SOMATIC STEM CELL POPULATIONS AND STUDIES ON THE FUNCTIONAL ROLE AND REGULATION OF ABCG2

APPROVED BY SUPERVISORY COMMITTEE

Daniel Garry

Joseph Garcia

Jane Johnson

David Garbers

SOMATIC STEM CELL POPULATIONS AND STUDIES ON THE FUNCTIONAL ROLE AND REGULATION OFABCG2

by

MARY KATHERINE TUNISON

THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2005

SOMATIC STEM CELL POPULATIONS AND STUDIES ON THE FUNCTIONAL ROLE AND REGULATION OF ABCG2

MARY KATHERINE TUNISON, M.S.

The University of Texas Southwestern Medical Center at Dallas, 2005

Daniel Garry, M.D., Ph.D.

ATP binding cassette transporters use ATP to transport substances such as sterols, peptides, drugs and other toxins across membranes. Abcg2 is a member of the G family of transporters that was identified in breast cancer cells due to its ability to efflux chemotherapeutic drugs. Abcg2 can also efflux Hoechst 33342, resulting in a side population phenotype when stained cells are sorted by FACS. Recent studies have suggested that Abcg2 may be a marker for stem and progenitor cells. This paper presents experiments that were undertaken to further evaluate the functional role and regulation of Abcg2. Preliminary data obtained from a microarray performed on main and side population cells indicated that side population cells were enriched for genes that are important in cytoprotection and cell cycle control. To confirm this observation, cell cycle analysis was performed on C2C12 myoblasts transfected with an Abcg2 overexpressing plasmid. Those cells that overexpressed Abcg2 and consequently effluxed Hoechst 33342 dye were arrested in G0/G1 phase of the cell cycle, consistent with the microarray results. To determine whether Abcg2 played a cytoprotective role I attempted to measure the viability of main population versus side population cells when exposed to the oxidative

compound menadione. These experiments were inconclusive, most likely due to limitations of the cell line used. To assess the regulation of Abcg2 I examined evolutionary conservation between the upstream regions of mouse and human Abcg2. Conserved regions were identified up to 11kb upstream of the mouse gene. In these regions, putative binding sites were discovered for transcription factors that are involved in stem and progenitor cell regulation. Present studies support that Abcg2 is a marker for stem and progenitor cells. Future studies will uncover additional functional roles of the transporter.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abcg2: ATP binding cassette, subfamily G, member 2

AGM: Aorta-gonad-mesonephros

BCRP1: Breast cancer resistance protein

DMD: Duchenne muscular dystrophy

EGF: Epidermal growth factor

EGFP: Enhanced Green Fluorescent Protein

FACS: Fluorescence activated cell sorting

FTC: Fumitremorgin C

GFAP: Glial fibrillary acid protein

HSCs: Hematopoietic stem cells

KSL: c-Kit^{+}, Sca-1^{+}, Lin^{-}

MDR: Multidrug resistance protein

MP: Main population

NSCs: Neural stem cells

PI: Propidium iodide

SP: Side population

SPOC: Skeletal-based precursors of cardiomyocytes

SSCs: Somatic stem cells

YFP: Yellow fluorescent protein

Introduction

In the process of creating an organism, two cells fuse into one. This is then followed by the massive cell division that is required to create a complex multicellular creature. As the number of cells increase, numerous genetic programs are activated, transcribing proteins which differentiate these cells into endothelial, muscle, liver, and all the other cell types found in the adult organism. Pluripotent cells present in the early embryo, called embryonic stem cells, divide many times to produce the cells that will differentiate into the various cell types. The cells of the adult organism continue to function until they are injured or undergo apoptosis. One of the most widely studied questions in biology asks what cells present in the mature organism can replenish these cell populations. It is thought that most tissues of the body contain their own specific population of cells that divide and differentiate to replace the dead cells; these are called somatic stem cells (SSCs).

Somatic stem cells are defined by two criteria: proliferation, the ability to self-renew and plasticity, the ability to give rise to differentiated cells with a specific function. The fate of a given stem cell depends on whether it undergoes asymmetric or symmetric division. In asymmetric division, the multipotent stem cell divides, giving rise to one stem cell and one cell that will differentiate. In

symmetric division, both daughter cells will be the same type of cell, either both stem cells or both differentiating cells (Mayani 2003).

While stem cells provide a source of differentiated cells for their respective tissues, it is also thought that stem cells retain an inherent plasticity, or ability to differentiate into other cell types. There are two hypothesized methods of somatic stem cell plasticity: dedetermination and transdifferentiation. In the former, a stem cell would first have to revert to a more primitive stem cell that is less committed to a specific program, and then differentiate into cells of a different type. In the latter, a stem cell of one type would begin to follow the differentiation path of another type of cell, without first reverting to a primitive state (Mayani 2003). Whichever path a stem cell takes, another important distinction to make is differentiation vs. fusion. Some studies claim that transplanted cells have fused with endogenous tissue rather than assume a different cell fate (Terada 2002; Ying 2002; Wang 2003). For instance, Wang et. al. showed that the donor bone marrow cells that were responsible for repopulation of the liver had fused with endogenous hepatocytes, as evidenced by the resulting aneuploid karyotypes. Studies such as these illustrate that it is important to closely examine cell phenotypes when addressing the issue of plasticity. However, it may still be beneficial if cells fuse rather than differentiate if, through fusion, they are still able to contribute to the repair of the tissue. For instance, when satellite cells in skeletal

muscle are activated for repair, their mechanism of action is to fuse with existing myofibers (Hawke and Garry 2001).

Scientists have developed multiple strategies to address the question of differentiation vs. fusion. One strategy is to transplant a labeled stem cell, such as one expressing GFP or β-gal so that it can be traced, and examine the cells for tissue specific markers (Orlic 2001; Lagasse 2000). Another method includes injecting male cells into a female recipient and evaluating the tissue of interest for the presence of the Y chromosome in the new cells (Theise 2000). A third method is to inject cells that contain a tissue-specific promoter driving a transgene that will only be expressed if it transdifferentiates into the target tissue.

Although work is being undertaken to identify, characterize, and isolate stem cells in each tissue, much of it focuses on hematopoietic, skeletal muscle, epithelial, neural, and cardiac stem cells. In addition to identifying stem cells, scientists are also trying to discover the limits of each stem cell population's plasticity, and utilize this ability to treat disease and injury.

Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are perhaps the most widely studied stem cell population. They arise from the aorta-gonad-mesonephros (AGM) region in the mouse embryo around embryonic day 10 and are believed to colonize the fetal liver and bone marrow (de Bruijn 2000). The adult bone marrow is the main site of hematopoiesis where the HSCs give rise to all blood cell types, though they only comprise 0.005 - 0.01% of all bone marrow cells (Mayani 2003). Upon stimulation, HSCs differentiate to become granulocytes, monocytes, megacaryocytes, erythrocytes, and lymphocytes. This multipotency can be demonstrated through the use of the colony forming assay, in which unfractionated bone marrow is plated onto a semisolid media and allowed to form clonogenic colonies of the various myeloid and lymphoid lineages (Ogawa 2002).

