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THE ROLE OF STEM CELL ANTIGEN-1(Sca-1) IN MUSCLE AGING

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ABSTRACT OF THESIS

THE ROLE OF STEM CELL ANTIGEN-1(Sca-1) IN MUSCLE AGING

Muscle aging is associated with a decrease in the number of satellite cells and their progeny, muscle progenitor cells (MPCs) that are available for muscle repair and regeneration. However, there is an increase in non-immuno-hematopoietic cells (CD45 negative) in regenerating muscle from aged mice characterized by high stem cell antigen-1(Sca-1) expression. In aged regenerating muscle, 14.2% of cells are CD45^{neg} Sca-1^{pos} while 7.2% of cells are CD45^{neg} Sca-1^{pos} in young adult muscle. *In vitro*, CD45^{neg} Sca-1^{pos} cells over express genes associated with fibrosis, potentially controlled by Wnt2. These cells are proliferative, non-myogenic and non-adipogenic, and arise in clonally-derived MPCs cultures from aged mice. Both *in vitro* and *in vivo* studies suggest that CD45^{neg} Sca-1^{pos} cells from aged muscle are more susceptible to apoptosis than their MPCs, which may contribute to depletion of the satellite cell pool. Therefore, with age, a subset of MPCs takes on an altered phenotype, which is marked by high Sca-1 expression. This altered phenotype prevents these cells from participating in muscle regeneration or replenishing the satellite cell pool, and instead may contribute to fibrosis in aged muscle.

KEYWORDS: Stem cell antigen-1, Hematopoietic Stem Cells, Skeletal muscle stem cells, Muscle Aging, Fluorescent Activating Cell Sorting

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04/14/2008

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THESIS

Sonia Angela Richards-Malcolm

The Graduate School
University of Kentucky

2008

THE ROLE OF STEM CELL ANTIGEN-1(SCA-1) IN MUSCLE AGING

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Health Sciences
at the University of Kentucky

By

Sonia Angela Richards-Malcolm
Lexington, Kentucky
2008

Director: Dr. Charlotte A. Peterson, Joseph Hamburg Endowed Professor and
Associate Dean for Research

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DEDICATION

This Master's Thesis is dedicated to my husband

Newman Malcolm

Who in every way exemplifies

A supportive and true friend

Thanks for your love, understanding, support and patience

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The following thesis, while an individual work, benefited from the insights, expertise and teamwork of several individuals. First, I would like to thank my thesis chair, Professor Charlotte A. Peterson, who was not only the key advisor throughout the thesis process, but also a mentor. She provided timely, instructive comments, criticisms and evaluations at every stage of the thesis process allowing me to complete this project on schedule. I am indebted to Professor Gary Van Zant for his collaborative input and expertise in stem cells. Professor Anne-Stiene-Martin and Assistant Professor, Oliver Oakley have taught me several courses, but helping me to understand Hematopoiesis and Flow Cytometry by each respectively, became the backbone of the research.

All four individuals mentioned above complete my thesis committee, each provided insights and critically read the manuscript, which guided and challenged my thinking, substantially improving the finished product. Next, I wish to thank Mats Hidestrand (Peterson's laboratory) and Amanda Waterstrat (Van Zant's laboratory) for initiating experiments involving muscle cell isolation, antibody preparation and RNA isolation and Barry Grimes (Van Zant's laboratory) for assistance with FACS analysis.

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CHAPTER 1

Introduction

Stem Cells

There are approximately 10-100 trillion cells in an average human body. Stem cells are rare cells, approximately 5×10^4 per tissue is found in all multi-cellular organisms (Kindt 2007). These uniquely unspecialized cells are capable of self renewal through mitotic cell division and give rise to a diverse range of differentiated and specialized cell types that are needed to maintain tissue growth, homeostasis, and repair throughout an organism's life span (Till and Mc 1961). The two main types of mammalian stem cells are: embryonic stem cells that are found in blastocysts and adult stem cells that are found in adult tissues (Cheng 2008). Embryonic stem cells can differentiate into all types of specialized embryonic tissues and are thus considered to be totipotent. Pluripotent adult stem cells, identified in essentially all mammalian tissues, can give rise to many differentiated cells types and have been hypothesized to have the capacity to differentiate outside their tissue of origin (Krtolica 2005; Shizuru, Negrin et al. 2005).

Adult stem cells reside in a variety of locations throughout the body and may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury (Weissman 2000; Kenyon and Gerson 2007). Upon activation, stem cells can undergo either symmetric or asymmetric cell divisions (Fuller 2006; Holmes and Stanford 2007). In symmetric cell division, either two new stem cells or two differentiating cells are produced. In contrast, asymmetric cell division, results in one

new stem cell, which either may return to quiescence or continue to divide, and a progenitor cell, which is more restricted in differentiation potential, often referred to as multipotent. Progenitor cells typically proliferate, and depending on environmental cues, specialize into the appropriate differentiated cell type. Finally, stem cells may also undergo apoptosis, resulting in depletion of the stem cell pool. Detailed studies involving stem cells from bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, and liver have shown that upon manipulation in vitro, they can be induced to differentiate into a number of different cell types (Weissman 2000; McKinney-Freeman 2002). Regenerative medicine is based on the development of these techniques to control stem cell fate so that they may be used to treat many serious diseases.

Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the best characterized adult stem cell population (Wilson and Trumpp 2006; Huang, Cho et al. 2007). HSCs, which are derived from whole bone marrow, have been shown to be capable of contributing to the regeneration of skeletal muscle, cardiac muscle, liver, and multiple epithelial tissues (McKinney-Freeman 2002; Wong, Lowes et al. 2007). However, due to the rarity of HSCs, which account for less than 0.5% of a mouse bone marrow cells, the identification and isolation of pure populations of HSCs in sufficient numbers has been challenging (Spangrude, Heimfeld et al. 1988; Harrison, Astle et al. 1989; Muller-Sieburg and Riblet 1996; Reya, Morrison et al. 2001; Kamminga and de Haan 2006). For many years, HSC properties were inferred from cell-based tissue repair assays (Montarras, Morgan et al. 2005; Shizuru, Negrin et al. 2005). More recently, with the development of specific antibodies against cell surface

antigens, researchers have been able to isolate and identify HSCs from both mouse and human. Identification of unique markers on the surface of HSCs provided the basis for the Fluorescence-Activated Cell Sorting (FACS) technique, by far the most effective and most widely used method for identification and isolation of stem cells (Spangrude, Heimfeld et al. 1988; Shizuru, Negrin et al. 2005; Wong, Lowes et al. 2007; Cheng 2008).

One of the markers whose expression defines HSCs in mice is stem cell antigen-1 (Sca-1). The Sca-1 gene is found on chromosome 15 and is a Ly-6A/E member of the Ly-6 multigene family of glycosyl phosphatidylinositol (GPI)-anchored membrane proteins, originally identified as an antigen upregulated on activated lymphocytes (Spangrude, Aihara et al. 1988; Spangrude, Heimfeld et al. 1988; Miles, Sanchez et al. 1997; Bradfute, Graubert et al. 2005; Holmes and Stanford 2007). It is regulated in a complex fashion during hematopoietic cell differentiation. (A detailed discussion of the progression of HSC restriction and differentiation is presented in Chapter 2 under Stem Cell Hierarchy.) The expression of Sca-1 is down regulated (Akashi, Traver et al. 2000) as HSCs differentiate into common myeloid progenitors (CMPs) which give rise to monocytes and macrophages, neutrophils, basophils eosinophils, erythrocytes, megakarocytes. As HSCs commit to the lymphoid progenitors, which give rise to T, B, and natural killer (NK) cells, Sca-1 expression is decreased (Kondo, Weissman et al. 1997; Ito, Li et al. 2003). Sca-1 expression is turned off at an early stage of thymocyte differentiation, and then re-expressed on mature single-positive medullary thymocytes and peripheral T cells (Bamezai 2004). The expression is further upregulated in activated lymphocytes (van de Rijn, Heimfeld et al. 1989). Expression of Sca-1 occurs outside the

hematopoietic system in the form of stem, progenitor and differentiated cell types in a wide variety of tissues and organs, which makes it ideal for routine use in combination with negative selection against mature markers for enrichment of stem and progenitor cells (Bamezai 2004; Holmes and Stanford 2007). The *in vivo* functions of Sca-1 are unknown, however, *in vitro* studies suggest that it plays a key role in cellular activation (Gumley, McKenzie et al. 1995).

CD45, also known as the common leukocyte antigen, (T220 and B220 in mice) is a protein tyrosine phosphatase located in all hematopoietic cells except erythrocytes and platelets (Thomas 1989; Barford, Flint et al. 1994; Goodell, Brose et al. 1996; Gussoni, Soneoka et al. 1999). This protein has several isoforms and HSCs express one or more of these isoforms at specific stages of differentiation. CD45 is uniformly distributed in plasma membrane, constituting 10% of the cell surface protein of T cell and B cells, and facilitates signal transduction and cell activation (Virts, Barritt et al. 1997). It has been used as an exclusive marker of the immuno- hematopoietic lineage.

