THE EFFECTS OF EXERCISE ON HEMATOPOIESIS

THE EFFECTS OF EXERCISE ON HEMATOPOIESIS AND THE DEVELOPMENT OF THE HEMATOPOIETIC STEM CELL NICHE

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Lay Abstract

Exercise affects many different tissue types throughout the body. The hematopoietic system, through which the body produces blood cells, is no exception. How exercise as a stimulus is able to influence this system is poorly understood. Here, we demonstrate that exercise is able to stimulate skeletal muscle to produce erythropoietin, a potent hematopoietic growth factor. As well, we demonstrate that exercise is capable of mobilizing hematopoietic stem cells from the bone marrow to the blood. We then describe how exercise is able to increase hematopoietic activity outside the bone marrow. Finally, we show that exercise affects marrow in only certain bone marrow cavities. Our findings demonstrate that exercise is able to influence hematopoiesis in a myriad of ways. As well, our findings highlight potential commonalities in the means by which exercise exerts these influences.

Abstract

Exercise has been shown to influence nearly every tissue type in the body, including the hematopoietic system. The means by and the extent to which exercise is able to do this is unknown. Here, we investigated the effects of skeletal muscle and exercise on several components of hematopoiesis. Firstly, we investigated exercise induced changes in skeletal muscle endocrine signalling. We demonstrated that exercise increased skeletal muscle hypoxia, leading to HIF1α and HIF2α stability, resulting in increased expression of erythropoietin. As well, myoblasts in culture were shown to express and release erythropoietin in response to hypoxia. Secondly, we measured mobilization of hematopoietic cells during exercise. We demonstrated that exercise greatly increased the number of hematopoietic stem cells in circulation. The quantity of mobilization was dependent on exercise intensity, did not depend on fitness levels, and was at peak immediately post exercise. Thirdly, we measured levels of extramedullary hematopoiesis following exercise. Exercise increased spleen hematopoietic stem cell content. Furthermore, expression of genes associated with hematopoietic homing, adhesion, quiescence, and growth were all increased in the spleen following exercise. Finally, we examined bone marrow in the appendicular and axial skeleton of aged animals. Here, exercise increased bone marrow cellularity and reduced bone marrow adiposity in the appendicular skeleton of aged mice. However, lumbar vertebral marrow cellularity and skeletal muscle expression of hematopoietic cytokines in these mice was unaffected by exercise. Taken together, these results demonstrate that exercise is a potent mediator of hematopoietic homeostasis. Several themes recurrent in these and other studies lead to insight in how exercise is able to exert influence on hematopoiesis, namely: tissue specific changes in hematopoietic growth and homing factor expression, the ability of mechanical forces felt during exercise to alter the bone marrow niche microenvironment, and increased flux of hematopoietic stem cells through their various bodily niches.

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List of Abbreviations

1. M-CSF: macrophage colony stimulating factor
2. G-CSF: granulocyte colony stimulating factor
3. GM-CSF: granulocyte macrophage colony stimulating factor
4. EPO: erythropoietin
5. IL3: interleukin 3
6. HSC: hematopoietic stem cell
7. MSC: mesenchymal stem cell
8. CAFC: cobblestone area forming cell
9. LTC-IC: long term culture initiating cell
10. CFU: colony forming unit
11. CFU-GEMM: CFU granulocyte erythrocyte monocyte megakaryocyte
12. CFU-GM: CFU granulocyte monocyte
13. BFU-E: burst forming unit erythrocyte
14. CD: cluster of differentiation
15. LSK: lineage negative Sca1 positive c-kit positive
16. EMH: extramedullary hematopoiesis
17. PBMC: peripheral blood mononuclear cell

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Chapter 1 – Introduction

Sedentary behaviour is part of our modern lifestyle. The act of sitting in front of a computer to work, sitting on a couch to watch television, and sitting while traveling from place to place are all common in a normal routine (1). For most, daily physical activity is a workplace obligation or recreational choice. In the past, regular physical activity was mandatory for survival; planting crops, cutting wood, hunting animals, and fighting predators, all without the use of machines. Given that regular physical activity was a constant variable during the lengthy span of human development (2) it is no surprise that sedentary behaviour has been linked to a host of pathologies. Cancer, diabetes, hypertension, immune deficiencies, osteoporosis, and cardiovascular disease have all been tied to a lack of physical activity (3,4). That bone mineral density in early and medieval human skeletons was much higher than it is today is suggestive of the importance of exercise (5,6). While exercise may not be strictly mandatory for survival, it should at least be considered highly recommended for continued quality of life.

1.1 – Exercise

Though any physical activity done on a regular basis can be advantageous and healthy, adopting a defined exercise training regime can be much more valuable. In general, there are two types of exercise – aerobic or endurance type exercise and anaerobic or resistance type exercise. While not mutually exclusive activities, many individuals choose to focus on one type or another. Each type of exercise is associated with particular bodily adaptations and, as such, the body of an individual who is highly aerobically trained is different than that of an individual who is highly anaerobically trained (7). Aerobic exercise training is generally associated with activities such as walking, running, swimming, and cycling. The ability of the body to uptake, transport, and utilize oxygen are the limiting factors for aerobic exercise capacity (8). As such, these exercises build up aerobic capacity more so than muscular strength. Resistance exercise is associated with activities that apply resistance to muscular contractions, such as weight lifting. Muscle strength is the limiting factor for resistance type exercise. As a result, these types

of activities increase muscle size and strength (9). As this work focuses on bodily adaptations to aerobic exercise, adaptations to resistance exercise will be discussed in no further detail.

1.1.1 – Adaptations to Aerobic Exercise

Aerobic exercise tends to increase physical endurance by improving the efficiency of the heart, lungs, and circulatory system (10). These changes result in an increase in the ability of an individual to transport and utilize oxygen, further increasing aerobic exercise capacity (11). Specifically, there are three categories of adaptations associated with aerobic exercise; adaptations to ventilator capacity, adaptations to circulatory ability, and adaptations to muscle efficiency.

During aerobic exercise, oxygen first enters the body through the lungs. Increases in ventilatory capacity thus allow for increases in aerobic performance. Larger lung tidal volume, the normal amount of air displaced between inhalation and exhalation, facilitates increased access to oxygen (12). As well, increases in pulmonary diffusing capacity allow for greater ability of oxygen in the lungs to diffuse to the blood (13). Following oxygen diffusion to blood, it is transported through the body via red blood cells. Here, another adaptation helps to increase oxygen transport; increases in cardiac output. The stroke volume of the heart, rather than heart rate, increases (10); this increases oxygenated blood flow throughout the body. After oxygenated blood reaches working skeletal muscle, adaptations to muscle capillary density allow for shorter oxygen diffusion distances and a better supply of oxygen to muscle mitochondria (14). Increased red blood cell transit time through increased numbers of capillaries also helps to facilitate increased oxygen extraction from blood to muscle (15). Once inside muscle, oxygen is used for aerobic metabolism. Increased muscle oxygen utilization efficiency results in increased energy production and increased aerobic capacity. Increases in efficiency follow from an upregulation of oxidative enzymes in the mitochondria along with increased mitochondrial biogenesis (16). As well, metabolic changes within muscle result in increased reliance on oxidative fat metabolism as a fuel source and decreased reliance on anaerobic glycolysis, furthering aerobic capacity (17).

Of particular interest to this thesis are blood based adaptations to aerobic exercise training. Aside from adaptations associated with oxygen input, oxygen diffusion, and oxygen usage efficiency, aerobic exercise also leads to adaptations in blood itself. The primary role of blood is to transport oxygen throughout the body. Hemoglobin, found inside the cytoplasm of red blood cells, has the ability to bind oxygen. Oxygen from the lungs diffuses into the haemoglobin of red blood cells and later, when arterial blood is delivered to working skeletal muscle, oxygen diffuses from the haemoglobin into muscle fibres. The ability of blood to carry oxygen is equally as important as the ability of lungs to extract oxygen from the atmosphere or the ability of muscle to utilize it during aerobic metabolism. Blood based adaptations account for much of the increased oxygen transport capability seen following exercise training (18)

An increased hematocrit is commonly associated with endurance exercise training. While various groups have reported differing opinions on whether white cell mass changes with endurance training, red blood cell mass has been shown on many occasions to increase (8). Supplementary to this, many groups have reported increased blood volume as well as increased blood hemoglobin content with endurance training (19). Increased red cell volume and hemoglobin content serve to increase the capability of blood to transport oxygen, resulting in increased exercise performance (20). These blood based adaptations are even more prevalent in altitude training models. Endurance training in environments with reduced oxygen concentrations can strongly stimulate these changes (21). Blood adaptations in hypoxic environments are associated with increased production of kidney EPO (22). In mice, changes in kidney expression of EPO and increased red blood cell production have also been noted during aerobic training at sea level (23). Indeed, exogenous erythropoietin supplementation is one means by which athletes are able to artificially boost their hematocrit and increase their exercise performance (24). Aerobic exercise in untrained individuals can also lead to intravascular hemolysis of older, less flexible, red blood cells through sheer stress as these cells are forcefully circulated around the body and through small capillaries (25,26). Rupture of old red cells leads to increases in erythropoiesis and the development of a pool of younger, more flexible erythrocytes, better able to absorb and transport oxygen (27).

1.1.2 – Skeletal Muscle

Physical activity and exercise relies on the ability of skeletal muscle to generate force. Regular aerobic and resistance exercise cause skeletal muscle to undergo many adaptations. Skeletal muscle adaptations to exercise have been studied and defined in great detail, but are not the focus of this thesis. Of particular interest, however, is the ability of skeletal muscle to express and produce many different types of growth factors. Also of note is the ability of skeletal muscle to greatly upregulate expression of many types of growth factors following exercise. Growth factors and cytokines produced by skeletal muscle have been called myokines – myocyte cytokines. A multitude of these factors have been shown to be released into circulation from skeletal muscle, including IL6, IL8, IL13, IL15, BDNF, LIF, FGF21, Irisin, CCL2 , and CX3CL1 (28–31). This list is by no means comprehensive; as many as 188 factors have been identified as secreted during myogenesis in a muscle cell line (32). Given that the combined mass of skeletal muscle in the body outweighs all other organs (33), with the exception of adipose tissue in severely obese individuals, the ability of skeletal muscle to act as an endocrine organ should not be underestimated. As an example of this, one study found that following a marathon run in adult athletes IL6 levels in blood increased by 8000 fold (34). Skeletal muscle has also been shown to express cytokines specifically associated with blood cell development. Following an endurance exercise training program in mice, skeletal muscle has been shown to express IL3, M-CSF, GM-CSF, and EPO (23).

1.1.3 – Health Benefits of Exercise

Aside from adaptations to exercise specifically focused on aerobic capacity and muscle strength, exercise has been associated with a wide range of health benefits. Negative adaptations associated with sedentary behaviour often mirror positive adaptations associated with regular physical activity and exercise (35). Well known health benefits associated with exercise include increases in cardiovascular health (36), better blood pressure control in hypertensive patients (37), decreases in obesity (38), decreased risk of type II diabetes (39), increased glucose control in individuals with diabetes (40), and mitigation of sarcopenia (41). However, the health benefits of exercise extend to almost every tissue type.

In the brain, exercise has been shown to improve cognitive function (42), promote neuron survival (43), and stimulate neurogenesis (44). In eyes, exercise has been shown to protect retinal function and structure from light induced damage (45). Long term exercise training has also been shown to slow age related hearing loss and cochlear degeneration (46). Risk of olfactory impairment is also decreased in individuals exercising at least once a week (47). As well, endurance exercise attenuates age associated changes to skin in humans and mice (48). Increases in bone mineral density and decreases in fracture risk following exercise are also well documented (49). Health benefits of exercise even go so far as to reduce the prevalence of periodontitis in young adults (50).