Although there is not one specific marker for HSCs, there are ways of identifying populations of cells that are highly enriched in HSCs. Cells are identified using flow cytometry to detect the presence or absence of surface markers. Studies have shown that the most primitive HSCs have a $CD34^-$, c-Kit^+, $Sca\text{-}1^+$, Lin^- phenotype, also known as $CD34^-$ KSL (Smith 1991; Spangrude 1995; Osawa 1996; Uchida 1996). This population of cells has been demonstrated to contain single cells with the ability to reconstitute the hematopoietic system of lethally irradiated mice, but in only approximately 20% of

recipients (Osawa 1996). Recently, Matsuzaki et al. discovered a way to further purify HSCs that utilizes the ability of a population of cells, termed the side population, to efflux the DNA binding dye Hoechst. The $CD34^-$ KSL cells with the greatest dye efflux ability, or Tip-SP, exhibited multilineage, long-term competitive repopulation in over 90% of recipients (Matsuzaki 2004).

The ability to isolate and purify HSCs may offer a better therapy for alleviating or curing certain diseases that affect the blood and immune systems. In patients with cancer, the bone marrow can be extracted before treatment, purified, and injected back into the patient following treatment that destroys both cancerous and normal cells (Mayhall 2004). HSC transplant can also be used for autoimmune diseases such as multiple sclerosis and juvenile chronic arthritis. In cases such as these, the HSC transplant is used to rebuild the patient's immune system after ablative treatment (Wulffraat and Kuis 1999; Fassas 2002). Depending on the type of treatment, whether the HSCs are autologous – from the patient, or allogenic – from a matched donor, the symptoms of the disease can be alleviated or even be cured.

In addition to replacing the hematopoietic system, HSCs have also been shown to have the ability to become other cell types and repair damaged tissue. This has widespread implications in tissue repair, especially since bone marrow cells are more easily obtained than other stem cells. Some studies have shown that following injury,

bone marrow derived cells are present in the muscle of recipient mice (Camargo 2003; Corbel 2003). Other studies have shown that cells derived both from whole bone marrow and KSL purified HSCs can be found in the liver (Lagasse 2000; Theise 2000). Mezey et al. claim that cells derived from whole bone marrow can become neural cells by showing that donor cells from male mice are present in brain tissue and express the neural marker NeuN (Mezey 2000). In addition to becoming muscle, liver, and neural cells, studies have also claimed that bone marrow can regenerate injured heart and even become cardiomyocytes. Orlic et al. injected lin^- $c\text{-}kit^+$ EGFP male bone marrow cells into the infarcted left ventricle of female mice and found repair in 40% of recipients (Orlic 2001). Another study utilized an *in vitro* method to establish a cardiomyogenic cell line. Bone marrow stromal cells were cultured in the presence of 5-azacytidine, which induced spontaneous beating, formation of branched myotubes, and expression of cardiomyocyte-specific genes, such as myosin, actinin, and desmin (Makino 1999).

However, there are also several studies that find that HSCs do not transdifferentiate (Bjornson 1999). Menthena et al. showed, through the use of double immunohistochemical and immunofluorescent labeling of both liver and bone marrow derived cells that, in their hands, liver progenitor/oval cells derive from liver tissue and not from the donor bone marrow (Menthena 2004). One study

replicated the methods of HSC isolation and transplantation used by Orlic et al. but used phenotypic and lineage markers instead of immunofluorescence staining to detect cells. That study did not find that bone marrow stem cells differentiated into cardiomyocytes in either normal or infarcted hearts or that they participated in tissue regeneration (Murry 2004). In addition, Wagers et al. used lethally irradiated mice engrafted with a single GFP^+ HSC that was c-kit^+ $Thy1.1^{lo}$ Lin^- $Sca\text{-}1^+$ and examined various tissues 4-9 months after engraftment. The authors did not find any GFP^+ cells that either stained with tissue-specific markers, were negative for CD45, or exhibited a distinct morphology in kidney, skeletal or cardiac muscle, or lung tissues, and found only a few GFP^+ cells present in brain and liver (Wagers 2002).

Skeletal Muscle Stem Cells

Among the tissues of the body, skeletal muscle has a tremendous ability to regenerate and repair itself. This regeneration is accomplished by muscle progenitor cells termed satellite cells, so named for their location in muscle. Satellite cells are defined by their position adjacent to myofibers and surrounded by the basal lamina (Schultz and McCormick 1994). They can also be visually distinguished by their higher nuclear-to-cytoplasmic ratio and smaller nuclei when compared to myotubes (Schultz and McCormick 1994). In addition to visual distinctions, satellite cells are also characterized by several molecular markers. C-met, a receptor tyrosine kinase, was found to be expressed in all satellite cells, in both quiescent cells and cells that were in the period of activation and differentiation (Cornelison and Wold 1997). Although vascular cell adhesion molecule-1 (VCAM-1) is present during embryological development and is down-regulated in adult myotubes, it was shown to retain its expression in satellite cells (Jesse 1998). Another marker of satellite cells is an isoform of the winged helix transcription factor myocyte nuclear factor, FoxK1β. This isoform is predominantly expressed in satellite cells, and is downregulated as the satellite cells proliferate and differentiate (Garry 2000). The paired box transcription factor Pax7, another marker of satellite cells, is required for satellite cell specification (Seale 2000). While these and other proteins are markers for satellite cells, many are

also expressed in other tissues. It may be best to use a combination of these markers when identifying satellite cells.

Satellite cells facilitate repair and regeneration in response to muscle injury, whether caused by exercise, toxins, or disease. Once injury occurs, satellite cells become activated, begin to proliferate, and move to the injured area by chemotaxis (Schultz and McCormick 1994). At the site of injury, the activated satellite cells can then fuse to the damaged myofiber to repair it or fuse with each other to form new myofibers, while others satellite cells undergo self-renewal (Schultz and McCormick 1994). Satellite cells are able to repair and renew repeatedly, but eventually they lose this ability due to aging of the organism and disease (Jejurikar 2003). This loss of proliferative capacity is particularly seen in diseases such as Duchenne muscular dystrophy (DMD), an X-linked recessive disease caused by a null mutation at the dystrophin locus (Burghes 1987). Normally dystrophin binds to actin to link it to the sarcolemma. In DMD, dystrophin is absent in the skeletal muscle and therefore actin is not able to associate with the sarcolemma membrane, resulting in delicate myofibers that are more susceptible to injury (Rybakova 2000). Satellite cells are activated to repair the damaged tissue, but as the cells are repeatedly activated, their ability to proliferate and repair injured muscle is exhausted (Cossu 2000).