Another cell surface marker, CD34, is a transmembrane cell surface glycoprotein; it is known to be selectively expressed within the human (Civin, Strauss et al. 1984; Katz, Tindle et al. 1985) and murine (Baumheter, Singer et al. 1993; Krause, Ito et al. 1994) hematopoietic systems on stem and progenitor cells. It has been shown to play a role in adhesion and differentiation control. However, as the cells mature the expression of CD34 is lost (Wood 1997). Although it is commonly known as hematopoietic progenitor antigen it is also expressed on other cells, such as muscle stem

cells (see below), vascular endothelial cells (Fina, Molgaard et al. 1990; Baumheter, Singer et al. 1993) and certain classes of fibroblasts (Suda, Sudo et al. 1992) but it has been used most widely for enumeration and purification of HSCs.

Skeletal Muscle Stem Cells

Skeletal muscle is subjected to constant injury resulting from weight bearing, exercise, and trauma. Therefore, muscle requires an ever-available renewable source of cells for repair and regeneration. Satellite cells, quiescent muscle stem cells, reside beneath the basal lamina surrounding each myofiber, and are primarily responsible for postnatal muscle growth and repair (Bischoff and Heintz 1994; Bodine-Fowler 1994; Cossu and Biressi 2005; Shefer, Van de Mark et al. 2006). When freshly isolated satellite cells or intact myofibers are transplanted with their associated satellite cells to irradiated hosts, satellite cells can self-renew, replenish a depleted niche, and their progeny are capable of efficient regeneration of myofibers (Morgan, Hoffman et al. 1990; Gussoni, Soneoka et al. 1999; Kamminga, Akkerman et al. 2000; Partridge 2002; Kamminga, van Os et al. 2005; Fukada, Uezumi et al. 2007). However, satellite cells remain dormant in mature muscle and only enter the cell cycle in response to injury. The identification of markers of satellite cells lags behind HSCs, but they do express CD34⁺ cell surface marker and the paired-box transcription factor (Pax7). This transcription factor is specifically expressed in quiescent and newly activated satellite cells and plays a key role in controlling myogenic differentiation (Grounds and Yablonka-Reuveni 1993; Yablonka-Reuveni and Rivera 1994; Zammit, Heslop et al. 2002).

Pax7 is also important for cell survival, having anti-apoptotic functions. The absence of myogenic satellite cells in young *Pax7*^{-/-} skeletal muscle demonstrates a requirement for Pax7 in the function of the satellite cell lineage (Seale, Sabourin et al. 2000; Kuang 2006). Once activated, satellite cells proliferate extensively, and generate a pool of muscle progenitor cells (MPCs) which subsequently fuse and differentiate. In cases of minimal muscle damage, MPCs fuse with existing myofibers to repair them, which can result in hypertrophy upon repeated damage. Upon massive damage, MPCs fuse with each other to form new myofibers that replace those lost during degeneration. As the cycle begins and satellite cells are activated, they rapidly initiate expression of the myogenic regulatory factors (MRFs), MyoD and Myf5, which is accompanied by downregulation of CD34 and Pax7 (F. P. Moss 2005; Le Grand and Rudnicki 2007; Ueno and Weissman 2007).

The sequential activation of MRFs and repression of Pax7 is necessary for the progression of MPCs through myogenic differentiation (Cooper, Tajbakhsh et al. 1999; Le Grand and Rudnicki 2007). A small fraction of activated satellite cells will exit the cell cycle and return to the quiescent satellite cell state during muscle regeneration to maintain their numbers and the regenerative capacity of the muscle (Zammit, Relaix et al. 2006). Activated satellite cells destined to return to the quiescent satellite cell pool have been putatively identified as cells that lose MyoD or Myf5 expression and maintain Pax7 expression (Megency, Kablar et al. 1996; McKinnell, Ishibashi et al. 2008). Sca-1 also shows heterogeneity in expression in activated satellite cells and it has been proposed that Sca-1 is present on satellite cells that will self-renew as opposed to those that will give

rise to MPCs (Mitchell, Mills et al. 2005; Fukada, Uezumi et al. 2007; Le Grand and Rudnicki 2007).

Aging in Stem Cells

Aging has been associated with qualitative and quantitative changes in stem cells. HSC aging has been linked to a slower and incomplete response to hematopoietic stress, such that mature blood and immune cells are no longer produced at the same rate at which they are lost. The age-dependent decline in the activities and/or number of HSCs has been proposed to contribute to decreased immunity (Linton and Dorshkind 2004), increased incidence of bone marrow failure (Lichtman and Rowe 2004), hematological neoplasia (Lichtman and Rowe 2004), and moderate anemia (Beghe, Wilson et al. 2004; Guralnik, Eisenstaedt et al. 2004), all common effects of aging that occur in humans. Experimentally, the ability of HSCs to home and engraft the bone marrow is significantly diminished with age (Lorenzon, Bandi et al. 2004). However, some studies reported that although HSC function clearly declines with age, the number of HSCs does not necessarily decline. In some strains of mice, the number of HSC actually expands with advancing age and this age-dependent expansion of HSCs is a transplantable, cell-autonomous property (Morrison, Wright et al. 1997; Mitchell, Mills et al. 2005; Roeder, Kamminga et al. 2005; Kamminga and de Haan 2006; Chambers, Shaw et al. 2007; Sharpless and DePinho 2007).

Aging in skeletal muscle is associated with a significant decline in mass, strength, and endurance in both human and animal models (Cartee 1995; Musaro, McCullagh et al. 1999; Karakelides and Nair 2005). Fewer myofibers are present in aged compared to young adult muscle and those remaining are atrophied and show increased susceptibility

to contraction-induced injury (Edstrom and Ulfhake 2005; Zammit 2006; Zammit, Relaix et al. 2006). Muscle regeneration is impaired with age and it has been proposed to be the result of impaired satellite cell function, as well as a decrease in satellite cell number (Shefer, Van de Mark et al. 2006). Preliminary data from Dr. Peterson's laboratory suggest that Sca-1 expression increases in aged MPCs in vivo and in vitro (Hidestrand 2008 in press). This is in contrast to HSCs where Sca-1 deficiency appears to hasten age associated changes in stem cells (Holmes and Stanford 2007). Therefore the goal of this project was to characterize Sca-1 expressing cells in muscle as a function of age.

Research Question (Hypothesis)

Sca-1 is a HSC marker that is not expressed in quiescent muscle satellite/stem cells. However, it is expressed in a subset of MPCs in young adult muscle and it has been proposed to identify MPCs that will return to the quiescent satellite cell pool. Sca-1 expressing cells appear more abundant in aged muscle but appear to lack myogenic differentiation capacity. Therefore, I set out to test the hypothesis that Sca-1 marks a unique population of non-immuno-hematopoietic cells that increases in abundance in aged muscle that may contribute to impaired muscle regeneration potential during aging.

CHAPTER 2

Background/Literature Review

Adult Stem Cells

Stem cell research began in the 1960s, when researchers discovered that stem cells were able to self renew and give rise to one or more progeny (Till and Mc; Becker, Mc et al. 1963; Siminovitch, McCulloch et al. 1963). This description of adult stem cells continues to be the definition of choice, even in the 21st century, as current researchers continue to show that the hallmarks of stem cells are: self-renewal, capacity to maintain the frequency of stem cells in a given tissue and differentiation to maintain that tissue's physiological function (Krtolica 2005). These attributes are found to be important in both tissue repairs after injury and during the normal process of aging (Lorenzon, Bandi et al. 2004; Rando 2006; Bellantuono and Keith 2007). Scientists have found adult stem cells in many more tissues than was once thought possible, maintaining regenerative organs, such as blood, skin, liver, skeletal muscle and intestinal tissue (Wong, Lowes et al. 2007). This has led scientists to contemplate whether adult stem cells could be the gold standard for treating many devastating diseases.

Therapeutic Potential of Adult Stem Cells

The therapeutic potential of embryonic stem cells remains limited at this time due to ethical concerns and the lack of federal funding for research. However, adult stem cell research is fast becoming center stage in the biomedical field as a potential therapeutic tool. Therefore understanding the basic biological properties of mammalian stem cells is of paramount importance. The main driving force for stem cell research is the growing

demand for regenerative medicine, which offers hope for the treatment of many devastating degenerative diseases such as diabetes, Parkinson's disease, and Alzheimer's disease (McKinney-Freeman 2002; Wong, Lowes et al. 2007). Cell therapy has also been explored for muscle degenerative diseases such as Duchenne muscular dystrophy (DMD) and muscle atrophy during aging (sarcopenia) (Ivanova, Dimos et al. 2002; Cheng 2008).

Satellite cells and their progeny, known to play a role in muscle growth and regeneration are the primary cell types used for treating muscular dystrophies (Sebillé 2005). From clonal analysis (monitor the progeny of individual cells), it was revealed that the yield and proliferative capacity of MPCs in DMD was down regulated. From the total number of MPCs obtained per gram of dystrophic muscle, only approximately 5 % were normal. This contributes to a large proportion (95%) of the cells that remain to give rise to clones of cells with altered cell morphology, increased doubling times, and poor growth potential leading to severely impaired muscle growth. (Pavlati, Thaloor et al. 1998). Normally, satellite cells can fully regenerate chemically destroyed myofibers at least 12 times in succession *in vivo*, while human MPCs give rise to an average of 10^8 progeny *in vitro* (Blau and Webster 1981). This proliferative potential is not just reserved for muscle regeneration but is also utilized for muscle growth. A 6-fold increase in muscle fiber diameter occurs in the first 13 years of human life, which is paralleled by an increase in the number of nuclei per unit fiber (Brooke and Engel 1969). Similar increases in the size and nuclear number of muscle fibers accompanies postnatal development in the mouse (Cardasis and Cooper 1975). Due to the absence of nuclear division within differentiated multinucleated myofibers, the additional nuclei must be

exogenous in origin and contributed by the division and fusion of adjacent satellite cells/MPCs (Stockdale and Holtzer 1961; Blau, Webster et al. 1983).