1.1.4 – Exercise and Hematopoiesis

Given that exercise training can affect beneficial changes in such seemingly unrelated tissues as eyes and ears, it is no surprise that exercise has also been shown to influence the process of hematopoiesis. Aside from the previously discussed adaptations in red blood cell production and health, focusing on increasing oxygen transport through the body, exercise has also been shown to affect other components of the hematopoietic system. Acute exercise mobilizes hematopoietic stem and progenitor cells (51). Exercise training increases bone marrow cellularity and hematopoietic stem cell content (23). Increased immune function is also associated with exercise training (52). Changes in bone marrow niche structure also occur following exercise training (23). However, the mechanism or mechanisms by which exercise influences hematopoiesis are less than clear. A more thorough review of previous findings that associate exercise with hematopoiesis is presented at the end of this chapter. As well, the current understanding of the links between exercise, skeletal muscle, and hematopoiesis will be discussed in more detail at the end of the chapter, following additional background discussion on the components of the hematopoietic system.

1.2 – Hematopoiesis

Hematopoiesis is the process by which blood cells are produced. It is estimated that nearly 50 billion mature blood cells are produced on a daily basis in order to maintain blood homeostasis (53). To

do this, the body maintains a population of 80 million hematopoietic stem cells (HSC) of which 10% are actively involved in proliferation and differentiation (54). In general, terminally differentiated hematopoietic cells are divided into two broad categories; cells of the myeloid lineages and cells of the lymphoid lineages (**Figure 1**). Myeloid cells are the oxygen carrying blood cells and the cells that form the basis of the innate immune system; megakaryocytes, erythrocytes, macrophages, neutrophils, and eosinophils. Lymphoid cells populate lymph tissues and form the basis of the adaptive immune system; T cells, B cells, natural killer cells, and all the variations thereof (53).

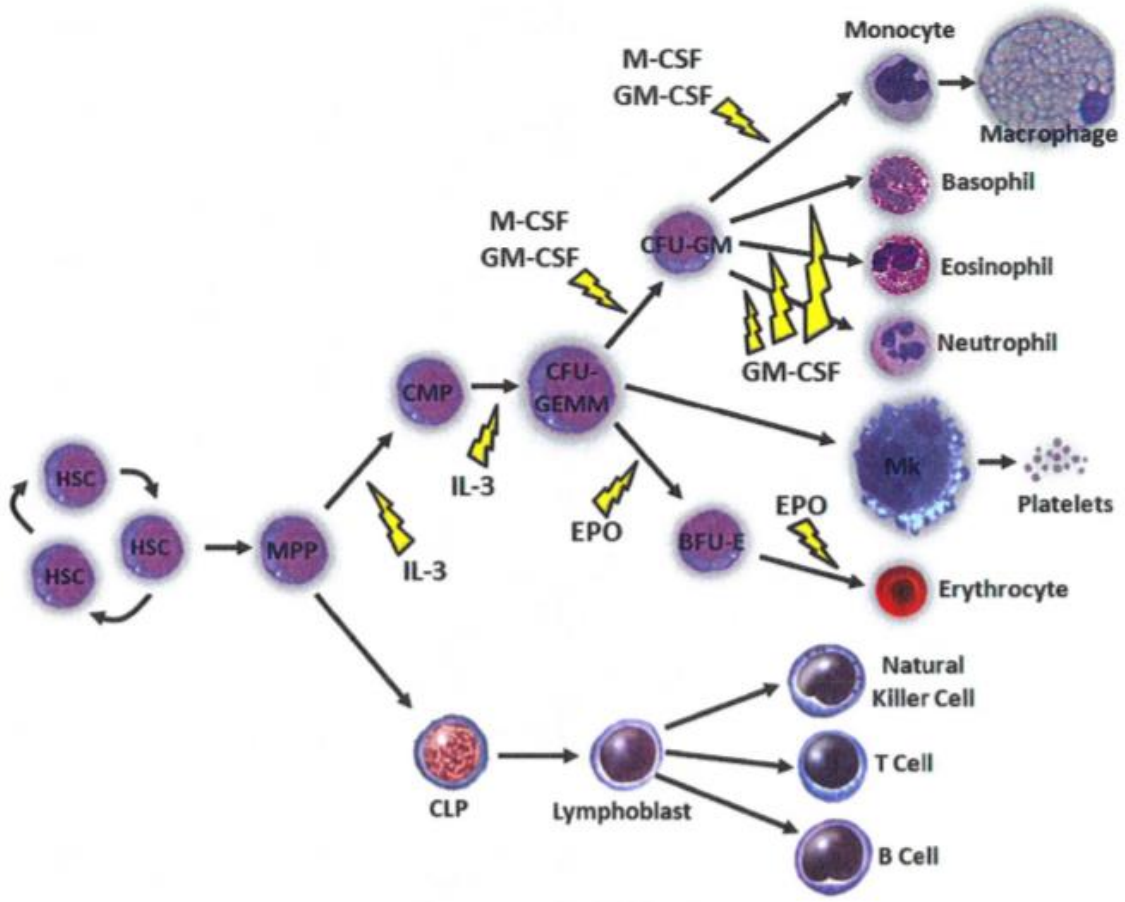

Figure 1: Hematopoiesis. Hematopoietic stem cells (HSC) differentiate into multipotent progenitors (MPP). These cells further differentiate into committed myeloid (CMP) and committed lymphoid progenitors (CLP). Hematopoietic growth factor involvement in this process is indicated.

Hematopoiesis has two distinct developmental phases - embryonic and adult. HSC are one of the first cell types to develop during embryogenesis (55). Cell niche shuffling characterizes embryonic

hematopoiesis. Cells from the developing mesoderm migrate to the posterior regions of the primitive streak. These cells become the first hematopoietic and endothelial stem cells in the developing yolk sac (56). Blood islands, multiple foci of primitive erythroid cells, begin to form on the yolk sac. Once the circulatory system develops, these blood islands give rise to primitive erythrocytes that are able to enter into circulation (55). As development continues, the yolk sac also begins to produce cells of white blood lineages and starts to release them into circulation (57). These circulating embryonic hematopoietic cells then begin to populate the fetal liver where they engraft and develop into adult hematopoietic stem cells (55). Colonization of the spleen with adult hematopoietic stem cells soon follows. Hematopoietic cells persist in these environments temporarily and soon begin to shift to the bone marrow cavities as they form (56). By birth, the majority of hematopoiesis shifts from the fetal liver and spleen to the bone marrow cavity. During post embryonic development the liver almost entirely loses its ability to serve as a hematopoietic organ (56). The spleen retains some hematopoietic capabilities during post embryonic development and, in some situations, can act as a secondary organ of hematopoiesis during adulthood.

1.3 – Hematopoietic Stem Cells

Hematopoietic homeostasis is maintained by self-renewal, proliferation, and differentiation of hematopoietic stem and progenitor cells. These cells exist on a spectrum from quiescent multipotent cells with a high proliferative capacity to actively proliferating unipotent cells that have an increasing likelihood of terminal differentiation. While a single more “primordial” HSC is capable of reconstituting the entire hematopoietic system of a hematopoietically impaired mouse (58), the majority of “regular” more differentiated HSC are unable to do this. In more realistic cases of bone marrow or peripheral blood mononuclear cell transplant, a spectrum of HSC are needed for successful reconstitution; lineage committed progenitors that immediately produce terminally differentiated blood cells, lineage committed HSC that can produce lineage committed progenitors, and true multipotent HSC that that can give rise to lineage committed HSC (59).

Early HSC are capable of giving rise to progenitor cells for both myeloid and lymphoid cell lineages (**Figure 1**). In mice, these can be classified as lineage negative, Sca1 positive, c-Kit positive (LSK) HSC using flow cytometry (60). In humans, CD34 positive CD38 negative cells, again measured with flow cytometry, are suggested to fill this role. Other CD34 positive cell types in humans, co-identified with additional CD markers, represent more committed progenitors. Cobblestone area forming cells, measured using cell culture, are similar to LSK and $CD34^{+}/CD38^{-}$ cells in their capacities (61). As these cells differentiate, they become more lineage restricted; committed myeloid progenitors, giving rise to cells only of the myeloid lineage, and lymphoid progenitors, giving rise to cells only of the lymphoid lineage. Colony forming unit granulocyte erythrocyte monocyte megakaryocyte (CFU-GEMM) cells, measured using cell culture assays, are known as common myeloid progenitors (62). As CFU-GEMM differentiate, lineage restriction continues until only unipotent cell types remain; CFU granulocyte monocyte (CFU-GM), CFU-G, CFU-M, burst forming unit erythroid (BFU-E), and CFU-E. BFU-E and CFU-E, for example, only give rise to cells of the erythroid lineage and are distinct from one another in their proliferative capacity (63).The colony forming unit moniker that these cells have been dubbed with speak to their functionality: single cells, capable of giving rise to a large colony of terminally differentiated cells, which are then identified in culture based on morphological characteristics. Aside from the cell types mentioned, many other hematopoietic progenitors exist. Lymphoid progenitors pertaining to this thesis are discussed in more detail in Chapter 3.

1.4 – Hematopoietic Stem Cell Niche

In order for HSC to function correctly they require an appropriate microenvironment. This is called the HSC niche. The primary hematopoietic niche in adult mammalian organisms is the bone marrow cavity. Within bone marrow, multiple cell types form a microenvironment that supports HSC function (**Figure 2**). These cell types allow for HSC attachment to a physical matrix and provide cues needed for quiescence, self-renewal, proliferation, differentiation, mobilization from the niche, and homing back to the niche (64). In bone marrow, two general niche domains exist; the endosteal niche and

the vascular niche. HSC are not stably attached to their niche, it is estimated in mice that 1-5% of the total pool of HSC enter circulation each day (65). These cells continually mobilize from their niche, circulate throughout the body, home back to their niche, and re-engraft. Outside of bone marrow, putative HSC niche structures have been identified but are less well understood than those in bone marrow.

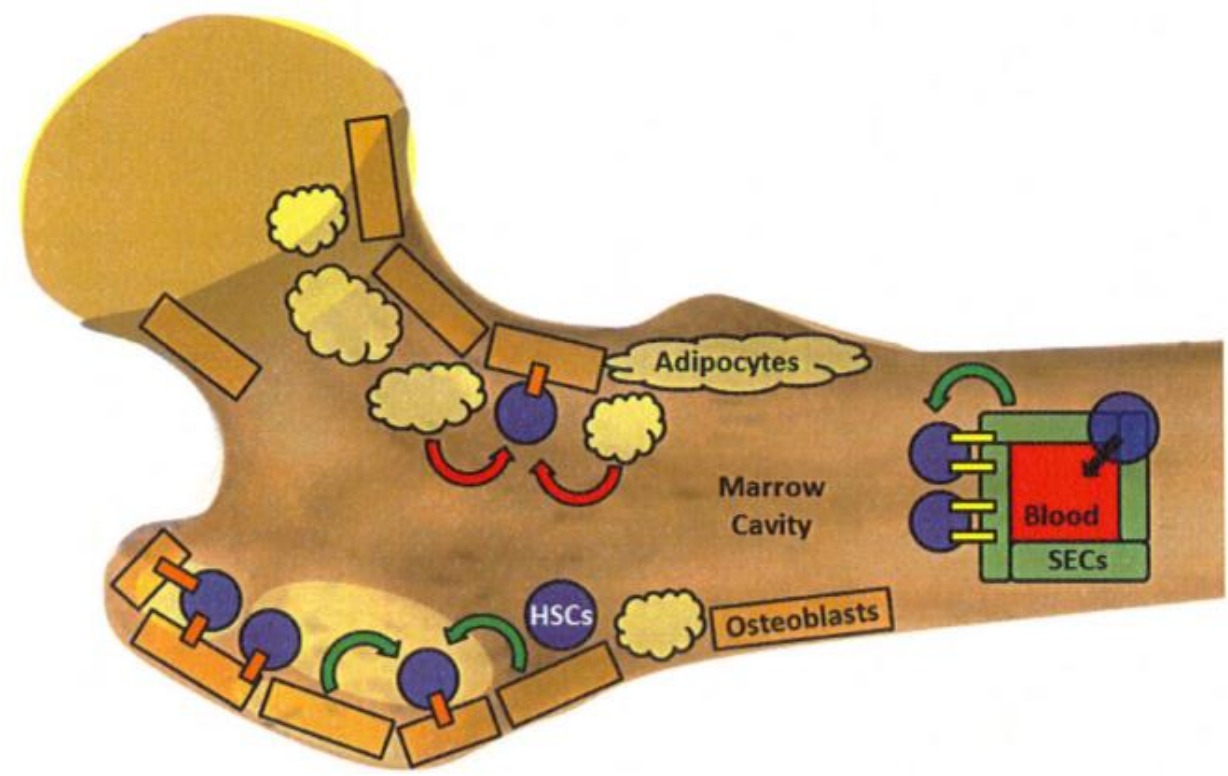

Figure 2: The Bone Marrow Hematopoietic Niche. The endosteal niche is made of osteoblast cell types, lining the endosteum, and sinusoidal endothelial cell types (SEC), adjacent to capillaries. These cells form attachments to hematopoietic stem cells (HSC) (yellow and orange indicators). Adipocytes can exert negative paracrine influences on HSC (red arrows), while osteoblasts and endothelial cells can exert positive influences through physical attachment and paracrine signalling. HSC are able to enter circulation through the vascular niche.