In addition to satellite cells, muscle also contains a pluripotent stem cell population termed the side population, or SP. Dual-wavelength flow cytometry is used to detect the cells based on their ability to efflux the DNA-binding dye Hoechst 33342 (Goodell 1996; Asakura 2002). Like HSCs, muscle SP are sca-1^+ lin^-, but they appear to be different from bone marrow SP in that they do not express c-kit and CD45 (Gussoni 1999). Studies have suggested that these cells can give rise to hematopoietic cells, satellite cells, and can contribute to myofiber formation (Gussoni 1999; Jackson 1999; Asakura 2002).

It has also been suggested that there is a population of skeletal muscle cells that can differentiate into cardiomyocytes. In this study, nonadherent cells were isolated from murine skeletal muscle and termed skeletal-based precursors of cardiomyocytes (SPOC). SPOC cells are $CD34^-$ $CD45^-$ and $c\text{-}kit^-$ and begin spontaneously beating in culture in the presence of epidermal growth factor (EGF). When injected into the peripheral circulation of mice with myocardial infarct, the GFP^+ SPOC cells were able to migrate to the infarct and 8% became cardiomyocytes that expressed phosphorylated cardiac myosin regulatory light chain (Winitsky 2005).

Epithelial Stem Cells

Epithelial cells are located in many areas of the body and are characterized by the speed at which they turn over. Rapidly self-renewing epithelial cells tend to act as a protective barrier and are located in the skin, intestine, and eye. Persistent epithelial cells do not turn over rapidly and are located in the liver, kidney, and mammary gland (Rizvi 2005). Each of these populations has its own stem cell compartment, consisting of quiescent stem cells and the proliferative transient amplifying daughter cells that become the differentiated epithelial cells (Rizvi 2005). While stem cell markers for epithelial cells are not well defined, in the rapidly self-renewing populations, stem cells can be identified by their ability to retain the DNA labels [3H]thymidine and BrdU (Mackenzie 1985; Morris 1985; Braun 2003). A recent study suggested that α6 integrin^{bright} CD71^{dim} cells may represent putative stem cells in the skin (Webb 2004). Although it is more difficult to locate stem cells in the persistent epithelium, studies have claimed to have identified BrdU label-retaining stem cells in kidney (Maeshima 2003).

Epithelial skin cells have been demonstrated to be multipotent. Juvenile or adult mouse tissue was cultured in epidermal growth factor and fibroblast growth factor and small floating spheres of cells were collected. They were then incubated in neural differentiating conditions and the cells began to express neural markers, including nestin, βIII-

tubulin, neurofilament-M, NeuN, and a neuron-specific Tα1 α-tubulin:nlacZ transgene. Some of the floating cells also began expressing glial markers GFAP and CNPase, or an oligodendrocyte precursor marker A2B5 (Toma 2001).

Skin stem cells are already being used in therapy. In burn patients, keratinocytes are extracted from a non-injured area, expanded in culture, and then grafted onto the burned areas. By using autologous cells, the danger of rejection is removed (Alonso 2003). Epithelial stem cells may be promising for use in other therapies as well. If stem cells can be coaxed to become other cell types, such as neural cells, then they can be readily obtained from the host and used to treat neurodegenerative disorders without the complications of graft vs. host disease.

Neural Stem Cells

Neural stem cells (NSCs) can give rise to three different major cell types: neurons, astrocytes, and oligodendrocytes (McKay 1997). Unlike hematopoietic and muscle stem cells, NSCs are isolated mainly based on clonal proliferation rather than cell markers. Cells are isolated from tissue and exposed to mitogens such as epidermal growth factor and fibroblast growth factor 2 in serum-deficient culture and NSCs are identified as small proliferative colonies (Vescovi 1993; Palmer 1995). However, there are markers to distinguish between the more differentiated cell types. Markers for the three major neural types include TUJ1 for neurons, glial fibrillary acid protein (GFAP) for astrocytes, and GalC for oligodendrocytes (Gage 2000). Scientists are beginning to use progenitor cell-selective reporter gene expression and surface antigen-based sorting to purify cells (Goldman 2005).

There are a variety of diseases affecting the central nervous system that may be treated with NSCs. One study showed that both fetal and adult human oligodendrocyte progenitor cells can develop into atrocytes and myelinating oligodentrocytes in a demyelinated mouse model (Windrem 2004). Studies like these may one day result in therapies for demyelinating diseases such as Tay-Sachs, adrenoleukodystrophy, and multiple sclerosis (Goldman 2005).

Like other stem cells, neural stem cells have been demonstrated to become other cell types. In a study by Bjornson, NSCs were

isolated from embryonic or adult forebrain of ROSA26 mice and cultured in epidermal growth factor and fibroblast growth factor 2. The cells were then injected into sublethally irradiated Balb/c mice and after 5-12 months analyzed for the presence of the lacZ gene. They detected a strong signal in mice injected with either embryonic, adult, or clonally derived NSCs. They also used clonogenic analysis and immunocytochemistry to determine whether the NSCs displayed hematopoietic characteristics and found that bone marrow isolated from NSC recipients displayed the ROSA26 specific antigen H-2K^{b} and formed granulocyte, macrophage, and B cell colonies (Bjornson 1999).

Cardiac Stem Cells

Until recently it was thought that a resident cardiac progenitor cell population might not exist. One reason for this was due to a seeming lack of regenerative capacity, especially after cardiac injury. However, the past few years have seen an increasing number of reports that claim to be able to isolate a cardiac progenitor population that can give rise to beating cardiomyocytes. Matsuura et al. purified a population of Sca-1^+ cells from adult murine heart that began spontaneously beating and expressed cardiac genes, including Nkx2.5, Gata4, and cardiac α-actin, after treatment with oxytocin (Matsuura 2004). Beltrami et al. identified a cardiac population that was Lin^- c-kit^+ and expressed Gata4, Nkx2.5, and Mef2. In differentiation medium, clones from an individual cell produced cells that expressed markers for three cardiac lineages: myocytes, endothelial cells, and smooth muscle cells. They also took GFP labeled precursors and injected them into the border region of infarcted heart and identified a band of new myocardium expressing GFP (Beltrami 2003). A third study isolated a population of cells that were Sca-1^+ and negative for Lin, c-kit, Flk-1, CD45, and CD34. They infused Sca-1^+ cells that contained an αMHC-Cre transgene into mice containing a floxed lacZ gene. After two weeks they saw Cre^+ cells, indicative of differentiation. Half of the Cre^+ cells were also positive for lacZ, suggesting fusion of donor cells with resident myocytes (Oh 2004).