Presently scientists in many laboratories are trying to find ways to grow adult stem cells in cell culture and manipulate them to generate specific cell types so they can be used to treat injury or disease. In the case of neurodegenerative diseases, potential treatments include replacing the dopamine-producing cells in the brains of Parkinson's patients (Stredrick, Stokes et al. 2004) and hippocampal neurons in Alzheimer's patients (Kelly and Ferreira 2007); for type I diabetes, developing insulin-producing cells (Zulewski 2007); and for the cardiovascular system, replacing scarred heart muscle following a heart attack (Dalton 2008). Hematopoietic stem cells (HSCs) are perhaps the best characterized adult stem cell population and have been used most successfully for therapeutic purposes (Wilson and Trumpp 2006). HSC transplantation (HSCT) can cure or treat hematopoietic failures, immune deficiencies, leukemias and certain other cancers (Cheng 2008). The two major types of HSCT are autologous and allogeneic, which are defined by the donor graft source. In autologous HSCT, the patients undergo myeloablative doses of chemotherapy and/or radiation designed to remove all malignancies from the body. These patients have a malignancy that has either proven to be resistant to the standard chemotherapy or considered to be high risk for relapse even though complete remission may have been achieved (Shizuru, Negrin et al. 2005). The doses given to patients are very high and the bone marrow is ablated in the process. Therefore the patient must undergo a bone marrow harvest of their own stem cells prior to treatment and complete their regimen with transplantation. However, only a fraction of patients are cured using autologous HSCT because in many patients, the disease recurs as

the HSCs of patients with leukemia are often contaminated with cancer-initiating cells or so-called cancer stem cells (see Stem Cell Hierarchy section, below). In 2000, three separate clinical trials were carried out for patients with multiple myeloma (Michallet, Philip et al. 2000), non-Hodgkin's lymphoma (Vose, Zhang et al. 2001), and metastatic breast cancer (Negrin, Atkinson et al. 2000). The goal of the trials was to purify HSCs, and thereby reduce the burden of occult malignant cells. In all three diseases, harvested bone marrow (BM) or mobilized peripheral blood (MPB) were known to contain a significant percentage of contaminated malignant cells. Reports from the studies showed that purified HSCs resulted in excellent removal of the tumor cells.

Allogeneic HSCT is also used for high risk, relapsed or chemotherapy refractory malignancies, especially in patients with early hematopoietic progenitor cancer such as chronic myeloid leukemia (CML) or bone marrow failure states (aplastic anemia). These patients undergo myeloablative doses of chemotherapy and/or radiation and then are transplanted with stem cells from an allogeneic donor. These are members from the same species that are genetically different, but are matched at the human leukocyte antigen (major histocompatibility complex), called H2 in mouse, a complex of genes encoding cell surface molecules that are required for antigen presentation to T cells and the cause of graft rejection. The allogeneic HSCT patient is at risk for graft-versus-host-disease primarily because the transplant is not genetically identical and they contain mature immune cells that can respond against the host-specific antigens and cause graft-versus-host rejection (Shizuru, Negrin et al. 2005). For both autologous and allogeneic HSCT, chemotherapy and/or radiation may not eradicate the resident cancer stem cell (see Stem Cell Hierarchy section below), which renders the diseases essentially incurable. For

example, in CML, a well known HSC cancer, Bcr-Abl tyrosine kinase inhibitors have been used as the first-line treatment (Druker 2002). Although HSCT is successful, CML can recur after long periods of remission due to reactivation of a dormant cancer stem cell (Ren and Mu 2005). Bone marrow or mobilized peripheral blood are the most commonly used sources of HSCs for transplantation in the treatment of leukemia and is most effective when the leukemia is in remission. However, there is an alternative source of allogenic HSCs, human umbilical cord blood (UCB). It has been identified to contain a rich source of primitive HSCs (Gluckman and Locatelli 2000). However, this type of transplant is especially important for patients unable to match either related or unrelated donors (Hofmeister, Zhang et al. 2007). It improves donor availability for transplantation because frozen and stored UCB can be made available on demand. Infectious agents, such as cytomegalovirus (CMV), are rarely found in the new born compared to adults. UCB cell transplants have a lower incidence and severity of GVHD than conventional BMT, allowing successful transplantation in the HLA-mismatched recipient (Mao, Wang et al. 2004). However, due to the limited sample size which results in low number of HSCs, it is primarily used for pediatric patients (Hofmeister, Zhang et al. 2007).

Stem cell research has also played a key role in gene therapy, a technique for correcting defective genes responsible for disease development or delivering a therapeutic gene product (Bordignon and Roncarolo 2002). Due to their self-renewing and differentiation properties, HSCs are ideal vehicles for delivery of therapeutic transgenes for expression in their progeny. Examples of success using combinational stem cell and gene therapies to treat inherited disorder are: adenosine deaminase in deficient patients and myoblast therapy for delivery of dystrophin (Payne, Oshima et al.

2005; Seville 2005), the missing gene in DMD. Cellular cardiomyoplasty, a procedure involving the transplantation of exogenous cells into the heart to regenerate diseased myocardium, has also been used deliver therapeutic genes, and improve cardiac function (Bonaros, Rauf et al. 2007). Vascular endothelial growth factor (VEGF) is known to play a key role in blood vessel formation and the mobilization of stem cells from the bone marrow. It has been delivered to exert a mitogenic effect on cardio- myocytes (Springer 2002).

Stem Cell Hierarchy

Due to their ability to self renew, adult stem cells are maintained at roughly stable levels throughout adult life. However, when there is an increased demand for a particular cell type, stem cells divide and go through a series of specializations such that the initial pluripotent stem cell gives rise to progeny with differing proliferative capacities and increasingly restricted potential (Uher 2000). This has been most thoroughly described for HSCs, where it has been shown that in mice, one HSC can completely restore the erythroid population and the immune system following irradiation, while in humans, less than 10% of a donor's total volume of bone marrow can provide enough HSCs to completely restore the recipient's hematopoietic system (Kindt 2007) .

In mice, bone marrow cells are sub- divided into long-term self renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs) and multipotent progenitors (MPPs) that lack self- renewal properties. The LT-HSCs give rise to the ST-HSCs, which give rise to the MPPs (Weissman 2000). Highly purified HSCs can be isolated by FACS using the cell surface markers $c\text{-kit}^+$ $\text{Thy-1.1}^{\text{low}}$ $\text{lineage}^{-/\text{low}}$ $\text{Sca-1}^{\text{pos}}$ (Christensen and

Weissman 2001). LT-HSCs fulfill the criteria of HSCs due their ability to give rise to the lymphoid and myeloid lineages indefinitely after transplantation into lethally irradiated recipients. ST-HSCs have more limited self-renewal capacity and are capable of giving rise to these lineages for 8–12 weeks (Zipori and Honigwachs-Sha'anani 1992; Christensen and Weissman 2001). The MPPs then further sub-divide to form progenitor cells that become committed to either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). The myeloid progenitor cells differentiate to form either granulocyte/macrophage progenitors (GMPs) or erythroid/megakaryotic progenitor cells (MEPs). The GMPs terminally differentiate to form dendritic cells, granulocytes and macrophages, while the MEPs terminally differentiate to form erythrocytes and megakarocytes. Similarly, CLPs differentiate to form dendritic cells, Pro-B progenitors, Pro-T progenitors and Pro-NK progenitors. These progenitors finally form mature B cells, T cells and Natural Killer cells, respectively (Morrison, Shah et al. 1997). In young adult mice, approximately 8% of the LT-HSCs enter cell division per day randomly, however half their progeny must remain LT-HSCs to maintain the steady state level (Weissman 2000).

This elaborate hierarchy has important implications for cancer biology. As DNA replication is prone to error, cell division provides the opportunity for the genome to suffer mutations, some of which may result in cancer. Stem cells have a long life span with the ability to survive in severe environments, to stay quiescent in the G₀ phase of the cell cycle most of the time, and to self-renew, making them susceptible to accumulation of mutations over time potentially leading to formation of a true cancer stem cell (CSC) (Cheng 2008). However, in the hematopoietic system, keeping the number of LT-HSCs

low and their frequency of division low, the chance of a catastrophic event leading to formation of a true CSC is also low (Kindt 2007).