1.4.1 – Endosteal Hematopoietic Niche

The endosteal niche occupies the space immediately adjacent to bone. HSC that reside here physically attach to osteoblasts that line the endosteum (66). They receive physical signals from these cells, through adhesion molecules and other cell to cell receptor contacts, and also receive paracrine signals from these cells, in the form of cytokines and growth factors (55). Osteoclast type cells in this

environment have been reported to aid in release of HSC from the endosteal niche and HSC mobilization (67). HSC associated with the endosteal niche tend to be quiescent, long lasting, multipotent cell types with high proliferative capacities (66). Ablation of osteoblasts from the bone marrow cavity reduces the number of HSC found in the endosteal niche (68). Conversely, increasing the number of osteoblasts in the bone marrow increases the number of hematopoietic cells found there (69). Changes to mesenchymal stem cell (MSC) differentiation potential, the cell type that gives rise to osteoblasts, adipocytes, and other cell types within the bone marrow cavity, also has a direct impact on HSC number (70). Controlling bone marrow osteoblast content, or increasing MSC osteogenesis potential, is a probable means of increasing bone marrow hematopoietic cell content.

1.4.2 – Vascular Hematopoietic Niche

HSC and endothelial cells are highly related; hematopoiesis and vascularization occur concurrently during development and HSC and endothelial cells both form from a common progenitor, the hemangioblast (71). In adult bone marrow, HSC form close associations with endothelial cells that make up the sinusoidal endothelial vessels within the marrow cavity (55). Located throughout bone marrow around blood vessel structures, these associations are known as the vascular hematopoietic niche. There are vertebrate species, such as zebrafish, where hematopoiesis does not occur in association with bone. In these species all hematopoietic cells are associated with endothelial cells (66). HSC in association with the vascular niche tend to be actively undergoing self-renewal, proliferation, and differentiation, in contrast to the quiescent HSC found in the endosteal niche (72). These cells are also more likely to mobilize into circulation and return back to the marrow cavity, given their localization next to sinusoidal blood vessels (73). Just as with the endosteal niche, endothelial cells regulate hematopoietic cells through physical attachment and paracrine signalling (73). Similar to osteoblasts in the endosteal niche, deletions of surface proteins that attach hematopoietic and endothelial cells together results in large scale ablation of bone marrow cellularity (66). As well, infusion of endothelial progenitor cells brings

about increased hematopoietic reconstitution in lethally irradiated animals (74), indicating the importance of endothelial cells in supporting marrow hematopoiesis.

1.4.3 – Bone Marrow Adipose Tissue

Early in development, bone marrow is filled with HSC and hematopoietic cells. With aging, adipose tissue in marrow accumulates until it accounts for 50-70% of the bone marrow volume in healthy adult humans (75). So called "red marrow" is highly cellular and hematopoietically active, while yellow adipocyte rich marrow contains much less hematopoietic tissue and is relatively inactive (76). Marrow MSC can undergo adipogenesis, osteogenesis, as well as chondrogenesis (77). It is thought that MSC adipogenesis competes with osteogenesis within marrow. Indeed, increases bone marrow adipose tissue often coincides with decreases in bone mass (75). Stimuli that tend to promote adipogenesis will repress osteogenesis, and stimuli that promote osteogenesis will supress adipogenesis (78,79). In relation to hematopoietic function, adipocytes act as negative regulators of hematopoiesis. Mice with genetically or pharmacologically impaired adipogenesis show accelerated hematopoietic recovery after bone marrow ablation (80). Adipocytes are also unable to maintain HSC survival and self-renewal (81). As well, marrow adipocytes release factors that directly interfere with hematopoietic function (82). Thus, as when impairing adipogenesis through genetic or pharmacological means, interventions that reduce bone marrow adiposity tend to increase bone marrow hematopoietic cell content (23).

1.4.4 – Extramedullary Hematopoiesis

Hematopoiesis in the spleen still occurs at some level during adulthood, especially in mice (83). In the spleen, myeloid HSC reside within the red pulp and lymphoid HSC, that form immune cells, reside within the white pulp. When hematopoiesis in bone marrow is impaired by a medical condition, with aging, loss of blood, or when a haematological disorder develops, extramedullary hematopoiesis (EMH) is often upregulated in the spleen, and on rare cases, in the liver (84,85). In this way, the spleen serves as a backup hematopoietic organ. It is thought that this is because the spleen contains a niche like structure to support the development of HSC (86,87). What this niche looks like precisely, and what cell types it is

composed of, is still poorly understood. A vascular like niche within the spleen composed of endothelial cells is one promising candidate (87), although many cell types found in the spleen have been shown to support hematopoiesis to some extent. Expression of hematopoietic homing and growth factors is also noted in the spleen (88). Further discussion regarding EMH is found in Chapter 4.

1.5 – Hematopoietic Growth Factors

Aside from an appropriate niche structure, HSC require many growth factors and signalling molecules in order to differentiate and function correctly (**Figure 1**). These factors can be released by cell types residing directly in the bone marrow niche, by hematopoietic or immune cells, or into circulation from various tissues and organs. For example, specific control of erythropoiesis is highly dependent on EPO, which is released and moderated by the kidney, while broad control of hematopoiesis is dependent on IL3, which is released by immune cells and various other cell types within bone marrow (89). Families of hematopoietic cytokines include the interleukins (ILs), the colony stimulating factors (CSFs), chemokines (CCL and CXCL), and interferons (IFNs). There are also singular cytokines that are of great importance to hematopoiesis; EPO, thrombopoietin (TPO), Flt3 ligand (Flt3L), and stem cell factor (SCF) are amongst the most vital. These factors control everything; HSC quiescence, self-renewal, proliferation, differentiation, mobilization, and homing. Synergistic application of growth factors can combinatorially influence hematopoietic cells and result in stronger cell responses (90). Addition of exogenous factors can drastically change hematopoietic homeostasis: G-CSF can greatly increase HSC content in blood by mobilizing these cells from bone marrow (91), while EPO can specifically increase red cell content in blood by increasing erythropoiesis (92). In general, the serum profile of an individual, with hematopoietic cytokine and growth factor concentrations tightly balanced and precisely controlled, gives the body exacting control over the process of hematopoiesis (89). Understanding changes and alterations in hematopoiesis is often predicated upon understanding stimuli that change the expression and release of hematopoietic growth factors. More in depth discussion of hematopoietic factors relevant to this thesis are found in the following chapters.

1.6 – Hematopoietic Health

Given the massive turnover that hematopoietic cells undergo on a daily basis, hematopoiesis must be precisely controlled and highly regulated. Failures in regulation result in perturbations in HSC function, typically followed by severe health consequences. Several types of hematopoietic disorders exist, broad examples include: leukemias, myeloproliferative disorders, lymphomas, anemias, and immune deficiencies. A simplistic categorization can be used to describe most disorders – too much blood cell production or not enough blood cell production. Chronic myeloid or myelogenous leukemia, for example, involves deregulation of a myeloid progenitor or progenitors, in most cases through a genetic mutation, which results in over proliferation and mass production of myeloid cells (93). In contrast, aplastic anemia is categorized by the inability of the body to produce enough blood cells, often caused by immune cell mediated destruction of bone marrow and HSC (94). Of course, not all hematopoietic disorders can be characterized by too many or too few hematopoietic cells. Sickle cell anemia, for example, is characterized by a mutated form of hemoglobin that distorts red blood cell shape and reduces red blood cell functionality (95).

Treatment of many hematological disorders necessitates temporary replenishment or permanent replacement of malfunctioning cell types. Where anemias are concerned, regular blood transfusions can alleviate most symptoms. Healthy transfused cells temporarily assume the function of unhealthy cells. However, transfusions need be regular: terminally differentiated transfused cells will slowly die off and cannot be naturally replaced. This is because no healthy HSC exist in these disorders to replenish them. In myeloproliferative disorders where malfunctioning HSC are endangering an individual due to mass proliferation, more drastic treatments are needed. These malfunctioning HSC must be fully or partially eliminated and subsequently replaced. Here, a bone marrow or peripheral blood mononuclear cell (PBMC) transplant is required. First, the recipient undergoes partial or full myeloablative conditioning with chemotherapy and/or radiation. New and healthier HSC are then transplanted in order to reconstitute

the hematopoietic system of the recipient. The reconstituted hematopoietic system of the recipient should then, hopefully, be free of malignant HSC and only contain normally functioning HSC.

Rather than collection and transplantation of bone marrow directly, hematopoietic transplantation today is done through collection of HSC circulating in peripheral blood. In its natural state, bone marrow is a much richer source of HSC than peripheral blood. In order to collect sufficient numbers of HSC from peripheral blood, as certain threshold doses of HSC are required for a successful transplantation, HSC must first be mobilized into circulation. Mobilization is done through pharmacological means. Typically, multiple injections of G-CSF, a hematopoietic growth factor that is also a potent mobilizing agent, are given to an individual across the course of several days. When enough HSC are found to be in circulation, peripheral blood is collected and processed before being introduced to a recipient.

1.7 – Questions and Themes in Exercise and Hematopoiesis

Aerobic exercise leads to adaptations that increase the ability of the body to transport oxygen. Among these known adaptations, changes in hematopoietic cell content have been observed. Further study has demonstrated that both exercise training and acute exercise can influence hematopoiesis and HSC in more meaningful ways. As well, exercise has been shown to influence many systems that have a direct effect on hematopoiesis. Below, a short list summarizes available knowledge on the subject.

Exercise training has been shown to:

- Increase hematocrit and blood hemoglobin content (8)
- Increase skeletal muscle expression of hematopoietic growth factors (23)
- Reduce kidney EPO expression (23)
- Increase bone marrow HSC content (23)
- Increase circulating HSC levels (23)
- Increase the survival of exercise trained recipients post bone marrow transplant (96)
- Decrease marrow adiposity (23)

- Shift MSC differentiation towards osteogenesis and away from adipogenesis (23)
- Increase innate immune function (52)

Acute exercise has been shown to:

- Temporarily increase HSC mobilization to blood (51,97)
- Increase skeletal muscle expression of factors associated with hematopoiesis (30)
- Change spleen expression of factors associated with hematopoiesis (97)

Despite this summary, many questions remain. By what mechanism or mechanisms is exercise able to affect these changes? Does exercise affect any additional components of the hematopoietic system? How do disease states or age play a role in the ability of exercise to exert these changes? Is the fact that exercise can exert these changes useful or practical in any way? When pondering these questions, and the above summary of results, several themes emerge.

The first theme involves the ability of exercise to change expression of hematopoietic associated factors in tissue types throughout the body – exercise changing the secretome. Be it skeletal muscle, spleen, kidney, or when measured in blood, exercise changes expression of factors associated with hematopoietic development. How precisely does it do this though? No compelling explanations have been given. No mechanistic data has been presented. This is a severe limitation, as hematopoietic growth factors play a very important role in the regulation of the hematopoietic system.