The heart, like most other tissues, also contains a side population of cells (Hierlihy 2002; Martin 2004; Pfister 2005). These cells were shown to be positive for sca-1 and negative for c-kit, CD34, CD45, and CD31 (Martin 2004), although Pfister et al. also identified a $CD31^+$ SP fraction. $CD31^+$ cells occupied a distinct portion of the SP arm from the $CD31^-$ fraction, although the $CD31^+$ cells were shown to lack the ability to differentiate into cardiomyocytes (Pfister 2005). All three studies utilized a co-culture method to demonstrate differentiation ability. GFP^+ SP cells were co-cultured with wild type cardiac cells and after incubation were examined for markers of cardiac differentiation. This assessment showed that GFP^+ cells were positive for connexin 43 (Hierlihy 2002; Pfister 2005), alpha-actinin (Martin 2004), Gata4 and MEF2C, and 10% began spontaneously beating at a low frequency (Pfister 2005).

While it appears that cardiac SP cells are able to differentiate into cardiomyocytes, it is unclear whether they are also able to differentiate into other cell types. Martin et al. plated cardiac SP cells in methylcellulose media and found that they were able to form hematopoietic colonies (Martin 2004). However, Hierlihy et al. found that myocardial cell suspensions formed limited hematopoietic colonies in methylcellulose. In addition, while cardiac SP cells could contribute to myotubes by fusing to co-cultured primary-derived skeletal muscle myoblasts, SP cells driving a skeletal muscle specific myf5/lacz

transgene did not express β-gal or myogenin when co-cultured with C2C12 myoblasts (Hierlihy 2002).

Cardiovascular disease is the leading cause of death in the United States. In 2002, there were 928,000 deaths from cardiovascular disease, 1.6 times more than deaths from cancer (American Heart Association 2005). One of the main factors contributing to failure after injury is the loss of cardiomyocytes (Smits 2005). Currently scientists are focusing on the promise of stem cell transplant to regenerate the injured tissue by looking at both cardiac progenitor cells and stem cells from other tissues that appear to have the ability to contribute to cardiac repair (Makino 1999; Orlic 2001; Winitsky 2005).

Abcg2

In 1996 Margaret Goodell isolated a new population of cells from bone marrow. Whole bone marrow had been stained with the DNA binding dye Hoechst 33342 and FACS sorted using red and blue emission wavelengths. They observed a group of cells that was Hoechst^{low} and created a side arm of cells branching off of a main population of brightly staining cells. These cells were enriched for hematopoietic stem cell activity and could be eliminated after incubation with verapamil, an inhibitor of the multidrug resistance protein (MDR), which had been shown to efflux dyes from cells (Goodell 1996). Side population cells have since been found in many adult tissues, including skeletal muscle, brain, mammary tissue, liver, and lung, and appear to represent a progenitor cell population (Asakura 2002; Murayama 2002; Alvi 2003; Summer 2003; Wulf 2003).

MDR is a member of the ATP binding cassette (ABC) family of membrane transporters. These transporters use energy from binding ATP to transport various substances across the plasma membrane, endoplasmic reticulum, and mitochondrial membrane (Dean 2001). The 49 members of the ABC family transport sterols, peptides, bile salt, drugs, and other toxins (Dean 2001). One of the most recently discovered ABC transporters is Abcg2, also known as breast cancer resistance protein (BCRP1). Abcg2 was first discovered in the human MCF-7 breast cancer line through its ability to confer resistance to

drugs such as doxorubicin, daunorubicin, and mitoxantrone (Doyle 1998). Abcg2, unlike most other members of the ABC transporter family, is a half-transporter with six transmembrane domains and functions as a homodimer (Doyle 1998).

The ability of cells to efflux Hoechst dye is conferred by Abcg2, and was demonstrated by overexpressing the protein in both bone marrow and skeletal muscle. The presence of side population cells could be reversed by adding either the broad inhibitor reserpine, or the Abcg2 specific inhibitor fumitremorgin C (FTC) (Zhou 2001; Martin 2004).

Several studies have suggested that Abcg2 expression may be a marker for stem or progenitor cells. SP cells isolated from bone marrow have been shown to be able to reconstitute lethally irradiated mice (Goodell 1996), regenerate muscle in the mdx mouse (Gussoni 1999), and regenerate cardiomyocytes (Jackson 2001). Based on these studies and preliminary data, I undertook experiments to further evaluate the functional role and regulation of Abcg2 in stem cells.

The preliminary data for my studies came from a microarray performed on main and side population cells. To generate these populations, C2C12 myoblasts were transfected with either empty vector or constitutively active Abcg2. The cells were then sorted by dual-wavelength FACS and the Hoechst^{low} SP and Hoechst^{bright} MP cells were collected. RNA from these cells was isolated, labeled, and

hybridized to an Affymetrix gene chip containing over 20,000 genes. The resulting microarray data showed Abcg2 overexpressing cells have a greater than 2-fold increase in mRNA levels of many genes that are important in cytoprotection and cell cycle control.

Unigene name	SP signal strength	Fold Change	MP signal strength
Upregulated in SP cells			
activating transcription factor 3	1665.8	9.19	132.5
GRO1 oncogene	660.6	6.96	91.6
proliferin 2	7272.4	5.66	854.7
N-myc downstream regulated 1	3291.1	5.28	574.5
cyclin-dependent kinase inhibitor 1A (P21)	1254.1	4.92	261.4
glutathione S-transferase, alpha 2 (Yc2)	551.2	4.29	121.3
glutathione S-transferase, alpha 4	1086.7	4.00	252.3
serine protease inhibitor 13	303.5	4.00	60.1
Metallothionein 2	4775.7	3.03	1478.6
cyclin G2	303.8	2.14	144.5
Downregulated in SP cells			
cell division cycle 6 homolog (S. cerevisiae)	177.2	-2.00	513.4

Figure 1. Disregulated genes in C2C12 SP cells

These genes include glutathione S-transferase and metallothionein 2, which reduce hydroperoxides and regulate zinc homeostasis in response to nitrosative stress, respectively, and cyclin G2, which is a negative regulator of cell cycle progression.

Since stem cells are a reserve for adult tissues and only need to divide to replace dead cells, they spend most of their time in the G0, or quiescent stage of the cell cycle. Finding upregulated genes that play a negative role in cell cycle progression prompted me to examine the

cell cycle of SP cells. C2C12 myoblasts were transfected with empty vector or Abcg2, and MP and SP cells were isolated by FACS. Cells were stained with the DNA binding dye propidium iodide (PI) and analyzed by flow cytometry. Cells that are in S or G2/M phases of the cell cycle have more DNA and therefore will bind more PI, resulting in a brighter signal. 40-50% of cells transfected with empty vector or main population cells were in G0/G1 phase of the cell cycle, whereas 80% of cells overexpressing Abcg2 were in G0/G1. Therefore it appears that SP cells, like stem cells, are quiescent.