The confinement of rapid proliferation to ST-HSCs and MPPs with limited life span and limited self-renewal capability, assures that mutations in these cells will likely not give rise to new cancer. The current stem cell theory of cancer postulates that only CSCs, not most of the remaining constituent cancer cells, are responsible for tumorigenesis, progression, and metastasis (Komuro, Saihara et al. 2007). The CSCs can generate their descendants, including progenitors and relatively differentiated cancer cells, producing the heterogeneity of the cancer. Further, CSCs survive conventional anticancer therapies that target only the rapidly dividing cancer cells, whereas their descendants (most remaining constituent cancer cells) are killed, which may be strongly related to treatment failure, as well as cancer relapse (Asakura, Seale et al. 2002). It should also be noted that the key for homeostatic regulation of stem cells depends on the dynamic niche maintaining a balance between proliferation signals and anti-proliferation signals. Any genetic mutation that leads stem cells to become independent of growth signals, or to resist antigrowth signals, will cause the stem cells to undergo uncontrolled proliferation and possible tumorigenesis. (Diabira and Morandi 2008).

Stem Cell Markers

As introduced in Chapter 1, HSCs and their progeny are characterized by the array of cell surface antigens that they express. Clonogenic pluripotent mouse HSCs and progenitor cells are contained within the CD 34^{neg} Sca-1^{pos} CD45^{neg} c-kit^{pos} (CD117) lineage (Lin)^{-/low} and Thy-1.1^{low}(CD90) population of bone marrow (Ito, Li et al. 2003;

Abedi, Foster et al. 2007). It is suggested that this population of cells may represent universal pluripotent stem cells residing at different levels in multiple murine tissue cells (Grounds and Yablonka-Reuveni 1993). Lineage (Lin) markers are cell surface markers that mark differentiated peripheral blood cells (e.g. CD4-and CD8-). The marker, c-kit, or CD117, is another transmembrane glycoprotein and a member of the receptor tyrosine kinase subclass III family that is expressed on a number of cell types, including HSCs, mast cells, germ cells, and melanocytes (Wong, Lowes et al. 2007). It is important in the proliferation and differentiation of hematopoietic cells and is coexpressed on 50–70% of CD34⁺, HSCs (Dooley, Oppenlander et al. 2004; Sharma, Cabana et al. 2008). Thy-1 (CD-90) is a rather promiscuous molecule. It is expressed by several different cell types, and, among others, it is present on the surface of stem cells. It is expressed on 10–40% of CD34⁺ cells, the non-expressing population being HSCs (Christensen and Weissman 2001; Wong, Lowes et al. 2007).

Sca-1 in Muscle

In muscle, although 99% of quiescent satellite cells do not express Sca1, it is expressed in myogenic progenitor cells (MPCs) where it plays a role in regulating proliferation, cell cycle exit, differentiation, and fusion (Asakura, Seale et al. 2002). Primary MPCs heterogeneously express Sca-1 and those that are Sca-1^{pos} divide slower and form myotubes less readily than their Sca-1^{neg} counterparts (Mitchell, Mills et al. 2005). Blocking Sca-1 by antibodies or down regulating expression via antisense, inhibited MPC fusion and promoted proliferation. Therefore, in the absence of Sca-1, proliferation and differentiation are favored at the expense of self-renewal. This has led to the proposal

that Sca-1 expressing MPCs represent “reserve cells” destined to return to quiescence thereby maintaining the satellite cell pool (Zammit and Beauchamp 2001).

Other populations of cells expressing Sca-1 have been isolated from muscle tissue, leading to the hypothesis that there exists a more primitive stem cell population in muscle that gives rise to satellite cells, such that a stem cell hierarchy may also exist in muscle (Cossu and Biressi 2005). Polesskaya reported that CD45^{pos}Sca-1^{pos} muscle-derived cells give rise to Pax7^{pos} myogenic cells in response to Wnt signaling during regeneration. However, this has not been confirmed and it is generally accepted that CD45 is restricted to immuno-hematopoietic cells and is used to distinguish these cells from myogenic cells in muscle (Polesskaya, Seale et al. 2003; Sharma, Cabana et al. 2008).

Endothelial cells found in the blood vessels of muscle readily express Sca-1 (Tamaki, Akatsuka et al. 2002). It is possible that they are a source of satellite cells as endothelial cells from the retina have been shown to activate early satellite cell markers and undergo myogenic differentiation when transplanted into muscle (Kirillova, Gussoni et al. 2007). Pericytes (mural cells), another type of cell from the vasculature system, may replenish the satellite cell pool when satellite cells are depleted during injury or in neuromuscular degenerative disorders such as DMD (Dellavalle, Sampaolesi et al. 2007). Pericytes isolated from postnatal human skeletal muscle blood vessels can differentiate into muscle fibers in vitro and participate in muscle regeneration in vivo in host mice. The following are characteristics of pericytes compared to satellite cells: (1) they are located under the endothelium of small vessels, whereas satellite cells are located underneath the basal lamina of muscle fibers; (2) their growth requirements differ,

pericyte-derived cells undergo rapid senescence in cell culture media whereas satellite cells divide; (3) when placed in tissue culture under proliferation conditions, the pericytes-derived cells, unlike satellite cells do not express Pax7, MyoD or Myf5. Instead they express NG2 proteoglycan and alkaline phosphatase (ALP), both of which are not expressed in satellite cells. However, pericytes can undergo myogenic differentiation and express myogenic markers (MyoD and Myf5) (Dellavalle, Sampaolesi et al. 2007). Furthermore, pericyte-derived cells can cross the vessel wall, a feature presumed to be absent in satellite cell-derived myogenic progenitors, which make them potential candidates for cell therapy via systemic delivery to muscle (see Therapeutic Potential of Adult Stem Cells section, above) (Day, Shefer et al. 2007).

Aging

The most notable age-associated, functional change in HSCs, is the decreased ability to home and engraft in the bone marrow (Bordignon and Roncarolo 2002). This is true in rodents and humans; in a clinical setting increased donor age in bone marrow transplantation was found to be a predictor of transplant-related mortality. This suggests that the diminished reconstituting ability of HSCs from elderly donors is partly cell-autonomous. These observations further suggest a clinically overt decrease in HSC functioning with normal human aging (Morrison, Wright et al. 1997). Detailed analysis of aging effects on HSCs in rodents is complicated by significant differences between strains. The HSCs of the C57BL/6 (B6) mice are only minimally affected by aging because their numbers increase with age (de Haan and Van Zant 1999; Chen, Astle et al. 2000; Sudo, Ema et al. 2000) and these cells can outlive their original donor during serial

transplantation (Harrison 1979). In B6 mice, the homing properties of HSCs are altered, reducing their ability to engraft recipients (Morrison, Wandycz et al. 1996; Liang, Van Zant et al. 2005). The progenitor cells from older B6 mice have a relatively low cycling activity, whereas the stem cell pool increases with age and is relatively small (Kamminga and de Haan 2006).

Compared to the B6 mice, aged DBA/2 (D2) mice have a shorter life span. An increased cycling activity is shown by their progenitors and an apparent exhaustion of the stem cell pool during maturation (de Haan, Nijhof et al. 1997). The stem cell pool of D2 mice decreases upon aging and is relatively large, which suggests that rapidly dividing cells exhaust faster than progenitor cells. Thus, the maximum life span of different inbred mouse strains negatively correlates with the percentage of progenitors in the S-phase of the cell cycle (Roeder, Kamminga et al. 2005). The genetic differences with respect to cycling activity and stem cell pool population are still present when D2 and B6 cells co-exist in the same micro environment, suggesting these are cell intrinsic properties. In addition, differences between the growth advantage of D2 and B6 stem cell appear to be dependent on the actual state of the entire system and not a fixed cellular property (De Haan and Van Zant 1999).

In muscle, the relative roles of the aging environment compared to age-associated inherent changes in satellite cell function are less clear cut. When young or aged muscle tissue is transplanted into a young host muscle bed, regeneration is excellent (Brack, Conboy et al. 2007). The opposite is observed in an aged host, which indicates that a systemic effect could be occurring. Similarly, when parabiotic pairs of young and aged

mice sharing a common circulation are injured, the muscles of the aged partner showed improved regenerative responses when parabiosed with a young mouse, whereas regeneration in the young animal is impaired (Schultz and Lipton 1982; Dodson and Allen 1987; Taylor-Jones, McGehee et al. 2002; Barani, Durieux et al. 2003; Gallegly, Turesky et al. 2004).

Work in vitro suggests that there are also intrinsic changes in MPC function that contribute to age dependent muscle loss (Lorenzon, Bandi et al. 2004; Conboy, Conboy et al. 2005; Fulle, Di Donna et al. 2005). Alterations in rodent MPC metabolic activity and proliferative capacity occur with age. Further, changes in differentiation potential may contribute to intramyocellular lipid accumulation and muscle fibrosis, both processes regulated by Notch and Wnt signaling (Kida, Asahina et al. 2007; Brack, Conboy et al. 2008). The Wnt family of genes, encoding at least nineteen lipid modified signaling proteins, have been implicated in regulating proliferation and differentiation through the canonical β -catenin and non-canonical pathways (for review see (Huelsen and Behrens 2002). Changes in Wnt 10b expression with age contribute to increased adipogenic potential of MPCs and overall Wnt/ β catenin signaling increases in muscles as function of age (Vertino, Taylor-Jones et al. 2005) (Shimizu, Julius et al. 1997; Willert, Brown et al. 2003; Brack, Conboy et al. 2007).