The second theme involves the ability of exercise to effect changes in hematopoietic niche microenvironments – exercise altering the hematopoietic niche. Mainly in bone, by reducing marrow adiposity and increasing MSC osteoblast differentiation potential, but also in spleen, increasing spleen expression of factors associated with migration and homing. It has been suggested that changes in the structure of the bone marrow niche microenvironment could be a factor of the mechanical forces present during exercise. More information is presented on this in Chapter 5. As well, changes in the niche microenvironment could be a function of changes in the secretome following exercise; exercise leading to

release of factors that trigger niche redevelopment. Again, information regarding the mechanisms by which exercise changes these niche microenvironments is severely lacking.

The third theme involves increased HSC content in the bone marrow of exercise trained animals – exercise increasing HSC content. More than likely, the ability of exercise to increase HSC content in trained animals is a function of the first and second themes mentioned above. Exercise, somehow, changes the hematopoietic niche microenvironment to make it more suitable for HSC or to make more niche space available. Exercise also modifies the secretome, somehow, to promote HSC growth and development. Together, this leads to increased bone marrow HSC content following exercise. However, the questions posed above still remain: how does exercise trigger these changes?

The fourth theme involves the ability of exercise to increase circulating levels of HSC, either temporarily following acute exercise or at a new steady state level following an exercise training program. Increases in circulating levels of HSC following exercise could be a function of changes in the secretome, with exercise increasing the availability of factors that increase HSC egress from the hematopoietic niche. Temporary mobilization following acute exercise could certainly be explained by this. Increased mobilization could also be associated with the ability of exercise training to increase HSC content – all HSC leave their niche and mobilize on a regular basis, the more that are available the more that will be found in circulation. Again, very few studies have been performed on the ability of exercise to mobilize HSC to blood, especially regarding mechanistic information. Even the intensities of exercise, and kinetics of mobilization following exercise, are poorly documented. Finally, is the ability of exercise to mobilize HSC useful in a clinical setting? While some studies have identified the general quantity of HSC mobilized by exercise, no studies have looked at the ability of exercise to mobilize specific populations of HSC that are often associated with a successful PBMC graft.

1.8 – Purposes and Hypotheses

Hematopoiesis is controlled by the state of the various hematopoietic niche microenvironments throughout the body. Hematopoiesis is also controlled by the expression and production levels of

hematopoietic growth factors, released by multiple tissue and cell types. Finally, the availability and character of HSC within the body is directly relevant to hematopoietic function. In order to understand how exercise is able to influence hematopoiesis, an understanding of how exercise influences these variables is needed. Therefore, the studies included in this thesis are focused on these variables and examine the themes discussed above:

Chapter 2 evaluates the ability of exercise to regulate hematopoietic growth factor expression; specifically, the regulation of EPO expression in skeletal muscle.

- Expression of EPO in the kidney is regulated by hypoxia. Exercise is known to decrease skeletal muscle oxygen content. In this study, it should be expected that regulation of EPO expression will be mediated by skeletal muscle oxygen concentration and HIF1α and HIF2α stability.

Chapter 3 determines the ability of acute exercise to mobilize HSC from bone marrow to blood and the properties of exercise that are required for this to occur.

- Based on the literature available, moderate to low intensity exercise has not been reported to mobilize HSC into circulation. This could be a coincidence of study design or a physiological truth. Here, it is expected that only intense exercise will be able to mobilize HSC. As well, given that pharmacological mobilization regimes are based on a build-up of mobilizing agent within the body, cessation of activity should quickly result in a decrease in the number of mobilized cells.

Chapter 4 gauges the ability of exercise to upregulate splenic extramedullary hematopoiesis and examines how changes in the spleen, resultant from exercise, may allow for this.

- Endothelial cells, much like with the vascular niche in bone marrow, have been associated with the putative splenic hematopoietic niche. Additionally, exercise can decrease blood and skeletal muscle oxygen content, promote skeletal muscle angiogenesis and endothelial cell proliferation, and increase circulating levels of pro angiogenic factors such as VEGF. Based on this, it is expected that the endothelial hematopoietic niche in the spleen may be impacted by exercise. As

well, the ability of exercise to increase circulating HSC and endothelial progenitor cells may lead to seeding of the spleen with these cell types.

Chapter 5 examines bone marrow cellularity following exercise in two different marrow cavity locations, one of which should not experience significant mechanical stress during exercise.

- Mechanical stresses have been shown to increase osteoblast proliferation, increase MSC osteogenesis, and supress MSC adipogenesis. During treadmill running exercise in mice, mechanical stress is transmitted through appendicular bones but is not felt in the axial skeleton. In this study, it is expected that the mechanical forces felt during exercise should lead to the development of the endosteal niche in appendicular bones, increasing their cellularity. Conversely, as the vertebrae in the spine should not be subjected to the same level of mechanical stress, vertebral cellularity should not increase.

Finally, **Chapter 6** draws conclusions together from all of these studies in an examination of the ability of exercise to exert multiple stimuli that influence hematopoiesis in a global fashion.

1.9 – References

1. Brown WJ, Bauman AE, Owen N. Stand up, sit down, keep moving: turning circles in physical activity research? Br J Sports Med. 2009 Feb;43(2):86–8.

2. Egger GJ, Vogels N, Westerterp KR. Estimating historical changes in physical activity levels. Med J Aust. 2001 Dec 3;175(11–12):635–6.

3. Barnes AS. Obesity and Sedentary Lifestyles. Tex Heart Inst J. 2012;39(2):224–7.

4. Knight JA. Physical Inactivity: Associated Diseases and Disorders. Ann Clin Lab Sci. 2012 Jun 20;42(3):320–37.

5. Chirchir H, Kivell TL, Ruff CB, Hublin J-J, Carlson KJ, Zipfel B, et al. Recent origin of low trabecular bone density in modern humans. Proc Natl Acad Sci U S A. 2015 Jan 13;112(2):366–71.

6. Ekenman I, Eriksson SA, Lindgren JU. Bone density in medieval skeletons. Calcif Tissue Int. 1995 May;56(5):355–8.

7. Knuttgen HG. Strength training and aerobic exercise: comparison and contrast. J Strength Cond Res. 2007 Aug;21(3):973–8.

8. El-Sayed MS, Ali N, El-Sayed Ali Z. Haemorheology in exercise and training. Sports Med Auckl NZ. 2005;35(8):649–70.

9. Papa EV, Dong X, Hassan M. Resistance training for activity limitations in older adults with skeletal muscle function deficits: a systematic review. Clin Interv Aging. 2017;12:955–61.

10. Blomqvist CG, Saltin B. Cardiovascular adaptations to physical training. Annu Rev Physiol. 1983;45:169–89.

11. Zelis R. Human circulation: Regulation during physical stress by Lonng B. Rowell Oxford University press, New York (1986) 416 pages, illustrated, $39.95 isbn: 019-5040759. Clin Cardiol. 1987 Aug 1;10(8):432–4.

12. Chlif M, Chaouachi A, Ahmaidi S. Effect of Aerobic Exercise Training on Ventilatory Efficiency and Respiratory Drive in Obese Subjects. Respir Care. 2017 Jul;62(7):936–46.

13. Guazzi M, Reina G, Tumminello G, Guazzi MD. Improvement of alveolar-capillary membrane diffusing capacity with exercise training in chronic heart failure. J Appl Physiol Bethesda Md 1985. 2004 Nov;97(5):1866–73.

14. Laughlin MH, Roseguini B. Mechanisms for exercise training-induced increases in skeletal muscle blood flow capacity: differences with interval sprint training versus aerobic endurance training. J Physiol Pharmacol Off J Pol Physiol Soc. 2008 Dec;59 Suppl 7:71–88.

15. Dempsey JA, Fregosi RF. Adaptability of the pulmonary system to changing metabolic requirements. Am J Cardiol. 1985 Apr 26;55(10):59D–67D.

16. Holloszy JO. Regulation by exercise of skeletal muscle content of mitochondria and GLUT4. J Physiol Pharmacol Off J Pol Physiol Soc. 2008 Dec;59 Suppl 7:5–18.

17. Response to exercise after bed rest and after training. - PubMed - NCBI [Internet]. [cited 2017 Jul 10]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/5696236

18. Schmidt W, Prommer N. Impact of alterations in total hemoglobin mass on VO 2max. Exerc Sport Sci Rev. 2010 Apr;38(2):68–75.

19. Convertino VA. Blood volume: its adaptation to endurance training. Med Sci Sports Exerc. 1991 Dec;23(12):1338–48.

20. Joyner M. VO2MAX, blood doping, and erythropoietin. Br J Sports Med. 2003 Jun;37(3):190–1.

21. Heinicke K, Prommer N, Cajigal J, Viola T, Behn C, Schmidt W. Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man. Eur J Appl Physiol. 2003 Feb;88(6):535–43.

22. Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. Am J Physiol Renal Physiol. 2010 Jul;299(1):F1-13.

23. Baker JM, De Lisio M, Parise G. Endurance exercise training promotes medullary hematopoiesis. FASEB J Off Publ Fed Am Soc Exp Biol. 2011 Dec;25(12):4348–57.

24. Elliott S. Erythropoiesis-stimulating agents and other methods to enhance oxygen transport. Br J Pharmacol. 2008 Jun;154(3):529–41.

25. Telford RD, Sly GJ, Hahn AG, Cunningham RB, Bryant C, Smith JA. Footstrike is the major cause of hemolysis during running. J Appl Physiol Bethesda Md 1985. 2003 Jan;94(1):38–42.

26. Mairbäurl H. Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. Front Physiol. 2013;4:332.

27. Mairbäurl H, Humpeler E, Schwaberger G, Pessenhofer H. Training-dependent changes of red cell density and erythrocytic oxygen transport. J Appl Physiol. 1983 Nov;55(5):1403–7.

28. So B, Kim H-J, Kim J, Song W. Exercise-induced myokines in health and metabolic diseases. Integr Med Res. 2014 Dec 1;3(4):172–9.

29. Pedersen BK. Muscles and their myokines. J Exp Biol. 2011 Jan 15;214(Pt 2):337–46.

30. Carson BP. The Potential Role of Contraction-Induced Myokines in the Regulation of Metabolic Function for the Prevention and Treatment of Type 2 Diabetes. Front Endocrinol. 2017;8:97.

31. Catoire M, Mensink M, Kalkhoven E, Schrauwen P, Kersten S. Identification of human exercise-induced myokines using secretome analysis. Physiol Genomics. 2014 Apr 1;46(7):256–67.

32. Henningsen J, Rigbolt KTG, Blagoev B, Pedersen BK, Kratchmarova I. Dynamics of the Skeletal Muscle Secretome during Myoblast Differentiation. Mol Cell Proteomics. 2010 Nov 1;9(11):2482–96.

33. Janssen I, Heymsfield SB, Wang Z, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. J Appl Physiol. 2000 Jul 1;89(1):81–8.

34. Margeli A, Skenderi K, Tsironi M, Hantzi E, Matalas A-L, Vrettou C, et al. Dramatic Elevations of Interleukin-6 and Acute-Phase Reactants in Athletes Participating in the Ultradistance Foot Race Spartathlon: Severe Systemic Inflammation and Lipid and Lipoprotein Changes in Protracted Exercise. J Clin Endocrinol Metab. 2005 Jul 1;90(7):3914–8.

35. Thijssen DHJ, Maiorana AJ, O'Driscoll G, Cable NT, Hopman MTE, Green DJ. Impact of inactivity and exercise on the vasculature in humans. Eur J Appl Physiol. 2010 Mar;108(5):845–75.

36. Agarwal SK. Cardiovascular benefits of exercise. Int J Gen Med. 2012 Jun 22;5:541–5.

37. Fagard RH, Cornelissen VA. Effect of exercise on blood pressure control in hypertensive patients. Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol. 2007 Feb;14(1):12–7.