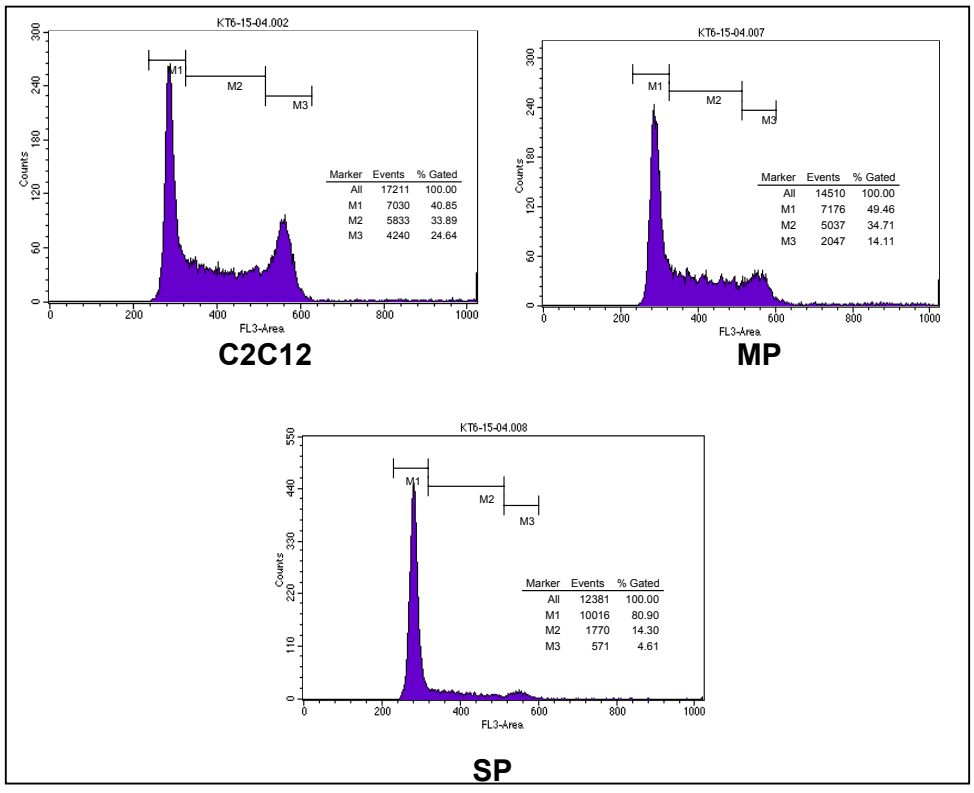

Figure 2. Cell cycle analysis of C2C12 cells either mock treated or transfected with GFP or Abcg2

Since array data identified several genes involved in oxidative stress reduction and cytoprotection, I attempted to determine whether cells that overexpressed Abcg2 were less susceptible to oxidative stress.

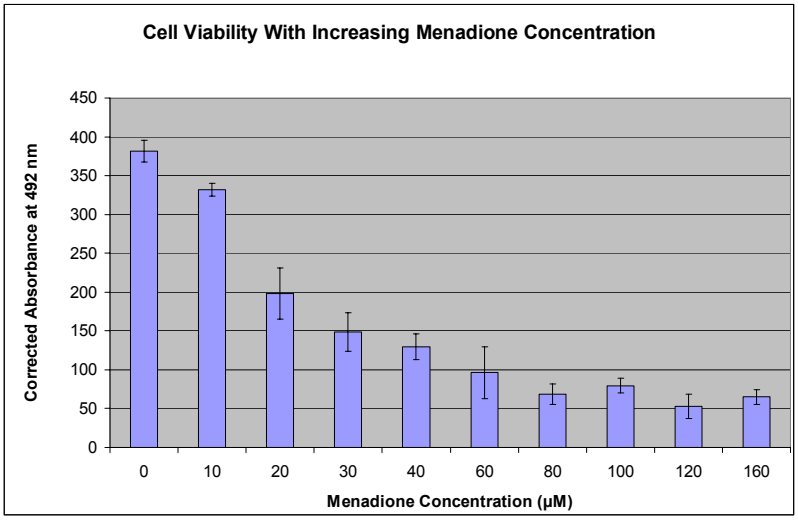

Figure 3. Cell viability of C2C12 cells with increasing menadione concentration

To do this, I first established a concentration of menadione at which there would be 50% cell death in C2C12 myoblasts. Menadione is a compound that produces the oxidative products O_2^-, hydroxyl radicals (OH·) and hydrogen peroxide during its metabolism. To test the viability I used a tetrazolium salt reagent that is reduced into a red formazan product by mitochondrial dehydrogenases of viable cells. The absorbance of the media is proportional to the number of viable cells. I then mock treated or transfected either GFP or Abcg2 into

C2C12 myoblasts, collected C2C12, GFP^-, GFP^+, MP and SP fractions, and exposed them to 20 μM menadione for 1 hour.

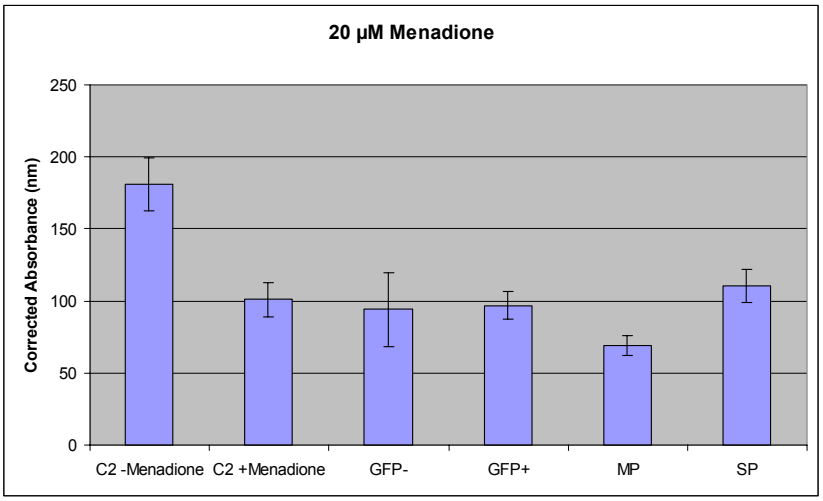

Figure 4. Cell viability of C2C12 cells either mock treated or transfected with GFP or Abcg2 with the addition of 20 μM menadione

From this data, Abcg2 is not protective against menadione-induced oxidative stress, but there should also be GFP, MP, and SP controls that were not exposed to menadione. The lack of cytoprotection seen in this assay may be due to limitations of C2C12 cells. Through personal communication I learned that C2C12 is not the best cell line to use in cell death studies due to variability in the death process. It was suggested that using hydrogen peroxide on mouse embryonic fibroblasts would be a better alternative and those studies are in progress.

To assess the regulation of Abcg2, I examined evolutionary conservation between the upstream regions of human and mouse Abcg2. Using the UCSC genome browser I identified conserved regions up to 11kb upstream of the mouse Abcg2 gene. I selected these regions in which to analyze conserved transcription factor binding sites that may play a role in regulating Abcg2 expression.