On the other hand, two recent studies (Shefer, Van de Mark et al. 2006; Collins, Zammit et al. 2007) show that satellite cell number is reduced with age, but the cells that remain retain proliferation and differentiation capacity. An age-dependent loss in cells destined for self-renewal from the satellite cell/MPC population would result in reduced

regenerative capacity. Based on this work, Dr. Peterson's lab set out to test the hypothesis that Sca-1 expressing MPCs may be absent or depleted in aged muscle following injury. Immunohistochemical analysis of aged muscles suggested that Sca-1 expressing cells were actually increased in muscle with age and these cells appeared to lack myogenic potential (Hidestrand 2008 in press). The goal of my study was to quantify Sca-1 expressing cells in muscle as a function of age and characterize them in vitro.

CHAPTER 3

Experimental Materials and Methodology

Animals

Six months old (young adult) and 23-24 months old (aged) female DBA/2JNIA mice were obtained from the National Institute of Aging (NIA) Aged Rodent Colony at Harlan Sprague Dawley (Indianapolis, IN) and housed in the animal facility at the University of Kentucky (UK) with access to food and water. They were maintained on normal chow. All studies were approved by the University Animal Care and Use Committee and overseen by the Division of Laboratory Animal Resources (DLAR) at the University of Kentucky.

Muscle – Derived Cell Isolation

Cells were isolated from muscles of both hind limbs of 6 young adult and 6 aged DBA/2JNIA mice by enzymatic digestion. Bones and tendons were removed, and the muscle tissue was thoroughly minced and then centrifuged for 2 minutes at a speed of 800 x g and resuspended in DMEM (Dulbecco's modified Eagle medium) (Invitrogen, Carlsbad, CA) with penicillin and streptomycin (p/s) (Invitrogen). The muscle was then digested for 30 minutes at 37°C in 5% CO₂ with 0.2% collagenase type II-filtered (Boehringer, Mannheim, Germany) which breaks down the native collagen to release cells, followed by 30 minutes in 0.5µ/mL of Dispase (Boehringer, Mannheim, Germany) a protease that is milder compared to collagenase. Following digestion, the cells were

pelleted by centrifugation for 3 minutes at 1000x g and resuspended in 10 ml of DMEM with 10 % fetal bovine serum (FBS, BioWhittaker, Walkerville, MD). The dissociated tissue was triturated briefly by using a 25-mL pipette and was then passed sequentially through 70- μ m and 40 μ m nylon mesh filters. Cells were counted manually using a hemocytometer (see detailed description below), which typically yielded 3×10^6 to 4×10^6 cells/mouse before the filtrate was incubated at 37 °C in 5% CO₂ for 6 hours. Cells were then centrifuged for 5 minutes at 900xg and resuspended in 1 ml of phosphate buffered saline (PBS).

Cell Counting

The hemacytometer counting chamber was used for counting cells on the inverted microscope at 10x and 40 x powers. It is constructed so that the distance between the bottom of the cover slip and the surface of the counting area of the chamber is 0.1mm. The surface of the chamber contains two square ruled areas separated by an H-shaped moat. These two squares are identical allowing the counter to duplicate the cell count. Approximately 20 μ L of the total cells obtained in the above experiment was diluted in media (1:50 dilution). In a new tube 20 μ L of the dilution was added to 20 μ L of trypan blue (discussion below). Approximately 10 μ L of the solution was placed on the hemacytometer covered by a coverslip and counted using at least two quadrants. The following calculation below was used to determine the total amount of cells.

$$\text{Grp1: } (\text{_____}/2) * 100 * (10,000) = \text{_____} \text{ cells/mL} * \text{___mL} = \text{_____} * \text{multiply}$$

All subsequent steps were performed on ice at 4 °C. Cells were counted for a second time using trypan blue. In a viable cell, trypan blue is excluded; however, it

traverses the membrane in a dead cell. Hence, dead cells are shown with a distinctive blue color under a microscope. The remaining cells were centrifuged for 5 minutes at 600 x g and then resuspended in 100uL viral supernatant 2.4G2 (HB197, ATCC, Manassas, VA) at a concentration of 5×10^6 cells/mL and incubated for 15 minutes in order to block nonspecific binding of the CD45 antibody. After incubation the cells were centrifuged for 5 minutes at 600 x g and resuspended in 3mL of PF buffer (PBS with 0.5% FBS).

Antibody Labeling

The primary antibodies of CD45.2 conjugated with Allophycocyanin (APC)-Alexa® Fluor750 and Sca-1, Ly-6A/E conjugated with Phycoerythrin (PE) were prepared and aliquoted in the following tubes:

1. Control Tube 1 : 75 μ L of PF media only
2. Control Tube 2: 75 μ L of PF and 15 μ L of CD45: APC: AF (1:5)
3. Control Tube 3: 75 μ L of PF and 0.5 μ L Sca-1: PE(1:160)
4. Sample Tube: 500 μ L of PF including 100 μ L CD45: APC-AF (1:5) and 3.125 μ L Sca-1: PE (1:160). The cells sample volume aliquot for all controls was determined by the following standard calculation :

$$(0.5 \times 10^6) * (\text{total volume/total cell count}) (* \text{multiply}).$$

Antibody CD45.2 is commonly used for the following mouse strains C57BL/6, BALB/c, DBA/1, and DBA/2. It is a 104 clone monoclonal antibody, which reacts with the mouse CD45 molecule. The conjugation with the fluorescent dye (APC)-Alexa® Fluor 750 produces photo-emission properties and compensation, which includes the peak emission

of 779 nm, a slightly higher mean fluorescent intensity and a more photo stable fluorochrome when compared to the similar clone conjugated antibodies. The Sca-1Ly-6A/E antibody is also commonly used in similar mouse strains previously listed above. It is a monoclonal antibody that reacts with both Sca-1Ly-6A.2 and Ly-6E.1 molecules. The conjugation of Sca-1Ly-6A/E antibody with phycoerythrin (PE), one of the most commonly-used fluorescent dyes for FACS analysis (see detailed discussion below) produces a huge absorption coefficient and almost perfect quantum efficiency. It emits at about 570 nm, one of the brightest dyes used today. However, it is sensitive to photo-induced oxidation; therefore, the dye and samples containing the dye must always be protected from light.

In order to determine the final volume of cells to be used for FACS analysis, a final count was done (the number of cells must be $\geq 1.0 \times 10^7$ mL), which yielded approximately 1.2×10^7 and 2.1×10^7 cells for the aged for the adult mice respectively. Cells were added to the four tubes described above and centrifuged for 4 minutes at 600 x g and resuspended in 0.5mL of PF buffer (process was repeated twice). Eppendorf collection tubes were prepared to collect samples after they were sorted. Immediately before the samples were subjected to FACS, a 1:3 dilution of propidium iodine (PI) in PBS was made and added to the tube. PI is commonly used for identifying dead cells in a population of cells and also acts as a counter stain in multicolor fluorescent techniques. Finally, due to the viscosity of the labeled cells, they are filtered through a 30 μ m filter and then approximately 2-4 mL were subjected to FACS analysis on a BD Biosciences FACS Vantage cell sorter. The aim was to collect Sca-1 positive and negative cells isolated from the CD45 negative pool, which were then cultured for

immunocytochemistry and/or for RNA isolation. The entire FACS analysis was repeated on both groups of 6 young adult and 6 aged mice twice.

Flow Cytometry for FACS Analysis

Since its inception more than 30 years ago flow cytometry has become an essential tool for both researchers and clinicians. This technique is used for counting, examining, and sorting microscopic particles suspended in a stream of fluid (Longobardi 2001). It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. A flow cytometer is similar to a microscope, except that instead of producing an image of the cell, flow cytometry offers high-throughput (for a large number of cells) automated quantification of set parameters (Shapiro 2003). Fluorescence-activated cell sorting/sorter (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest (Longobardi 2001). As the fluid containing the cells, which is entrained in the center, passes through a narrow orifice (~100 μ m in diameter) into the air, a beam of light (usually laser light) of a specific wavelength is directed onto this hydro-dynamically focused stream of fluid (Wiley-Liss 2007). At the same time, the flow chamber is vibrated by the attached piezoelectric crystal, at frequencies approximating 20,000Hz. The vibration produces a disturbance in the ejected stream. The disturbance grows very rapidly and the stream eventually breaks into individual droplets (20,000 drops per second)(Wiley-Liss 2007).

The steady vibration causes the drops to be uniform in size and spacing. Each cell that flows through the system will end up in a drop. A number of detectors are aimed at the point where the stream passes through the laser light beam; one in line or approximately 2^0 - 20^0 with the light beam (Forward Scatter or FSC), several perpendicular or 90^0 to it (Side Scatter or SSC) and one or more fluorescent detectors (Shapiro 2003).

Each suspended particle passing through the beam scatters the light in some way, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a lower frequency than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information about physical and chemical structure of each individual particle. FSC correlates with the cell volume and size of the cell. It is also more sensitive to surface properties of particles (cell ruffling) and will distinguish live from dead cells. SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness) (Wiley-Liss 2007).

Finally, if the measurement result indicates that the cell is to be sorted, a voltage is applied to the stream and a droplet begins to form. When the drop separates from the stream it will carry an electric charge. The voltage on the stream is immediately reduced to zero so that other drops will not be charged. Charges can be negative or positive, enabling the sorting of two categories of cells simultaneously. As the drop continues to move downward, they pass between two metal plates charged to a high voltage. The

drops now contain the selected charged cells and because of this they are deflected from the main stream of drops and collected in eppendorf tubes for visual examination or cultured for RNA isolation. Drops containing undesired cells are not charged and go directly into a waste tube (Shapiro 2003).