38. McInnis KJ. Exercise and obesity. Coron Artery Dis. 2000 Mar;11(2):111–6.

39. Colberg SR, Sigal RJ, Fernhall B, Regensteiner JG, Blissmer BJ, Rubin RR, et al. Exercise and Type 2 Diabetes. Diabetes Care. 2010 Dec;33(12):e147–67.

40. Francois ME, Little JP. Effectiveness and Safety of High-Intensity Interval Training in Patients With Type 2 Diabetes. Diabetes Spectr Publ Am Diabetes Assoc. 2015 Jan;28(1):39–44.

41. Naseeb MA, Volpe SL. Protein and exercise in the prevention of sarcopenia and aging. Nutr Res N Y N. 2017 Apr;40:1–20.

42. Gregory SM, Parker B, Thompson PD. Physical Activity, Cognitive Function, and Brain Health: What Is the Role of Exercise Training in the Prevention of Dementia? Brain Sci. 2012 Nov 29;2(4):684–708.

43. Ploughman M, Granter-Button S, Chernenko G, Attwood Z, Tucker BA, Mearow KM, et al. Exercise intensity influences the temporal profile of growth factors involved in neuronal plasticity following focal ischemia. Brain Res. 2007 May 30;1150:207–16.

44. van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A. 1999 Nov 9;96(23):13427–31.

45. Lawson EC, Han MK, Sellers JT, Chrenek MA, Hanif A, Gogniat MA, et al. Aerobic Exercise Protects Retinal Function and Structure from Light-Induced Retinal Degeneration. J Neurosci. 2014 Feb 12;34(7):2406–12.

46. Han C, Ding D, Lopez M-C, Manohar S, Zhang Y, Kim M-J, et al. Effects of Long-Term Exercise on Age-Related Hearing Loss in Mice. J Neurosci Off J Soc Neurosci. 2016 Nov 2;36(44):11308–19.

47. Schubert CR, Cruickshanks KJ, Nondahl DM, Klein BEK, Klein R, Fischer ME. Association of exercise with lower long-term risk of olfactory impairment in older adults. JAMA Otolaryngol--Head Neck Surg. 2013 Oct;139(10):1061–6.

48. Crane JD, MacNeil LG, Lally JS, Ford RJ, Bujak AL, Brar IK, et al. Exercise-stimulated interleukin-15 is controlled by AMPK and regulates skin metabolism and aging. Aging Cell. 2015 Aug;14(4):625–34.

49. Shanb AA, Youssef EF. The impact of adding weight-bearing exercise versus nonweight bearing programs to the medical treatment of elderly patients with osteoporosis. J Fam Community Med. 2014;21(3):176–81.

50. Al-Zahrani MS, Borawski EA, Bissada NF. Increased physical activity reduces prevalence of periodontitis. J Dent. 2005 Oct;33(9):703–10.

51. Morici G, Zangla D, Santoro A, Pelosi E, Petrucci E, Gioia M, et al. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. Am J Physiol - Regul Integr Comp Physiol. 2005 Nov 1;289(5):R1496–503.

52. Woods JA, Vieira VJ, Keylock KT. Exercise, inflammation, and innate immunity. Neurol Clin. 2006 Aug;24(3):585–99.

53. Fliedner TM, Graessle D, Paulsen C, Reimers K. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. Cancer Biother Radiopharm. 2002 Aug;17(4):405–26.

54. Silva AS, Anderson ARA, Gatenby RA. A multiscale model of the bone marrow and hematopoiesis. Math Biosci Eng MBE. 2011 Apr;8(2):643–58.

55. Huang X, Cho S, Spangrude GJ. Hematopoietic stem cells: generation and self-renewal. Cell Death Differ. 2007 Nov;14(11):1851–9.

56. Tavassoli M. Embryonic and fetal hemopoiesis: an overview. Blood Cells. 1991;17(2):269-281; discussion 282-286.

57. Palis J, Yoder MC. Yolk-sac hematopoiesis: the first blood cells of mouse and man. Exp Hematol. 2001 Aug;29(8):927–36.

58. Boggs DR, Boggs SS, Saxe DF, Gress LA, Canfield DR. Hematopoietic Stem Cells with High Proliferative Potential. J Clin Invest. 1982 Aug;70(2):242–53.

59. Zhang M, Huang B. The multi-differentiation potential of peripheral blood mononuclear cells. Stem Cell Res Ther. 2012 Nov 30;3(6):48.

60. Bryder D, Rossi DJ, Weissman IL. Hematopoietic Stem Cells. Am J Pathol. 2006 Aug;169(2):338–46.

61. Cobblestone area-forming cells, long-term culture-initiating cells and NODSCID repopulating cells in human neonatal blood: a comparison with umbilical cord blood [Internet]. [cited 2017 Jul 12]. Available from: http://www.nature.com/bmt/journal/v30/n9/full/1703714a.html

62. Zucali JR, Broxmeyer HE, Dinarello CA, Gross MA, Weiner RS. Regulation of early human hematopoietic (BFU-E and CFU-GEMM) progenitor cells in vitro by interleukin 1-induced fibroblast-conditioned medium. Blood. 1987 Jan;69(1):33–7.

63. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995 Oct 6;83(1):59–67.

64. Zhang J, Niu C, Ye L, Huang H, He X, Tong W-G, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003 Oct 23;425(6960):836–41.

65. Bhattacharya D, Czechowicz A, Ooi AGL, Rossi DJ, Bryder D, Weissman IL. Niche recycling through division-independent egress of hematopoietic stem cells. J Exp Med. 2009 Nov 23;206(12):2837–50.

66. Kiel MJ, Morrison SJ. Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol. 2008 Apr;8(4):290–301.

67. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med. 2006 Jun;12(6):657–64.

68. Bowers M, Zhang B, Ho Y, Agarwal P, Chen C-C, Bhatia R. Osteoblast ablation reduces normal long-term hematopoietic stem cell self-renewal but accelerates leukemia development. Blood. 2015 Apr 23;125(17):2678–88.

69. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003 Oct 23;425(6960):841–6.

70. Muruganandan S, Sinal CJ. The impact of bone marrow adipocytes on osteoblast and osteoclast differentiation. IUBMB Life. 2014 Mar 17;

71. Jaffredo T, Nottingham W, Liddiard K, Bollerot K, Pouget C, de Bruijn M. From hemangioblast to hematopoietic stem cell: an endothelial connection? Exp Hematol. 2005 Sep;33(9):1029–40.

72. Yin T, Li L. The stem cell niches in bone. J Clin Invest. 2006 May 1;116(5):1195–201.

73. Kopp H-G, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiol Bethesda Md. 2005 Oct;20:349–56.

74. Salter AB, Meadows SK, Muramoto GG, Himburg H, Doan P, Daher P, et al. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. Blood. 2009 Feb 26;113(9):2104–7.

75. Cawthorn WP, Scheller EL. Editorial: Bone Marrow Adipose Tissue: Formation, Function, and Impact on Health and Disease. Front Endocrinol [Internet]. 2017 [cited 2017 Jul 12];8. Available from: http://journal.frontiersin.org/article/10.3389/fendo.2017.00112/full

76. Meunier P, Aaron J, Edouard C, Vignon G. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. Clin Orthop. 1971 Oct;80:147–54.

77. Kokabu S, Lowery JW, Jimi E. Cell Fate and Differentiation of Bone Marrow Mesenchymal Stem Cells. Stem Cells Int. 2016;2016:3753581.

78. Hardouin P, Rharass T, Lucas S. Bone Marrow Adipose Tissue: To Be or Not To Be a Typical Adipose Tissue? Front Endocrinol [Internet]. 2016 [cited 2017 Jul 12];7. Available from: http://journal.frontiersin.org/article/10.3389/fendo.2016.00085/full

79. Fu L, Tang T, Miao Y, Zhang S, Qu Z, Dai K. Stimulation of osteogenic differentiation and inhibition of adipogenic differentiation in bone marrow stromal cells by alendronate via ERK and JNK activation. Bone. 2008 Jul 1;43(1):40–7.

80. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature. 2009 Jul 9;460(7252):259–63.

81. Corre J, Barreau C, Cousin B, Chavoin J-P, Caton D, Fournial G, et al. Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. J Cell Physiol. 2006 Aug;208(2):282–8.

82. Belaid-Choucair Z, Lepelletier Y, Poncin G, Thiry A, Humblet C, Maachi M, et al. Human bone marrow adipocytes block granulopoiesis through neuropilin-1-induced granulocyte colony-stimulating factor inhibition. Stem Cells Dayt Ohio. 2008 Jun;26(6):1556–64.

83. Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF. Roles of spleen and liver in development of the murine hematopoietic system. Exp Hematol. 2002 Sep;30(9):1010–9.

84. Galanello R, Origa R. Beta-thalassemia. Orphanet J Rare Dis. 2010 May 21;5:11.

85. Kim CH. Homeostatic and pathogenic extramedullary hematopoiesis. J Blood Med. 2010;1:13–9.

86. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. Blood. 2015 Apr 23;125(17):2621–9.

87. Inra CN, Zhou BO, Acar M, Murphy MM, Richardson J, Zhao Z, et al. A perisinusoidal niche for extramedullary haematopoiesis in the spleen. Nature. 2015 Nov 26;527(7579):466–71.

88. Yamamoto K, Miwa Y, Abe-Suzuki S, Abe S, Kirimura S, Onishi I, et al. Extramedullary hematopoiesis: Elucidating the function of the hematopoietic stem cell niche (Review). Mol Med Rep. 2016 Jan;13(1):587–91.

89. Metcalf D. Hematopoietic cytokines. Blood. 2008 Jan 15;111(2):485–91.

90. Koller MR, Bender JG, Papoutsakis ET, Miller WM. Effects of synergistic cytokine combinations, low oxygen, and irradiated stroma on the expansion of human cord blood progenitors. Blood. 1992 Jul 15;80(2):403–11.

91. Philpott NJ, Prue RL, Marsh JC, Gordon-Smith EC, Gibson FM. G-CSF-mobilized CD34 peripheral blood stem cells are significantly less apoptotic than unstimulated peripheral blood CD34 cells: role of G-CSF as survival factor. Br J Haematol. 1997 Apr;97(1):146–52.

92. Citartan M, Gopinath SCB, Chen Y, Lakshmipriya T, Tang T-H. Monitoring recombinant human erythropoietin abuse among athletes. Biosens Bioelectron. 2015 Jan 15;63:86–98.

93. Pierce A, Spooncer E, Ainsworth S, Whetton AD. BCR-ABL alters the proliferation and differentiation response of multipotent hematopoietic cells to stem cell factor. Oncogene. 2002 May 2;21(19):3068–75.

94. Young NS, Scheinberg P, Calado RT. APLASTIC ANEMIA. Curr Opin Hematol. 2008 May;15(3):162–8.

95. Roseff SD. Sickle cell disease: a review. Immunohematology. 2009;25(2):67–74.

96. De Lisio M, Baker JM, Parise G. Exercise promotes bone marrow cell survival and recipient reconstitution post-bone marrow transplantation, which is associated with increased survival. Exp Hematol. 2013 Feb;41(2):143–54.

97. Emmons R, Niemiro GM, Owolabi O, De Lisio M. Acute exercise mobilizes hematopoietic stem and progenitor cells and alters the mesenchymal stromal cell secretome. J Appl Physiol Bethesda Md 1985. 2016 Mar 15;120(6):624–32.

Chapter 2 – Skeletal Muscle Erythropoietin Expression Is Responsive to Hypoxia and Exercise

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Abstract

Purpose: Erythropoietin is responsible for regulating the growth and development of red blood cells. Reports conflict on whether skeletal muscle is able to produce erythropoietin and release it into circulation and if exercise affects this. We set out to determine how erythropoietin is regulated in skeletal muscle and to determine whether skeletal muscle-derived erythropoietin can stimulate erythropoiesis.