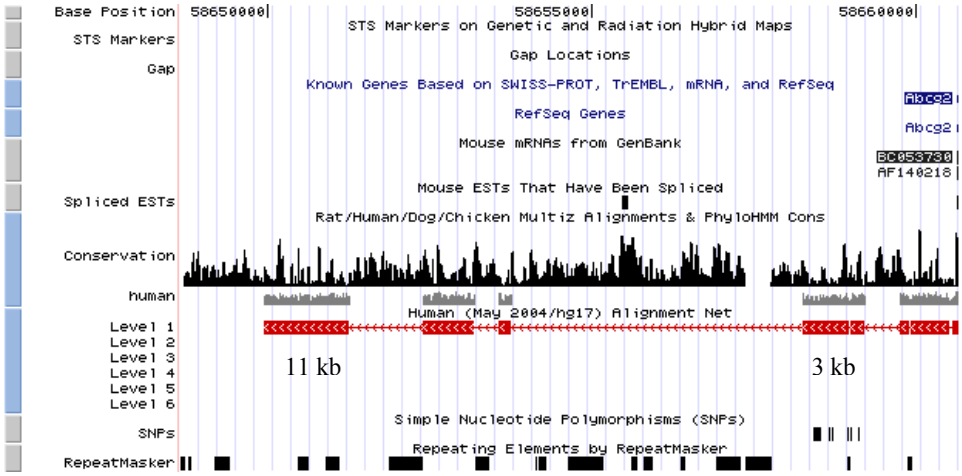

Figure 5. Evolutionary conservation of 11kb upstream of Abcg2 between mouse and human

Using the programs Consite and rVista, I identified conserved putative transcription factor binding sites. Many of these transcription factors are involved in stem and progenitor cell regulation. For example, aml-1 (runx-1) is required for definitive hematopoiesis in the fetus, is critical for normal hematopoiesis in the adult mouse, and can act as a transcriptional activator or repressor (Yamagata 2004; Growney 2005). HFH2 (FoxD3) is required for the maintenance of progenitor cells in the embryo (Hanna 2002) and is expressed in neural crest cells and

motorneuron progenitors in the developing spinal cord (Labosky 1998). Stat5a is required during the terminal stages of myeloid differentiation (Kieslinger 2000) and has been shown to activate transcription of Bcl-X(L) in erythroid cells to reduce apoptosis (Socolovsky 1999). Sox factors are also implicated in stem and progenitor cell regulation. Sox2 has been suggested to play a role in neural precursor proliferation and neural stem cell maintenance (Episkopou 2005), while Sox5 is

Kb upstream	Transcription Factor
10	hfh1
	broadcomplex 4
	sox 5
	sox 17
9.8	agl3
9.6	stat5a
	cap
	sox 17
	hfh3
9.4	sox 5
	c-fos
8	hnf3-beta
	athb-1
	athb5
	sox 17
	broadcomplex-4
	hunchback
7.8	stat5a
	cdxa
	athb-1
	nrf-2
	e74a
	freac-4

Kb upstream	Transcription Factor
7.4	aml-1
	athb-1
	hfh2
6.8	tbp
2.4-1.5	hfh-2
	hunchback
	e74a
	c-fos
	freac-4
	bzip910
1	hnf3-beta
	aml-1
	cf2-II
	agl3
	freac-4
	sox-17
	sox 5

Figure 6. Evolutionarily conserved transcription factors and their approximate location in the 11kb upstream region of Abcg2

important in chondrocyte differentiation and cartilage formation (Lefebvre 2001).

Based on transcription factor conservation data, I then undertook transcriptional analysis of the Abcg2 promoter utilizing the transcription factors sox2, sox5, and runx1. Two different promoter

fragments, extending from +165 bp to either 2.7 kb or 5.5 kb upstream, which I referred to as 3 Kb and 6 KB respectively, were cloned into the PGL3-Basic luciferase vector. The selection of these two fragments was made based on sequence conservation and ease of cloning. 100 ng of the promoter-luciferase construct was co-transfected with increasing concentrations of a plasmid containing the constitutively active transcription factor. Empty PGL3-Basic vector was co-transfected with each transcription factor as a control. Following a 24 hour incubation period, the cells were lysed and the extracts analyzed for a change in luciferase expression, indicating whether the transcription factor was able to activate or repress expression, or whether it had no effect. Unfortunately, the results from these experiments were uninterpretable because the background luciferase expression was very high. One interesting observation from these experiments was that in each case, the expression of the 6 kb promoter plasmid was reduced compared to the 3 kb plasmid of PGL3-Basic control. This could be due to either the large length of the inserted fragment or to a repressive element located somewhere between 2.7 and 5.5 kb upstream of the translational start site.

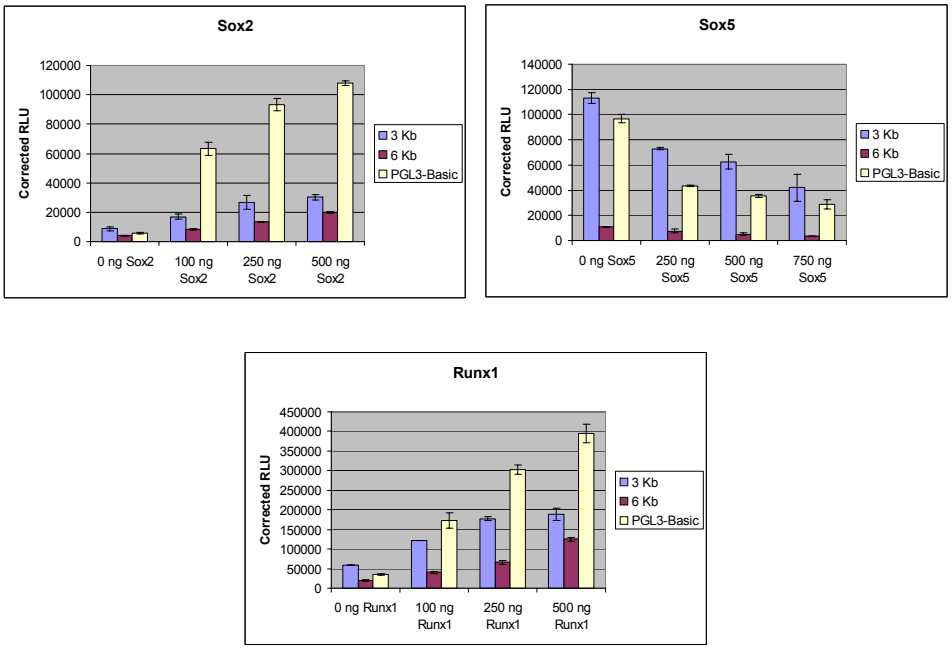

Figure 7. Transcriptional assays of C2C12 cells transfected with Abcg2 and either Sox2, Sox5 or Runx1