RNA Isolation

Following FACS, Sca-1 labeled (Sca-1^{POS}) and unlabeled (Sca-1^{NEG}) cells from the CD45 negative population were expanded on collagen-coated plates (Sigma, St. Louis, MO) and maintained in growth medium containing Ham's F-10 (BioWhittaker) supplemented with 20% FBS, 0.5% pen-strep and 5 ng/mL basic fibroblast growth factor (bFGF, Promega, Madison, WI) at 37°C in a humidified 5% CO₂-95% air atmosphere. To assess myogenic potential, cells were grown to confluence and then switched to differentiation medium consisting of DMEM with 2% horse serum (Hyclone, Logan, UT) and 0.5% pen-strep. Total RNA was prepared from primary cultures using the RNeasy RNeasy spin kit (Qiagen, Crawfordsville, IN) including DNase treatment technique, according to the manufacturer's recommendations. This kit disrupts cells in a solution containing guanidinium thiocyanate (lysis binding solution), a strong chaotropic denaturant which lyses cell membranes and rapidly inactivates cellular ribonucleases (Chirgwin, Przybyla et al. 1979; Chomczynski and Sacchi 1987). Approximately 10⁵ cells were disrupted by the addition of lysis binding solution and then transferred to a tube and vortexed to ensure complete cellular disruption. The lysate was then mixed with an ethanol solution, and applied to a silica-based filter which selectively binds mRNA and the larger ribosomal RNAs; however, very small RNAs such as tRNA and 5S ribosomal RNA are not bound. The filter was washed to remove residual DNA, protein, and other contaminants, and the

RNA was eluted in nuclease-free water containing a trace amount of EDTA to chelate heavy metals. The silica filter is housed in a small cartridge which fits into the RNase-free microfuge tubes supplied with the kit. The sample lysate, wash solutions, and elution solution are permeated through the filter by centrifugation or vacuum pressure. The entire RNA isolation procedure takes about 10 minutes. After elution from the filter, the RNA was treated with the ultra-pure DNase 1 solution provided with the kit by the manufacture to remove trace amounts of DNA. Finally, the DNase was inactivated and divalent cations removed. DNase treatment and inactivation takes about 30 minutes. This treatment is critical in the RT-PCR technique because no RNA isolation procedure can guarantee the complete removal of trace amounts of DNA below the limit of detection by RT-PCR. It is especially important that no DNA is present in RT-PCRs using primers that do not flank introns, or for genes that have processed pseudogenes, because the RT-PCR products from RNA and contaminating DNA cannot be distinguished by size in these cases. The DNase I solution was removed because it could degrade DNA made in the PCR. The DNase Inactivation Reagent is also used to remove divalent cations introduced by the DNase 1 Buffer. The quality of RNA was assessed with a Bioanalyzer nanochip assay (Agilent Technologies, Santa Clara, CA) and RNA concentration was determined with a spectrophotometer by measuring the absorbance at 260 nm.

Real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was analyzed using RT-PCR, the most sensitive technique for mRNA quantification that is routinely used. The technique is sensitive enough to enable quantitation of RNA from a single cell. Gene expression was quantified by real-time RT-PCR utilizing a standard curve from pooled cDNAs on an IQ5 icycler using SYBRGreen (BioRad, Hercules, CA). For these experiments 1 µg of RNA was converted to cDNA using the iScript reverse transcriptase kit (BioRad). Depending on the gene being analyzed 0.1-10 ng of cDNA/well was used for real-time analysis. In all cases the primer concentrations were held at 0.3µM and the annealing temperature was set at 60°C. Reactions were characterized by a single peak melting curve, 90-110% amplification efficiency of the standard curve, and no amplification of a non- template control. Samples were run in duplicate and experiments repeated 3 times. Each value was normalized to 18s ribosomal RNA using 18s F primer 5'- AATGAGCCATTCGCAGTTTC -3' and R primer 5'- CTCTGTTCCGCCTAGTCCTG -3'. All other primer sequences can be found at <http://pga.mgh.harvard.edu/primerbank/> using the following Genbank accession numbers: Cyclin D1, NM_007631; CyclinD2, NM_009829; Myc, NM_010849; Wnt2, NM_023653; Wnt7b, NM_009528; Wnt10, NM_011718; Twist1, NM_011658; Fibronectin, AF095690; CTGF, NM_010217. Results were analyzed using two-way Analysis of Variance (ANOVA) and are presented as means and standard deviations.

CHAPTER 4

Results

Quantification of Sca-1 expressing (Sca-1 pos) cells from adult and aged mice muscle

Immunohistochemical analysis of mice hind limb muscles showed that Sca-1 expressing cells increased in abundance with age (Hidestrand 2008 in press). To quantify the cells and characterize them in detail, hind limb muscles of adult (6 months old n= 6) and aged (23-24 months old, n= 6) female mice were enzymatically digested and mononucleated cells isolated. The muscle derived cells were counted (three times) at different stages throughout the experiment manually with a hemocytometer prior to Fluorescent Activating Cell Sorting (FACS). Table 1 show that muscles from the adult mice yielded a larger number (43.5×10^6) of cells overall compared to the aged muscles (23×10^6). However, significant cell loss was noted in both age groups during further processing such that cell number dropped by approximately half (11.5×10^6 cells from aged mice and 21.0×10^6 cells from adult). Finally, the cells yielded by both groups of mice are consistent with published data, which suggest that a typical yield of cells for two hind limbs from a mouse ranged between 3×10^7 - 4×10^7 .

Table 1: Summary of cells counted manually by hemocytometer prior to FACS Analysis

| Type of mice | Count 1 | Count 2 | Count 3 |
|--------------|-------------------|-------------------|-------------------|
| Aged | 2.3×10^7 | 1.2×10^7 | 1.2×10^7 |
| Adult | 4.4×10^7 | 2.2×10^7 | 2.2×10^7 |

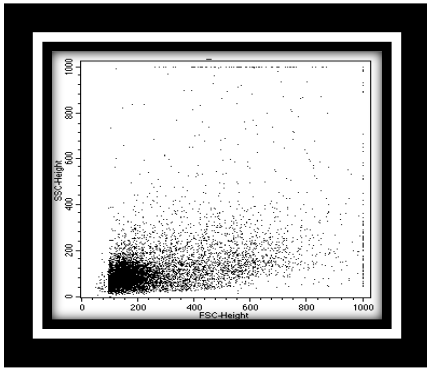


Figure1A.

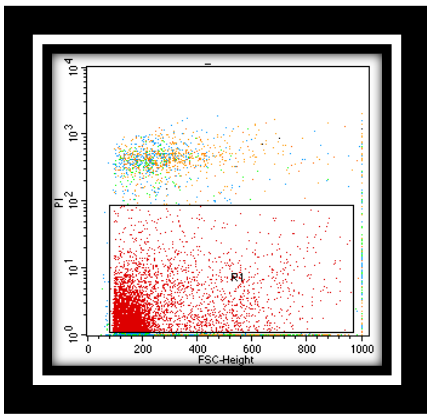


Figure1B.

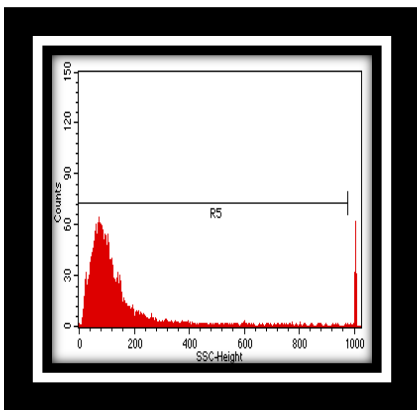


Figure1C.

Figure1: Fluorescent Activating Cell Sorting (FACS) parameters for CD45neg cells. The above figures illustrate a profile of all cells to be sorted based on size (FSC) and granularity (SSC) (A). The viable cell population (Region 1 in B) enriched for non-hematopoietic stem cells (CD45 negative, R5 in C) was selected using propidium iodide.

To identify Sca-1^{pos} cells derived specifically from muscle progenitors cells (MPCs), an antibody to CD45 was used to select against immuno-hematopoietic cells (CD45^{pos}). Several other cell parameters were also quantified during sorting (Figure 1). The forward scatter (FSC) on the x axis versus the side scatter (SSC) on the y axis in Figure 1A displays the profile for all cells collected based on their surface properties which includes size and granularity respectively. In Figure 1B the forward scatter versus propidium iodine (PI) allows for selection of viable cells (R1) excluding fragments (located on the far left), aggregates (located on far right) and nonviable cells (PI^{pos}). Finally, the histogram shown in Figure 1C is a representation of the CD45 negative cell population (R5) that was further sorted based on Sca-1 cell surface expression.

