.e., Carcinoembryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1). CEA-CAM-1-positive stem cells were located within the capsule of the spleen, along the splenic trabeculae, within the red pulp, and surrounding the penicillar arteries of the spleen in both the adult pig and in the adult rat. The results suggested that the totipotent stem cells are a resident population of stem cells within the splenic tissues. Since the totipotent stem cells are present in two different mammalian species, their presence might not be serendipitous. The spleen can be damaged in several ways, e.g., abdominal blunt force trauma, infectious diseases, medications, medical procedures, pregnancy, and hematological diseases, such as sickle cell disease, which depending on severity, can induce scarring, atrophy, and infarction, resulting autosplenectomy. Since totipotent stem cells have been utilized in transplantation models of disease to repair damaged organs, it is conceivable that their presence in the spleen is necessary for the repair of the various cellular components of the organ to forestall irreparable damage that might encourage autosplenectomy.

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