In addition to using transcriptional analysis to examine regulation of Abcg2, I am also creating constructs to use in the making of transgenic animals. I will have two mouse lines, with either an 11 kb or the 3 kb promoter fragment driving the expression of a yellow fluorescent protein (YFP). To examine whether there is expression of the transgene, I will mate male founders with wild type females and analyze the embryos and yolk sacs for fluorescent expression. I will look at the yolk sac because previous studies have shown that Abcg2 expression is strong in the yolk sac, so there should be a good chance that I should see expression of my transgene. The aim is to evaluate the upstream fragment of the Abcg2 gene that directs expression in

side population cells. Once identified, this promoter sequence can be used to drive a tetracycline-dependent transactivator that can activate a gene that is lethal to cells (Gallagher 2003). At points during development or after injury tetracycline can be administered and development or regeneration in the absence of side population cells can be examined. In addition, mice with fluorescent expression in side population cells could be mated to Abcg2 -/- mice. This would allow cells that would normally express Abcg2 to still be identified, since without Abcg2 the cells are not able to efflux the Hoechst dye and the side population cells can not be identified. These cells could then be examined for stem or progenitor cell ability in the absence of Abcg2.

Based on present studies, it appears that Abcg2 can be used as a marker for progenitor cell populations, which may be useful in therapeutic studies. What remains to be seen is exactly what kind of functional role Abcg2 plays. Studies have shown that Abcg2 is capable of effluxing chemotherapeutic drugs from cells, suggesting a protective role against xenotoxins (Burger 2004; Elkind 2005). Another study also suggests that Abcg2 can efflux porphyrins, a heme precursor (Jonker 2002). Future studies should elucidate whether Abcg2 plays an active role against oxidative stress, and whether it has any additional functions.

REFERENCES

Alonso, L. et al. (2003). "Stem cells of the skin epithelium." PNAS **100**: 11830-11835.

Alvi, A. J. et al. (2003). "Functional and molecular characterisation of mammary side population cells." Breast Cancer Research **5**(1): R1-8.

American Heart Association (2005). Heart Disease and Stroke Statistics - 2005 Update.

Asakura, A. et al. (2002). "Myogenic specification of side population cells in skeletal muscle." Journal of Cell Biology **159**(1): 123-135.

Beltrami, A. P. et al. (2003). "Adult cardiac stem cells are multipotent and support myocardial regeneration." Cell **114**(6): 763-776.

Bjornson, C. R. et al. (1999). "Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo." Science **283**(5401): 534-537.

Braun, K. M. et al. (2003). "Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis." Development **130**: 5241-5255.

de Bruijn, M. F. T. R. et al. (2000). "Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo." The EMBO Journal **19**(11): 2465-2474.

Burger, H. et al. (2004). "Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump." Blood **104**(9): 2940-2942.

Burghes, A. H. et al. (1987). "A cDNA clone from the Duchenne/Becker muscular dystrophy gene." Nature **328**: 434-437.

Camargo, F. D. et al. (2003). "Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates." Nature Medicine **9**(12): 1520-1527.

Corbel, S. Y. et al. (2003). "Contribution of hematopoietic stem cells to skeletal muscle." Nature Medicine **9**(12): 1528-1532.

Cornelison, D. D. W. and B. J. Wold (1997). "Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells." Developmental Biology **191**: 270-283.

Cossu, G. et al. (2000). "Myogenic stem cells for the therapy of primary myopathies: wishful thinking or therapeutic perspective?" Journal of Clinical Investigation **105**: 1669-1674.

Dean, M. et al. (2001). "Complete characterization of the human ABC gene family." Journal of Bioenergetics and Biomembranes **33**(6): 475-479.

Doyle, L. A. et al. (1998). "A multidrug resistance transporter from human MCF-7 breast cancer cells." PNAS **95**(26): 15665-15670.

Elkind, N. B. et al. (2005). "Multidrug transporter ABCG2 prevents tumor cell death induced by the epidermal growth factor receptor inhibitor Iressa (ZD1839, Gefitinib)." Cancer Research **65**(5): 1770-1777.

Episkopou, V. et al. (2005). "SOX2 functions in adult neural stem cells." Trends in Neurosciences **28**(5): 219-221.

Fassas, A. S. et al. (2002). "Hematopoietic stem cell transplantation for multiple sclerosis. A retrospective multicenter study." Journal of Neurology **249**(8): 1088-1097.

Gage, F. H. et al. (2000). "Mammalian neural stem cells." Science **285**(5457): 1433-1438.

Gallagher, A. R. et al. (2003). "Use of the tetracycline system for inducible protein synthesis in the kidney." Journal of the American Society of Nephrology **14**: 2042-2051.

Garry, D. J. et al. (2000). "Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF." PNAS **97**(10): 5416-5421.

Goldman, S. et al. (2005). "Stem and progenitor cell-based therapy of the human central nervous system." Nature Biotechnology **23**(7): 862-871.

Goodell, M. A. et al. (1996). "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo." Journal of Experimental Medicine **183**: 1797-1806.

Growney, J. D. et al. (2005). "Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype." Blood **106**(2): 494-504.

Gussoni, E. et al. (1999). "Dystrophin expression in the mdx mouse restored by stem cell transplantation." Nature **401**: 390-394.

Hanna, L. A. et al. (2002). "Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo." Genes and Development **16**(20): 2650-2661.

Hawke, T. J. and D. J. Garry (2001). "Myogenic satellite cells: physiology to molecular biology." Journal of Applied Physiology **91**: 534-551.

Hierlihy, A. M. et al. (2002). "The post-natal heart contains a myocardial stem cell population." FEBS Letters **530**: 239-243.

Jackson, K. A. et al. (1999). "Hematopoietic potential of stem cells isolated from murine skeletal muscle." PNAS **96**(25): 14482-14486.

Jackson, K. A. et al. (2001). "Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells." The Journal of Clinical Investigation **107**(11): 1395-1402.

Jejurikar, S. S. et al. (2003). "Satellite cell depletion in degenerative skeletal muscle." Apoptosis **8**(6): 573-578.

Jesse, T. L. et al. (1998). "Interferon regulatory factor-2 is a transcriptional activator in muscle where It regulates expression of vascular cell adhesion molecule-1." Journal of Cell Biology **140**(5): 1265-1276.

Jonker, J. W. et al. (2002). "The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria." PNAS **99**(24): 15649-15654.

Kieslinger, M. et al. (2000). "Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation." Genes and Development **14**(2): 232-244.

Labosky, P. A. et al. (1998). "The winged helix transcription factor Hfh2 is expressed in neural crest and spinal cord during mouse development." Mechanisms of Development **76**: 185-90.

Lagasse, E. et al. (2000). "Purified hematopoietic stem cells can differentiate into hepatocytes in vivo." Nature Medicine **6**(11): 1229-1234.