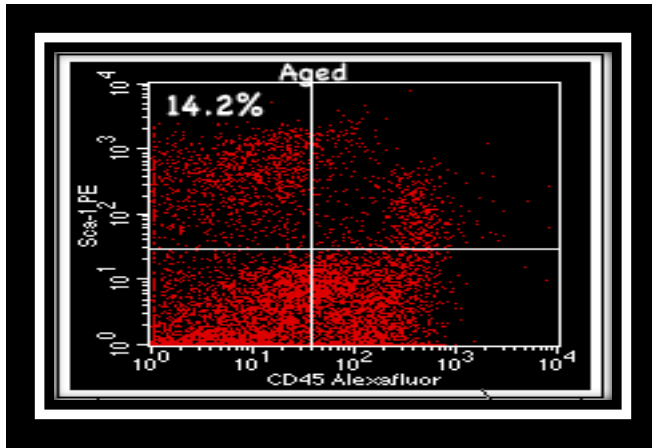


Figure 2A

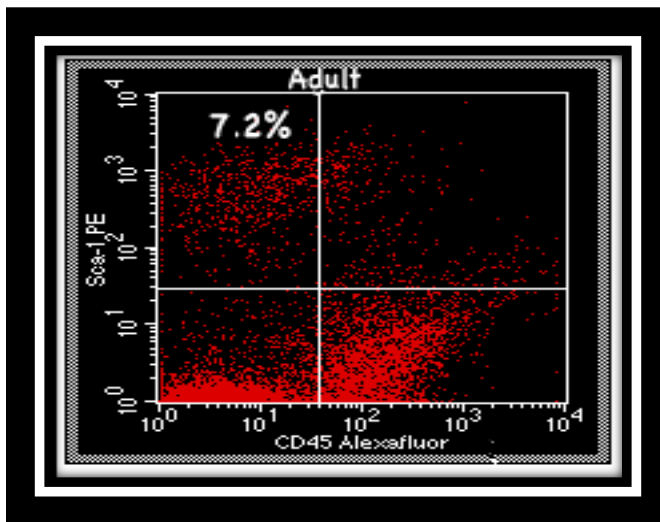


Figure 2B

Figure 2: FACS profile of CD45 and Sca-1 expressing and non-expressing cells in muscle from adult and aged mice

CD45 versus Sca-1 cells demonstrates an increased abundance of CD45^{neg}Sca-1^{pos} cells in muscle from aged (A) compared to adult (B) mice.

Sorting gates of the viable cells (R1, Figure 1B) were used to identify CD45^{neg} Sca-1^{pos} cells from adult and aged mice. In this representative analysis, repeated twice, 14.2% of the cells from aged mice were CD45^{neg} and Sca-1^{pos} (Figure 2A), while 7.2% of similar cells were observed in the adult mice ((Figure 2B). These results show that although total cell number decreases as a function of age, Sca-1-expressing non-immuno-hematopoietic cells are twice as abundant in aged compared to young adult muscle.

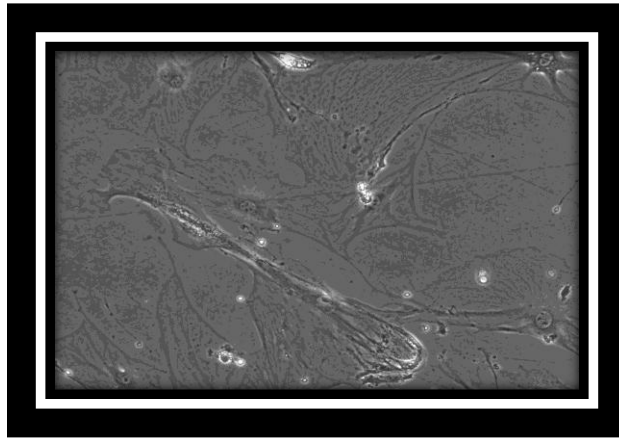


Figure 3A

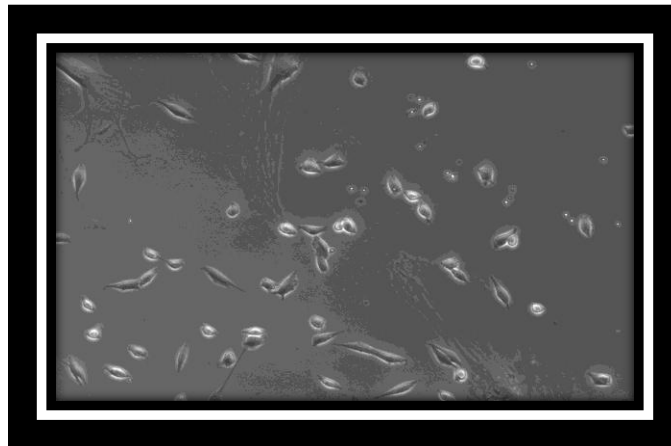


Figure 3B

Figure 3: In vitro characterization of Sca-1^{pos} and Sca-1^{neg} cells from aged and young adult muscle in culture.

Sca-1^{pos} (A) and Sca-1^{neg} (3B) cells from aged muscle in culture for approximately 2 weeks.

Previous results from Dr. Peterson's laboratory showed that although Sca-1 expressing cells in aged muscle were derived from MPCs, they lacked myogenic potential. Sca-1^{pos} cells from aged muscle did not express myogenic genes such as MyoD, and did not fuse or differentiate into myotubes (Hidestrand 2008 in press). To further characterize the Sca-1 expressing cells from aged muscle, the FACS isolated Sca-1^{pos} and Sca-1^{neg} cells, were expanded in culture. After approximately two weeks in culture, Sca-1^{pos} cells were quite large and flattened (Figure 3A) compared to Sca-1^{neg} cells (Figure 3B). The morphology of the Sca-1^{neg} cells was typical of normal MPCs. They readily differentiate in low serum- containing differentiation media; whereas the FACS sorted Sca-1^{pos} cells did not fuse to form myotubes.