Methods: Using an *in vitro* approach, we exposed proliferating and differentiated skeletal muscle cells to various forms of exercise-induced physiological stimuli and measured erythropoietin gene expression. To understand if skeletal muscle cells were able to stimulate erythropoiesis, independent of other cell types found in skeletal muscle, we used myoblast-conditioned media to treat bone marrow and to measure erythropoiesis through flow cytometry. We also measured erythropoietin expression and hypoxia in mice subjected to an exercise protocol designed to induce skeletal muscle oxygen stress.

Results: Hypoxia increased erythropoietin expression in C2C12 myoblasts, myotubes, and primary myoblasts *in vitro* by 50% to 130%. Bone marrow treated with media conditioned with hypoxic myoblasts for 24 h increased the number of Ter-119-positive cells by 32%. An erythropoietin-neutralizing antibody prevented this increase. Compared with unexercised controls, exhaustive exercise increased skeletal muscle HIF1α levels by 50% and HIF2α levels by 20%. Moreover, exercised skeletal muscle erythropoietin expression was 70% higher.

Conclusion: These results demonstrate that skeletal muscle produces erythropoietin in a hypoxia and HIF-dependent manner and that hypoxia-treated muscle is capable of stimulating erythropoiesis *in vitro*.

Introduction

Produced primarily by the kidney, erythropoietin (EPO) is known for its role in regulating erythropoiesis. EPO production is controlled by HIF1 and HIF2 availability. Under normoxic conditions HIF1α and HIF2α are ubiquitinated and degraded in an oxygen-dependent manner. During periods of hypoxia, interstitial fibroblasts in the kidney sense oxygen deficiency and stabilize these isoforms. They then heterodimerize with HIF1β, translocate to the nucleus, bind the hypoxia responsive element on the EPO promoter, and upregulate expression of EPO (9,21). Once produced and released into circulation, EPO interacts with the EPO receptor on immature erythrocyte precursors and promotes their survival and maturation (7). In a negative feedback loop, this increases red blood cell number, alleviates tissue hypoxia, and increases HIFα degradation, which reduces EPO expression. Through this mechanism, the kidney and the EPO serve as a sensor, maintaining oxygen homeostasis.

Because of the central role played by EPO and erythropoiesis in oxygen absorption, transportation, and utilization, many attempts have been made to determine whether exercise can increase circulating EPO and stimulate erythropoiesis, as exercise is strongly limited by these factors. The majority of studies conclude that, across a variety of types, durations, and intensities of exercise, increases in circulating EPO concentrations are not associated with exercise (4,12,13,19,24,25). However, when exercise is combined with hypoxia, or severe hypoxemia is induced by exercise, circulating concentrations of EPO increase (22,24). Very long-term and damaging exercise, capable of inducing hemolysis, has also been shown to increase circulating EPO and erythropoiesis (27).

Skeletal muscle experiences severe oxygen stress during exercise. The tissue adapts by stabilizing HIF1α, resulting in subsequent increases in oxidative capacity and angiogenesis, in an attempt to restore oxygen balance (17). As might be expected from this pathway, skeletal muscle has also been shown to express EPO. Not only that, exercise has been shown to increase EPO expression in skeletal muscle. Rundqvist et al. (23) had 10 males perform 65 min of cycle exercise. Although they were not able to demonstrate an increase in EPO expression post exercise, they were able to observe an increase in the

arteriovenous difference of circulating EPO. Ameln et al. (2) had males perform 45 min of one-legged knee extension exercise while blood flow to the leg was either restricted or left unrestricted. In both conditions, they were able to observe increased expression of skeletal muscle EPO 360 min post exercise. Finally, previous work from our laboratory demonstrated that in mice, treadmill trained on a regular basis for 2 months, basal skeletal muscle EPO expression was greater than sedentary controls. Perplexingly, EPO expression in the kidneys of treadmill trained mice was decreased when compared with sedentary controls (3).

Is it possible that skeletal muscle, in conjunction with exercise, is able to express EPO at physiologically relevant levels? While it seems that total serum EPO may not increase as a result of the exercise training, skeletal muscle, in conjunction with the kidney, may still be able to contribute to the EPO pool. Given these previous findings, we set out to understand how exercise and skeletal muscle contribute to the production of EPO, if myoblasts and myotubes themselves express EPO, how the process might be regulated, and whether any of this is able to exert a physiological effect on erythropoiesis.

Methods

Animals

Adult male C57Bl/6 mice (Jackson Laboratories), age 5 wk, were exercised. No more than 5 mice were housed per cage (27 × 12 × 15.5 cm). Animals were provided food and water *ad libitum*. Mice were maintained on a 12-h light–12-h dark cycle at 22°C ± 2°C. Ethics approval was granted by the McMaster University Animal Research Ethics Board and conformed to the guidelines of the Canadian Council on Animal Care.

Animal Exercise

Mice were exercised (n = 8) on an Exer 6 M Treadmill (Columbus Instruments Inc., Columbus, OH). The mice were allowed to acclimatize to the treadmill a week before exercise. The acute exercise protocol

used consisted of a 10-min warm-up at 12 m·min−1 followed by a 1-h exercise period at 16 m·min−1. Mice were encouraged to run using a mild electric shock. Resting control mice (n = 8) were exposed to the treadmill and similar encouragements on the same days as exercised mice but were not exercised.

Animal Sacrifice and Tissue Collection

Resting and exercised mice were killed immediately after their exercise period. Mice that were unable to continue were killed at once. Mice were euthanized via cervical dislocation. Lower-leg muscle groups were isolated and flash frozen in liquid nitrogen. This dissection was completed within 1 to 2 min to account for the short half life of HIF1α/2α (8). An additional group of resting mice (n = 6) were used for the erythropoiesis assay. These mice were euthanized via cervical dislocation, and both femurs and tibiae were excised, dissected of muscle and fat, and flushed with IMDM (Sigma-Aldrich, St. Louis, MO) with 2% fetal bovine serum (FBS) (Life Technologies Inc., Rockville, MD) to collect bone marrow. Mononuclear cells were then isolated with Ficoll-Paque (GE Healthcare Life Sciences, Mississauga, ON, Canada).

Cell Culture

C2C12 myoblasts (cat no. CRL-1772, ATCC) were grown at 37°C in 5% CO2 in growth media (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin; Life Technologies Inc.). Myoblasts were differentiated by switching to differentiation media (DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin; Life Technologies Inc.) for 5 d. Regular DAPI (Life Technologies) staining was performed to rule out any mycoplasma contamination.

***In Vitro* Model of Exercise**

Proliferating or differentiated C2C12 myoblasts (n = 8) were exposed to various physiological stimuli known to occur during aerobic type exercise. RNA was isolated after exposure to each condition. These stimuli are discussed in the following sections.

Mechanical stretch

Proliferating C2C12 myoblasts or terminally differentiated C2C12 myotubes were cultured on type I collagen-coated flexible-bottom plates (Flexcell International, Hillsborough, NC) in growth media or differentiation media, respectively. Proliferating cells were allowed to adhere for 12 h, whereas myotubes were induced to fuse for 7 d before mechanical stimulation. The stretch protocol, previously tested for this cell line (10), consisted of cyclic strain at 0.1 Hz (8 s of stretch alternated with 2 s of rest) of a 20% strain for a period of 4 h. Stretch was applied using the FX-4000™ Tension Plus™ (Flexcell International), a vacuum stretch apparatus coupled with computer software. Myoblasts and myotubes cultured in a similar fashion, but not exposed to mechanical stimulation, served as controls.

Hypoxia

Proliferating C2C12 myoblasts or terminally differentiated myotubes were exposed to 1% O2 and 5% CO2 for 4 h. This is a common treatment for this cell line, and no death was observed during this treatment (14). Proliferating C2C12 myoblasts or terminally differentiated myotubes that were cultured as normal served as controls. An enzyme-linked immunosorbent assay (ELISA; cat no. MEP00B, R&D Systems, Minneapolis, MN) was used to analyze EPO concentrations in normoxic and hypoxic-conditioned media for both proliferating C2C12 myoblasts and differentiated C2C12 myotubes, as well as uncultured growth and differentiation media.

Decreased pH

Proliferating C2C12 myoblasts or terminally differentiated C2C12 myotubes were exposed to regular growth media or differentiation media, respectively, at a pH of 6.6 for 4 h. This pH was chosen to mimic pH observed in muscle during exercise and recovery (1). No cell death was observed during this treatment. Regular growth or differentiation media (pH of 7.4) was used as a control.

Increased temperature

Proliferating C2C12 myoblasts or terminally differentiated C2C12 myotubes were exposed to a temperature of 41°C for 2 h. This is a mild, commonly used, heat shock protocol for this cell line, and no

cell death was observed during this treatment (15). Proliferating C2C12 myoblasts or terminally differentiated myotubes exposed grown at regular temperature (37°C) were used as a control.

Erythropoiesis Assay and Flow Cytometry

Proliferating C2C12 myoblasts in growth media were exposed to hypoxia for 4 h, as mentioned previously, and media from those cells were collected and filtered. Media from cells grown in typical conditions served as a control. Bone marrow, collected previously (n = 6), was then treated with either the hypoxic-conditioned or the control media and cultured at 37°C and 5% CO2 for 24 h or 72 h. Bone marrow cells were treated with or without an EPO-neutralizing antibody, at 0.5 μg·mL−1, and changed every 6 h, for the 24 h experiment (cat no. AF959, R&D Systems). Cells were then collected, washed, and stained as per manufacturer recommendation with TER-119 (cat no. 553672, BD Biosciences), a marker of murine hematopoietic cells in the erythroid lineage (11). After incubation with the primary antibody, cells were treated with a streptavidin FITC secondary antibody at 1:800 (cat no. SNN1008, Life Technologies), washed, and immediately analyzed by flow cytometry (Cyflow Space, Partec).

Reverse Transcriptase and Quantitative Polymerase Chain Reaction

Total RNA was isolated from exercised and resting gastrocnemius muscle and proliferating and differentiated C2C12 myoblasts using a combination TRIzol (Life Technologies Inc.) and total RNA kit (Omega Bio-Tek) method. RNA was reverse transcribed using a commercially available kit (high-capacity cDNA reverse transcription kit; Life Technologies Inc.) with a Mastercycler epGradient Thermal Cycler (Eppendorf Canada, Mississauga, Ontario). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) reactions were conducted using a Stratagene Mx3000P real-time PCR System (Agilent Technologies, Santa Clara, CA) or a Mastercycler epRealplex 2S qPCR (Eppendorf Canada) with epMotion 5075 (Eppendorf Canada). All samples were normalized to GAPDH, and fold changes in gene expression were calculated using the $\Delta\Delta Ct$ method (16). Some reports indicate that GAPDH is responsive to hypoxia and thus may not be an appropriate normalizing gene; however, there were no

observed differences in GAPDH expression in either skeletal muscle or cell lysate in any experimental condition. Previously published primer sequences were used for EPO (3).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

Muscle protein (50 μg per lane), isolated through homogenization of gastrocnemius samples in RIPA buffer (Sigma-Aldrich), was resolved using common sodium dodecyl sulfate–polyacrylamide gel electrophoresis conditions. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore). HIF1α was probed for with an HIF1α antibody (cat no. NB100-449, Novus Biologicals) at a concentration of 1:250 in 5% milk powder (Sigma-Aldrich). HIF2α was probed for with an HIF2α antibody (cat no. AF2997, R&D Systems) at 2 μg·mL−1 in 5% milk powder. In both cases, an actin antibody (cat no. A2066, Sigma-Aldrich) at 1:1000 in 5% milk powder was used as a loading control. An antirabbit horseradish peroxidase antibody (cat no. ab6721, Abcam) at 1:25,000 in 5% milk powder was used as a secondary antibody for HIF1α and actin, whereas an antigoat horseradish peroxidase antibody (cat no. A16142, Life Technologies Inc.) at 1:10,000 in 5% milk powder was used as a secondary antibody for HIF2α. Images were captured and analyzed with a FluorChem SP (ProteinSimple, San Jose, CA).