Lefebvre, V. et al. (2001). "L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway." Osteoarthritis Cartilage **Suppl A**: S69-75.

Mackenzie, I. C. et al. (1985). "Label-retaining keratinocytes and Langerhans cells in mouse epithelia." Cell and Tissue Research **242**(3): 551-556.

Maeshima, A. et al. (2003). "Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney." Journal of the American Society of Nephrology **14**(12): 3290-3292.

Makino, S. et al. (1999). "Cardiomyocytes can be generated from marrow stromal cells in vitro." Journal of Clinical Investigation **103**: 697-705.

Martin, C. M. et al. (2004). "Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart." Developmental Biology **265**(1): 262-275.

Matsuura, K. et al.(2004). "Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes." Journal of Biological Chemistry **279**(12): 11384-11391.

Matsuzaki, Y. et al. (2004). "Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells." Immunity **20**: 87-93.

Mayani, H. et al. (2003). "Biology of human hematopoietic stem and progenitor cells present in circulation." archives of Medical Research **34**: 476-488.

Mayani, H. et al. (2003). "A glance into somatic stem cell biology: basic principles, new concepts and clinical relevance." archives of Medical Research **34**: 3-15.

Mayhall, E. A. et al. (2004). "The clinical potential of stem cells." Current Opinion in Cell Biology **16**(6): 713-720.
McKay, R. et al. (1997). "Stem cells in the central nervous system." Science **276**(5309): 66-71.
Menthena, A. et al. (2004). "Bone marrow progenitors are not the source of expanding oval cells in injured liver." Stem Cells **22**: 1049-1061.
Mezey, E. et al. (2000). "Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow." Science **290**: 1779-1782.
Morris, R. J. et al. (1985). "Evidence that the centrally and peripherally located cells in the murine epidermal proliferative unit are two distinct cell populations." Journal of Investigative Dermatology **84**(4): 277-281.
Murayama, A. et al. (2002). "Flow cytometric analysis of neural stem cells in the developing and adult mouse brain." Journal of Neuroscience Research **69**(9): 837-847.
Murry, C. E. et al. (2004). "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts." Nature **428**(6983): 664-668.
Ogawa, M. et al. (2002). "Hematopoietic stem cell protocol." Methods in Molecular Medicine. Humana Pres.: 113.
Oh, H. et al. (2004). "Cardiac muscle plasticity in adult and embryo by heart-derived progenitor cells." Annals of New York Academy of Sciences **1015**: 182-189.
Orlic, D. et al. (2001). "Bone marrow cells regenerate infarcted myocardium." Nature **410**: 701-705.
Osawa, M. et al. (1996). "Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell." Science **273**(5272): 242-245.
Palmer, T. D. et al. (1995). "FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain" Molecular and Cellular Neuroscience **6**(5): 474-486.
Pfister, O. et al. (2005). "CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation." Circulation Research **97**(1): 65-61.
Rizvi, A. Z. et al. (2005). "Epithelial stem cells and their niche: there's no place like home." Stem Cells **23**: 150-165.
Rybakova, I. N. et al. (2000). "The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin." Journal of Cell Biology **150**(5): 1209-1214.
Schultz, E. and K. M. McCormick (1994). "Skeletal muscle satellite cells." Reviews of physiology, biochemistry and pharmacology **123**: 213-257.

Seale, P. et al. (2000). "Pax7 is required for the specification of myogenic satellite cells." Cell **102**(6): 777-786.
Smith, L. G. et al. (1991). "Clonal analysis of hematopoietic stem-cell differentiation in vivo." PNAS **88**(7): 2788-2792.
Smits, A. M. et al. (2005). "The role of stem cells in cardiac regeneration." J. Cell. Mol. Med **9**(1): 25-36.
Socolovsky, M. et al. (1999). "Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction." Cell **98**(2): 181-191.
Spangrude, G. J. et al. (1995). "Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function." Blood **85**(4): 1006-1016.
Summer, R. et al. (2003). "Side population cells and Bcrp1 expression in lung." American Journal of Physiology - Lung Cellular and Molecular Physiology **285**: L97-104.
Terada, N. et al. (2002). "Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion." Nature **416**: 542-545.
Theise, N. D. et al. (2000). "Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation." Hepatology **31**(1): 235-240.
Toma, J. G. et al. (2001). "Isolation of multipotent adult stem cells from the dermis mammalian skin." Nature Cell Biology **3**: 778-784.
Uchida, N. et al. (1996). "Searching for hematopoietic stem cells. II. The heterogeneity of Thy-1.1(lo)Lin(-/lo)Sca-1+ mouse hematopoietic stem cells separated by counterflow centrifugal elutriation." Experimental Hematology **24**(5): 649-659.
Vescovi, A. L. et al. (1993). "bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells." Neuron **11**: 951-966.
Wagers, A. J. et al. (2002). "Little evidence for developmental plasticity of adult hematopoietic stem cells." Science **297**: 2256-2259.
Wang, X. et al. (2003). "Cell fusion is the principal source of bone-marrow-derived hepatocytes." Nature **422**: 897-901.
Webb, A. et al. (2004). "Location and phenotype of human adult keratinocyte stem cells of the skin." Differentiation **72**(8): 387-395.
Windrem, M. S. et al. (2004). "Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain." Nature Medicine **10**(1): 93-97.
Winitsky, S. O. et al. (2005). "Adult Murine Skeletal Muscle Contains Cells That Can Differentiate into Beating Cardiomyocytes In Vitro." PLoS Biology **3**(4): 662-671.

Wulf, G. G. et al. (2003). "Cells of the hepatic side population contribute to liver regeneration and can be replenished with bone marrow stem cells." Haematologica **88**(4): 368-378.

Wulffraat, N. M. and W. Kuis (1999). "Autologous stem cell transplantation: a possible treatment for refractory juvenile chronic arthritis?" Rheumatology **38**(8): 764-766.

Yamagata, T. et al. (2004). "Runx1/AML1 in Normal and Abnormal Hematopoiesis." Int J Hematol **82**(1): 1-8.

Ying, Q.-L. et al. (2002). "Changing potency by spontaneous fusion." Nature **416**: 545-548.

Zhou, S. et al. (2001). "The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype." Nature Medicine **7**(9): 1028-1034.

VITAE

Mary Katherine Tunison was born in Olathe, Kansas on January 14, 1981, the daughter of Teresa L. Tunison and William A. Tunison. After completing her work at Olathe South High School, Olathe, Kansas in 1999, she entered Baylor University at Waco, Texas. She received the degree of Bachelor of Science with a major in Biology and minors in Chemistry and Spanish from Baylor University in May, 2003. In August, 2003 she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She was awarded the degree of Master of Arts in December, 2005.

Permanent Address: 3235 Carlisle St. #237
Dallas, Texas 75204