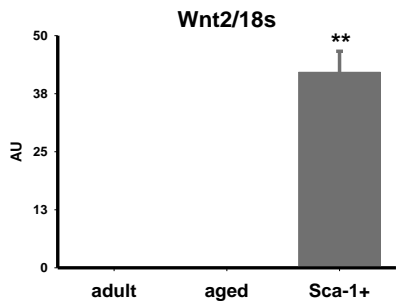


Figure 4A

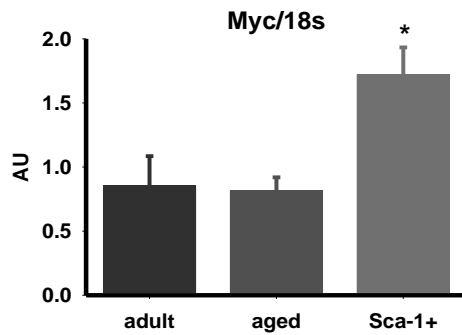


Figure 4B

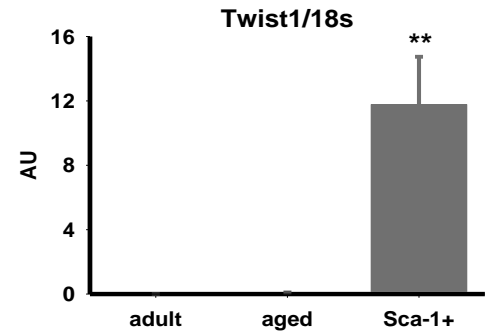


Figure 4C

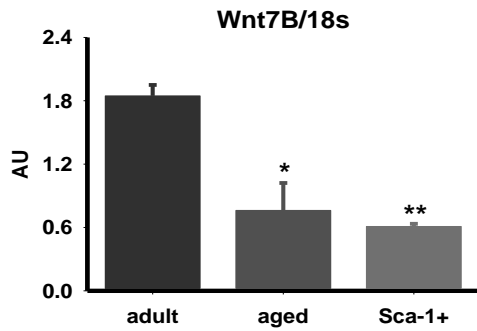


Figure 4D

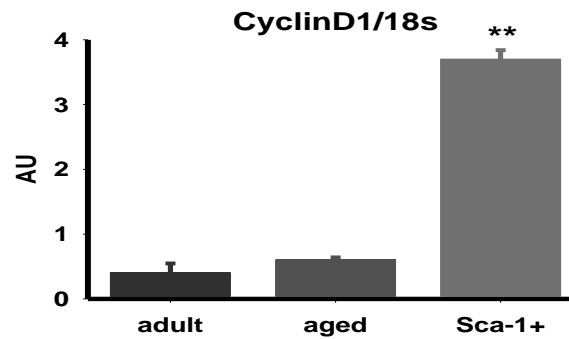


Figure 4E

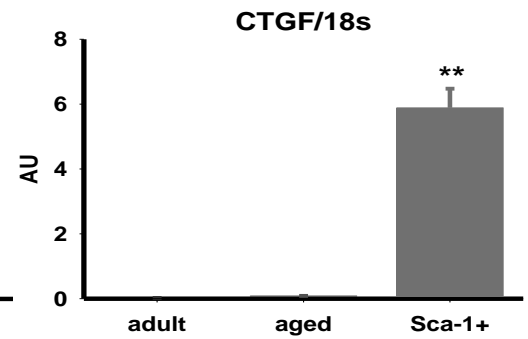


Figure 4F

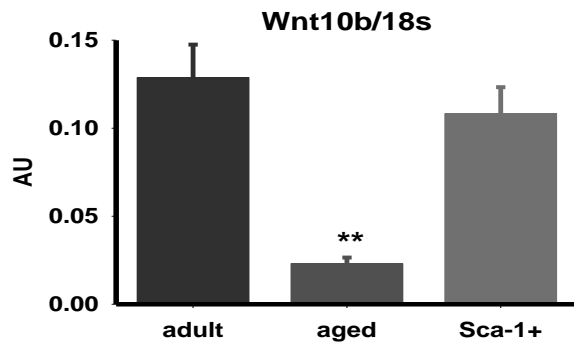


Figure 4G

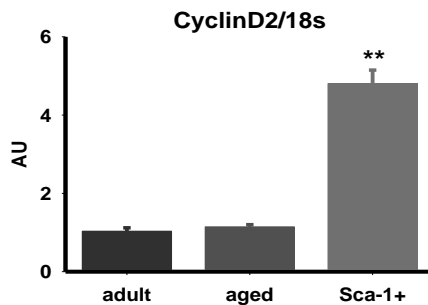


Figure 4H

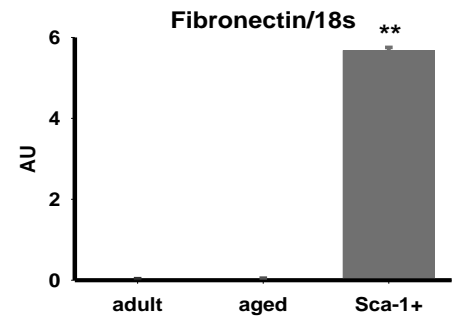


Figure 4I

Figure 4: Gene expression analysis of Sca-1 pos cells compared to Sca-1 neg MPCs from adult and aged muscle.

mRNA abundance for the indicated genes was quantified by real time RT-PCR. The data were analyzed by two-way Analysis of Variance (ANOVA) and expressed as different from aged mice ($p < 0.05$ is as * and $p < 0.005$ is assigned as **).

To characterize the cells further, RNA was isolated from Sca-1^{neg} adult (6 months-old) and aged (23-24 months-old) MPCs and from FACS isolated CD45^{neg} Sca-1^{pos} cells from 23-24 months-old mice (stable populations of Sca-1^{pos} cells from young animals were not available due to the dynamic expression of Sca-1 in these cells) and analyzed by real time reverse transcriptase PCR (Figure 4). The data presented in Figure 4 represent the means and standard deviations normalized to 18s, expressed as arbitrary units (AU). The following specific Wnt genes (Wnt2, 7b and 10b,) showed the greatest difference in gene expression between the cell types. Wnt2 (Figure 4A) was dramatically over expressed in Sca-1^{pos} cells which was associated with over expression of downstream target genes of canonical Wnt signaling such as c-myc (Figure 4B) and cyclins D1 and D2 (Figure 4E and 4H, respectively). Wnt7b mRNA (Figure 4D) was expressed at lower levels in both cell types from aged mice relative to young adult MPCs, potentially contributing to decrease myogenic potential in both cell types from aged mice. The Wnt10b gene (Figure 4G), that has been implicated in regulating adipogenesis was down regulated only in aged MPCs. Genes involved in fibrosis such as Twist (Figure 4C) connective tissue growth factor (CTGF, Figure 4F) and fibronectin (Figure 4I) were significantly over expressed in Sca-1^{pos} cells compared to MPCs.

CHAPTER 5

Discussion and Conclusion

Stem cell antigen-1(Sca-1) is expressed by stem, progenitor, and differentiated cell types in a wide variety of tissues and organs (Bamezai 2004; Holmes and Stanford 2007). In muscle, it has been proposed to play a role in controlling normal muscle satellite cell/MPC function (Fukada, Uezumi et al. 2007). However, these experiments have identified a subpopulation of cells ($CD45^{neg}Sca-1^{pos}$) that increase in abundance with age (Figure 2). This unique subpopulation of cells has an altered differentiation potential.

Quiescent satellite cells in young muscle do not express Sca-1 and give rise to MPCs that are heterogeneous for Sca-1 expression (Asakura, Seale et al. 2002). It has been proposed that Sca-1 may identify MPCs destined to return to the satellite cell pool (Mitchell, Mills et al. 2005). In aged muscle, cells expressing Sca-1 do not possess myogenic potential and may be undergoing apoptosis resulting in depletion of the stem cell pool in vivo (Hidestrand 2008 in press). However, not all cells are lost, and those that remain do not express myogenic markers and instead may contribute to muscle fibrosis with age, potentially controlled by Wnt signaling with β catenin as the integral component of the system (Willert, Brown et al. 2003; Brack, Conboy et al. 2007). Wnts are a major class of secreted morphogenic ligands with a role in generation, maintenance and /or differentiation of early progenitor cells in all multicellular organisms (Huelsen and Behrens 2002). They play a very important role in regulating osteogenesis, adipogenesis and myogenesis in adult tissues, subsequently controlling the fate of the resident stem cells (Lako, Lindsay et al. 2001; Vertino, Taylor-Jones et al. 2005). In the

absence of Wnt signaling, β -catenin is phosphorylated by glycogen synthase kinase (GSK), leading to rapid degradation. The ligands of Wnt bind to transmembrane receptors of the Frizzled family, leading to inactivation of GSK within the complex, thereby stabilizing β -catenin, allowing translocation to the nucleus where it regulates T-cell factor /leukocyte enhancing factor-dependent (TCF/LEF) gene transcription (Willert and Nusse 1998). Recent studies have shown that Wnts have distinct effects during limb myogenic differentiation (Van Den Berg, Sharma et al. 1998; Lako, Lindsay et al. 2001; Fevr, Robine et al. 2007). However, in contrast, Wnt signaling seems to inhibit adipogenic differentiation (Shimizu, Julius et al. 1997; Ross, Hemati et al. 2000; Bennett, Ross et al. 2002; Vertino, Taylor-Jones et al. 2005; Brack, Conboy et al. 2007; Fevr, Robine et al. 2007).

Due to these important roles of the Wnt signaling pathway it prompted us to examine this class of genes in Sca-1^{pos} cells. In our study, we found that Wnt7b, which promotes myogenic differentiation (Vertino, Taylor-Jones et al. 2005), is down regulated in both aged Sca-1^{neg} and Sca-1^{pos} cells, which may contribute to impaired myogenic potential of both cell populations. Wnt10b, which inhibits adipogenic differentiation (Ross, Hemati et al. 2000; Bennett, Ross et al. 2002), declines only in aged MPCs, potentially accounting for increased adipogenic potential specifically in those cells. As overall markers of Wnt/ β -catenin signaling, myc and cyclins D1 and D2 appear to be up-regulated in the Sca-1^{pos} cells, Wnt7b and 10b likely signal through non-canonical pathways to control differentiation potential.

Wnt2 (Figure 4A) was highly over expressed in Sca-1^{pos} cells that may be responsible for increased expression of the down stream targets of canonical Wnt/ β -

catenin signaling (Brack, Conboy et al. 2007). Moreover, genes associated with fibrosis, including Twist1 (Figure 4C), CTGF (Figure 4F), and fibronectin (Figure 4I) are over expressed in Sca-1^{pos} cells compared to both young adult and aged Sca-1^{neg} MPCs. In the kidney Wnt 2 is associated with fibrosis and directly regulates Twist 1 gene expression (Kida, Asahina et al. 2007). Twist also inhibits MyoD function which contributes to impaired myogenic potential (Hamamori, Wu et al. 1997). These results are consistent with a recent study done by Brack et. al which also shows that following injury in aged muscle, satellite cells/MPCs may contribute to fibrosis which is associated with increased canonical Wnt signaling. The data obtained from our experiments suggest that Sca-1 is a useful marker of this transition from myogenic to fibrogenic potential during aging.(Brack, Conboy et al. 2007). The fact that age-dependent differences in phenotype are stable in culture suggests that inherent changes in cell potential occur. However, satellite cell and MPC function are also clearly influenced by the aged muscle environment(Carlson and Faulkner 1989; Conboy, Conboy et al. 2003; Conboy, Conboy et al. 2005; Rando 2006).

The majority of the work presented and discussed in this paper is on mice; however, interesting parallels appears to exist with humans muscle progenitor cells. Presently, a human homolog is not yet identified, however, researchers analyzing Sca-1 mutant mice have demonstrated the following important stem cell concepts that are equally relevant to human health and disease: the importance of mesenchymal stem and progenitor cells to bone homeostasis and deterioration, the association between degenerative diseases and exhaustion of the stem cell pool, and the importance of the

stem cell stress responses to tissue maintenance (Holmes and Stanford 2007). Therefore, further experiments are required to determine if modulation of Sca-1 expression may alter the properties of the satellite cells/MPCs and lead to long-term consequences for muscle growth.

Finally, due to the growing demand for regenerative medicine, understanding the basic biological properties of mammalian stem cells, including Sca-1 is of paramount importance. This could offer hope for the treatment of many devastating degenerative diseases such as diabetes, Parkinson's disease, and Alzheimer's disease and muscular degenerative diseases such as Duchenne muscular dystrophy (DMD) and muscle atrophy during aging (sarcopenia).

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Kentucky Graduate Scholarship (2006-2008)
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Philanthropic and Educational Organization (P.E.O) International Peace Scholarship for Women Award (2007-2008)
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Publications:

Hidestrand Mats, **Richards-Malcolm Sonia**, Gurley Cathy M., Nolen Greg, Grimes Barry, Van Zant Gary and Peterson Charlotte A.

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