Proliferating Mouse Primary Myoblast EPO Expression

Five 8-wk-old C57Bl/6 mice (Jackson Laboratories) were euthanized via cervical dislocation. Primary myoblasts were isolated from the lower limb muscle of these mice using collagenase and dispase (20). Ham's F10 media (Sigma-Aldrich) supplemented with 20% FBS, 1% penicillin/streptomycin, and 2.5 ng·mL−1 bFGF (Life Technologies Inc.) was used to culture these cells. Myoblasts were purified to 99% positive using a Beckman Coulter MoFlo XDP with an α7-integrin antibody (cat no. K0046-5, MBL International), as described in more detail elsewhere (5). When the cells had grown to sufficient number, they were exposed to hypoxia for 4 h, RNA was isolated, and RT and qPCR on EPO was performed (same as previously mentioned). Normoxic primary myoblasts served as controls.

Statistics

Data are expressed as means ± SE with $P \leq 0.05$ considered significant. Statistical differences between resting and exercised groups were determined using two-tailed *t*-tests with SigmaPlot 11 (Systat Software). ELISA results are reported with standard deviation.

Results

EPO expression in myoblasts and myotubes is increased by hypoxia

Rundqvist et al. (23) previously demonstrated that skeletal muscle expresses EPO and can release it into circulation during exercise. However, the means by which exercise could stimulate skeletal muscle EPO production remained unknown. Moreover, whether skeletal muscle myoblasts and myotubes were responsible for expressing EPO, and not some other cell type within skeletal muscle, was also unclear. To determine how exercise might affect myoblast and myotube EPO expression, we tested these cell types isolated in culture against stimuli known to be associated with exercising muscle. When muscle is activated continuously a decrease in pH, an increase in temperature, exposure to hypoxia, and mechanical stretch all occur. Of the conditions tested, only hypoxia was able to stimulate an increase in EPO expression. In C2C12 myoblasts, exposure to 4 h of hypoxia increased EPO expression by 130% ($P < 0.05$) (Fig. 1A). In C2C12 myotubes, exposure to 4 h of hypoxia increased EPO expression by 50% ($P < 0.05$) (Fig. 1B). An ELISA was used to confirm the presence of EPO in the hypoxic-conditioned media. Hypoxic-conditioned growth and differentiation media contained 36.3 ± 12.1 pg·mL−1 and 39.7 ± 9.5 pg·mL−1 of EPO, respectively, while normoxic and control uncultured growth and differentiation media contained no detectable EPO ($P < 0.05$). No other stimuli were able to alter EPO expression in a significant manner.

EPO expression in proliferating primary myoblasts

C2C12 myoblasts are a transformed cell line, and some believe that they have gained multiple features generally not associated with skeletal muscle. To verify the physiological relevance of the C2C12

findings and to ensure that EPO expression was not such a feature, EPO expression was measured in proliferating primary myoblasts exposed to hypoxia. Proliferating primary myoblasts subjected to 4 h of hypoxia demonstrated a 128% ± 6.6% increase in EPO expression, when compared with normoxic proliferating primary myoblast controls ($P < 0.05$).

Exercise stabilizes HIFα isoforms and increases EPO expression in skeletal muscle

Given that skeletal muscle myoblasts and myotubes were able to express EPO directly, and did so in response to hypoxia, we set out to determine the mechanism by which this might occur. EPO expression is classically regulated in the kidney by HIFα isoform stabilization in response to hypoxia. Given that muscle also uses HIF1α and HIF2α to adapt to hypoxia, by increasing oxidative capacity and stimulating angiogenesis, EPO may be regulated in a similar fashion as well. To test this, we exercised mice until exhaustion and measured EPO expression and HIF1α and HIF2α levels in muscle. Confirming previously observed findings, exercise training was able to increase skeletal muscle expression of EPO by 70% ($P < 0.05$) (Fig. 2C). Moreover, HIF1α levels in exercised muscle were increased by 50% ($P < 0.05$) (Fig. 2A), and HIF2α levels were increased by 20% ($P < 0.05$) (Fig. 2B). Both HIFα isoforms were nearly undetectable in resting muscle (see Figure, Supplemental Digital Content 1, HIF1α western blot representative image, http://links.lww.com/MSS/A649; see Figure, Supplemental Digital Content 2, HIF2α western blot representative image, http://links.lww.com/MSS/A650).

Hypoxia can induce myoblasts to stimulate erythropoiesis

Previously our group had demonstrated that long-term exercise training increased basal levels of EPO expression in skeletal muscle (3). Moreover, we observed large increases in circulating erythrocyte progenitors after exercise. What remained unknown, however, was whether muscle itself, some other cell type within muscle, or some other tissue in general was responsible for this observation. To measure the capacity of skeletal muscle in isolation to stimulate erythropoiesis we exposed proliferating myoblasts to hypoxia and used the media from those cells to assay erythropoiesis in whole bone marrow. Ter-119 is a marker of murine hematopoietic cells in the erythroid lineage (11). We observed a 32% increase ($P <$

0.05) in the number of Ter-119-positive cells 24 h after incubation with media conditioned by hypoxic myoblasts (Fig. 3A). When an EPO-neutralizing antibody was added to the hypoxic media, this increase was completely blocked. In a separate experiment, we observed a 67% increase ($P < 0.05$) in the number of Ter-119-positive cells 72 h after incubation with media conditioned by hypoxic myoblasts (Fig. 3B) (see Figure, Supplemental Digital Content 3, 24 h Ter-119 flow cytometry representative plot, http://links.lww.com/MSS/A651; see Figure, Supplemental Digital Content 4, 72 h Ter-119 flow cytometry representative plot, http://links.lww.com/MSS/A652).

Discussion

In this study, we have shown that both myoblasts and differentiated myotubes express the hematopoietic cytokine EPO in vitro. Of all the stimuli tested, only hypoxia was able to alter EPO expression in these cell types (Fig. 1). Importantly, myoblasts exposed to hypoxia were able to produce EPO at physiologically meaningful levels; erythropoiesis was upregulated in bone marrow treated with media taken from hypoxic myoblasts, and this increase was ablated with the use of an EPO-neutralizing antibody (Fig. 3). Using an exhaustive aerobic exercise protocol to promote skeletal muscle hypoxia, we were able to confirm these findings in vivo. Increased skeletal muscle HIF1α and HIF2α levels were mirrored with increased skeletal muscle EPO expression (Fig. 2).

Very little is known about the ability of skeletal muscle to produce EPO. A handful of studies exist; however, the data are equivocal. Rundqvist et al. (23) were able to demonstrate that skeletal muscle expresses EPO and that an exercising leg can release EPO into blood. During recovery, they noted absorption of serum EPO into the exercised leg (23). Perplexingly, they were unable to detect changes in EPO expression or protein content within muscle after exercise. Schwandt et al. (1991) performed a similar study, measuring serum EPO levels in well-trained men before, during, and after a marathon run. They were able to measure an increase in serum EPO 3 h into the run, and impressively, higher than basal levels of EPO were measured 31 h after the run (26). Moreover, Ameln et al. (2) demonstrated increased skeletal muscle EPO expression post exercise. In a study by our group, where animals were exercised

trained on a treadmill several times a week for 12 wk, we were able to measure a 93% increase in basal skeletal muscle expression of EPO (3). Strangely, in the same animals, we were also able to measure a 22% decrease in basal kidney levels of EPO.

These studies seem to suggest that exercise can increase skeletal muscle EPO expression and release of EPO into circulation. The intensity and duration of exercise also seems to have an effect, with longer training periods or more intense bouts of exercise resulting in measureable increases in EPO expression. The addition of systemic hypoxia, or hypoxemia through intense exercise, also seems to promote skeletal muscle EPO expression. Several questions, however, still remained unanswered.

The first unknown is whether myoblasts and myotubes within skeletal muscle express EPO, or if some other cell type within skeletal muscle is responsible for the observed increases in expression. In the kidney a very specialized population of fibroblasts is responsible for EPO production. These cells are found in close association with peritubular capillaries of the nephron and as a result are able to closely monitor blood oxygen levels. When levels fall, the HIFα isoforms in these cells stabilize and initiate a signalling cascade that results in increased EPO expression. Similar to the kidney, myoblasts and myotubes are found in proximity to capillaries and rely on oxygen for their ability to provide energy. HIF1α and HIF2α in these cells are also stabilized in hypoxic conditions and can help the muscle adapt to hypoxia. Here we report that both C2C12 myoblasts and myotubes are directly able to express EPO (Fig. 1), as well as proliferating primary myoblasts.

Secondly, no attempts have been made to understand how the observed increases in skeletal muscle EPO expression are regulated. In several of the studies mentioned previously, only long-term training or very intense exercise was able to invoke increases in EPO expression. Given these discrepancies, a mechanistic approach was used to determine how exercise might increase EPO expression. To this end, we used known individual components of exercise and tested them in isolation. Performing the experiment in this fashion allowed us to maximally stress each potential pathway to elicit a result. Increased temperature, decreased pH, mechanical stretch, and hypoxia were used as each of these stimuli

is associated with exercise. Although this is a crude means of mimicking exercise in vitro, we feel that these conditions cover the majority of important physiological stimuli occurring during an exercise bout.

It was observed that only hypoxia was able to increase EPO expression in both C2C12 myoblasts and myotubes, by 130% (Fig. 1A) and 50% (Fig. 1B), respectively. Further testing revealed that hypoxia also increased EPO expression in proliferating primary myoblasts by 128%. The release of EPO from C2C12s was confirmed by an ELISA, demonstrating 36.3 ± 12.1 and 39.7 ± 9.5 pg·mL−1 EPO in the hypoxic proliferating and differentiated conditions, respectively, with no detectable EPO in any control condition. This result was not altogether surprising, given that hypoxia serves as the classical regulator of EPO expression in the kidney. This also falls in line with the previous exercise studies mentioned. Only very intense or very long-term exercise, with or without the addition of systemic or local hypoxia, was able to increase EPO expression. In these protocols of exercise, the muscle would become hypoxic, HIFα isoforms would stabilize, and increased EPO expression would be observed.

As EPO expression is regulated in the kidney by HIFα isoforms, we wanted to design an exercise protocol capable of inducing severe oxygen depletion in an exercising muscle. A protocol of this type should be able to promote hypoxia and to allow for the stabilization of skeletal muscle HIFα isoforms. To this end, we exercised animals for 1 h or until they were unable to continue. The animals were then killed immediately. Confirming the previously mentioned finding, and the findings of some of the previous studies, intense exercise was able to increase EPO expression (Fig. 2A) and was able to induce hypoxia severe enough to stabilize skeletal muscle HIF1α (Fig. 2A) and HIF2α levels (Fig. 2B). Given that only hypoxia was able to increase EPO expression in vitro and EPO expression was associated with an increase in HIF1α and HIF2α availability in vivo, we conclude that, similar to the kidney, EPO expression in skeletal muscle is regulated in a hypoxia and HIFα-dependent manner.

Finally, whether skeletal muscle expression of EPO serves a physiologically relevant purpose has thus far remained unknown. In the case of the human studies discussed previously, or our previous work where exercise was able to increase skeletal muscle expression of EPO and increase numbers of

circulating erythrocyte progenitors, any biological system responsive to exercise could have been the underlying mechanism responsible for these observations. No isolation of stimuli was possible. Although muscle expresses EPO and is responsive to exercise, nothing suggests that muscle-derived EPO is able to modulate erythropoiesis in any physiologically relevant manner. To answer this question, we analyzed the system independent of any other variables. C2C12 myoblasts were exposed to hypoxia, their media were collected and filtered, and that media were used to treat bone marrow isolated from healthy mice. In designing the experiment this way, the direct ability of myoblasts to stimulate erythropoiesis was tested. The result was a 32% increase in bone marrow erythropoiesis at 24 h (Fig. 3A) and a 67% increase at 72 h (Fig. 3B), measured by Ter-119 and analyzed with flow cytometry. Supporting this result, an EPO-neutralizing antibody completely ablated the observed increase at 24 h. Therefore, myoblasts exposed to hypoxia were directly able to stimulate erythropoiesis.

This result falls in line with findings from our previous study (3). In that study, we observed a basal increase in expression of skeletal muscle EPO, a basal decrease in expression of kidney EPO, and a higher steady state level of circulating erythropoietic progenitors. If the kidney-derived EPO had solely been responsible for regulating erythropoiesis, and muscle was unable to release EPO into circulation, we previously would have observed a decrease in circulating erythropoietic progenitors. These results, along with our other work demonstrating that exercise training can increase bone marrow transplant recipient survival (6), speak to the importance of exercise on the health of the hematopoietic system and the potential clinical importance of exercise on hematopoietic health. Our current results reinforce our previous theory; exercise and skeletal muscle can act to upregulate erythropoiesis and bolster hematopoiesis.

The ability of skeletal muscle to adapt to chronic hypoxia is a well-observed phenomenon. During periods of hypoxia, muscle can upregulate angiogenic factors such as VEGF, increasing capillarization to provide more oxygenated blood to the muscle. Moreover, muscle fiber type can shift, resulting in increased glycolytic capacity and decreased need for oxygen (18). Both these adaptations are mediated by

HIF1α. That exercising muscle can also use HIF1α and HIF2α to increase expression of EPO is not surprising. This only serves as another layer of adaptation, where muscle produces additional EPO, EPO stimulates erythropoiesis, and additional oxygen can be carried to the muscle by an increased number of erythrocytes. These results demonstrate that, together with the kidney, skeletal muscle may act as an atypical regulator of erythropoiesis.

2.1 – Figure 1

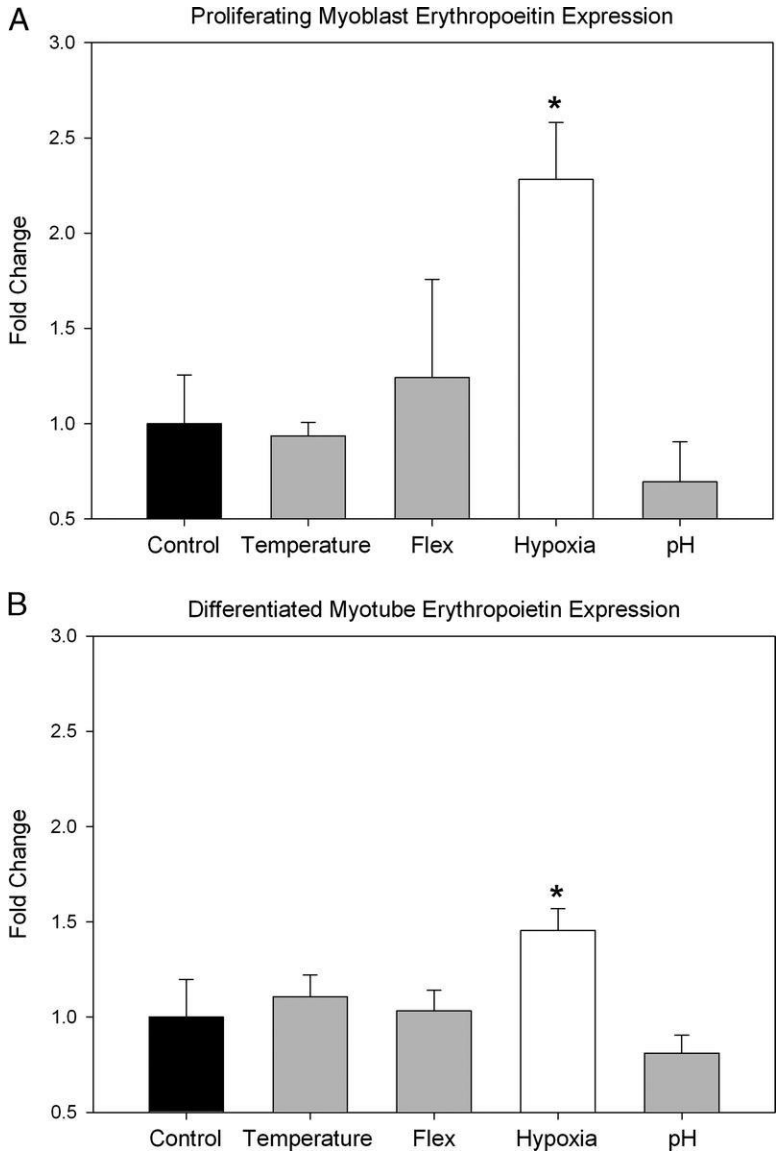


Figure 1: EPO expression is increased by hypoxia in myoblasts and myotubes. EPO expression in proliferating myoblasts and differentiated myotubes in response to increased temperature, mechanical stimulation, hypoxia, and decreased pH. In both proliferating myoblasts and differentiated myotubes, no stimulus other than hypoxia was able to elicit a change in EPO expression. A. In proliferating myoblasts, hypoxia increased EPO expression by 130% ($P < 0.05$). B. In differentiated myotubes, hypoxia increased EPO expression by 50% ($P < 0.05$). Data are presented as fold change from control.

2.2 – Figure 2

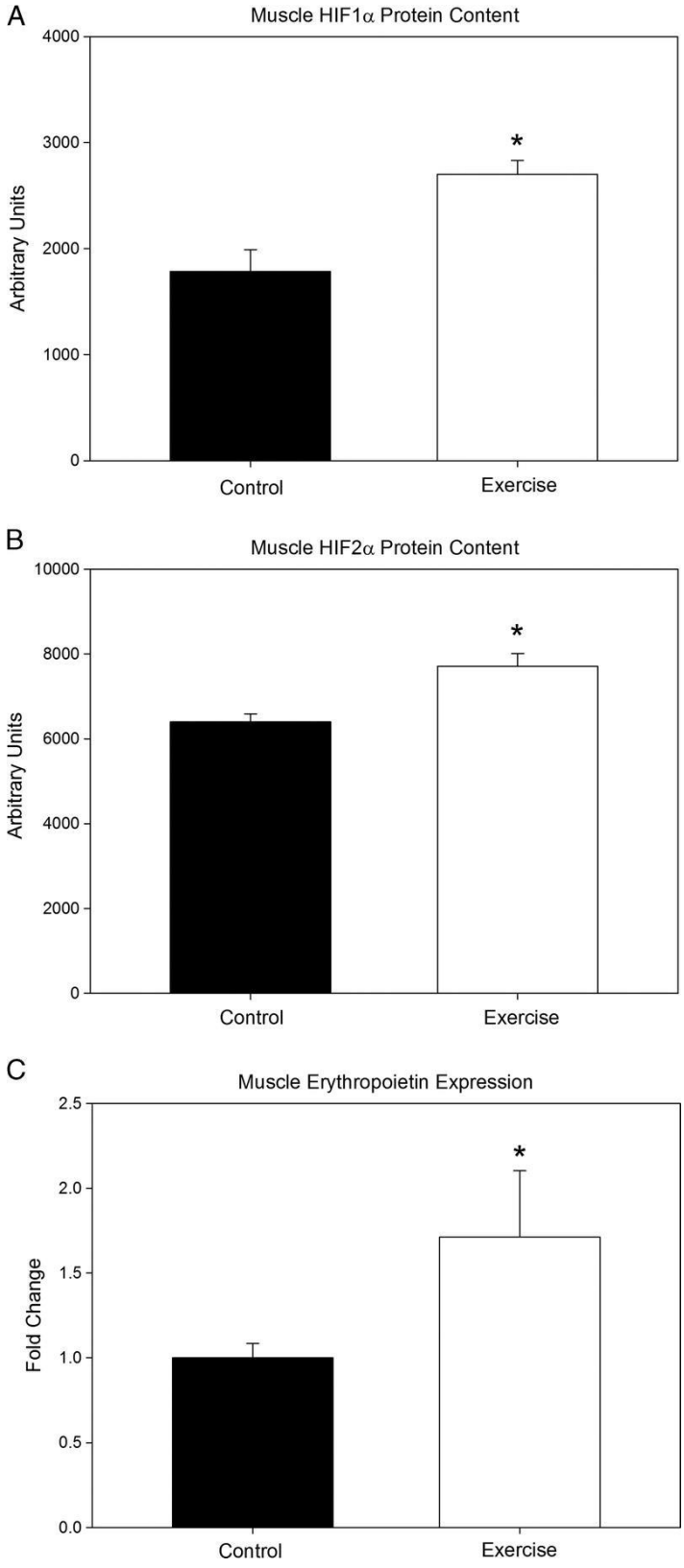

A
Muscle HIF1α Protein Content
Arbitrary Units
4000
3000
2000
1000
0
*
Control
Exercise
B
Muscle HIF2α Protein Content
Arbitrary Units
10000
8000
6000
4000
2000
0
*
Control
Exercise
C
Muscle Erythropoietin Expression
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Exercise

Figure 2: Effects of exercise on skeletal muscle EPO expression and HIFα levels. Effects of intense aerobic exercise on EPO expression and HIFα levels in skeletal muscle. Animals were exercised for 1 h or until exhaustion. A. HIF1α levels in the gastrocnemius were increased by 50% ($P < 0.05$); data are expressed as arbitrary units. B. HIF2α levels in the gastrocnemius were increased by 20% ($P < 0.05$); data are expressed as arbitrary units. C. EPO expression in the gastrocnemius was increased by 70% ($P < 0.05$); data are expressed as fold change from control.

2.3 – Figure 3

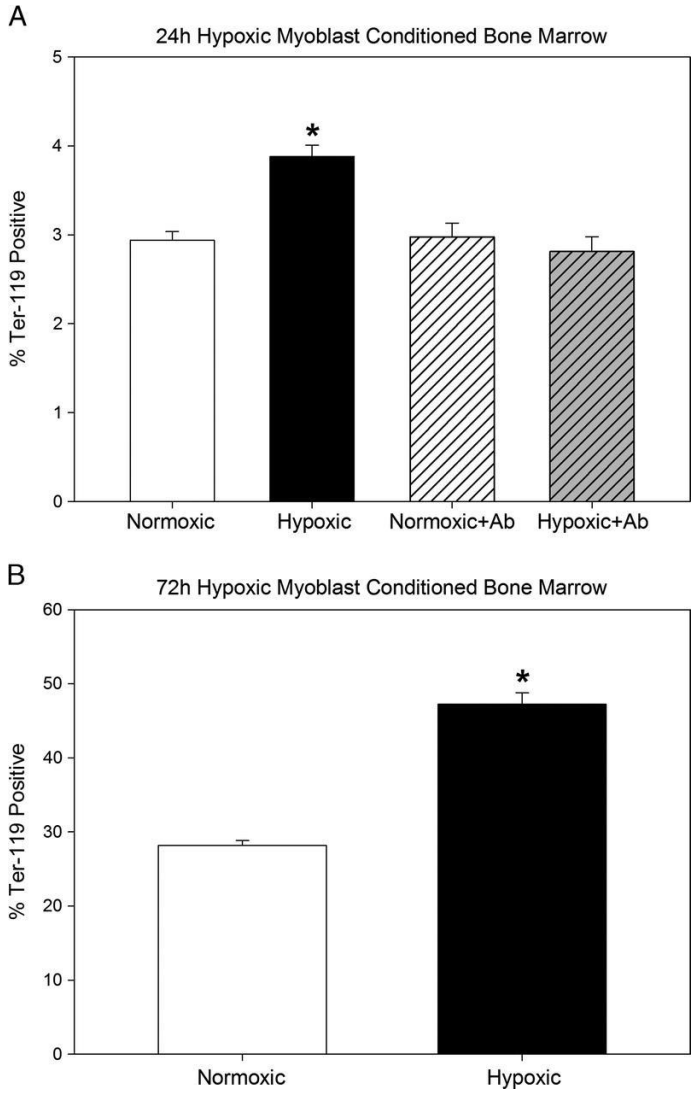
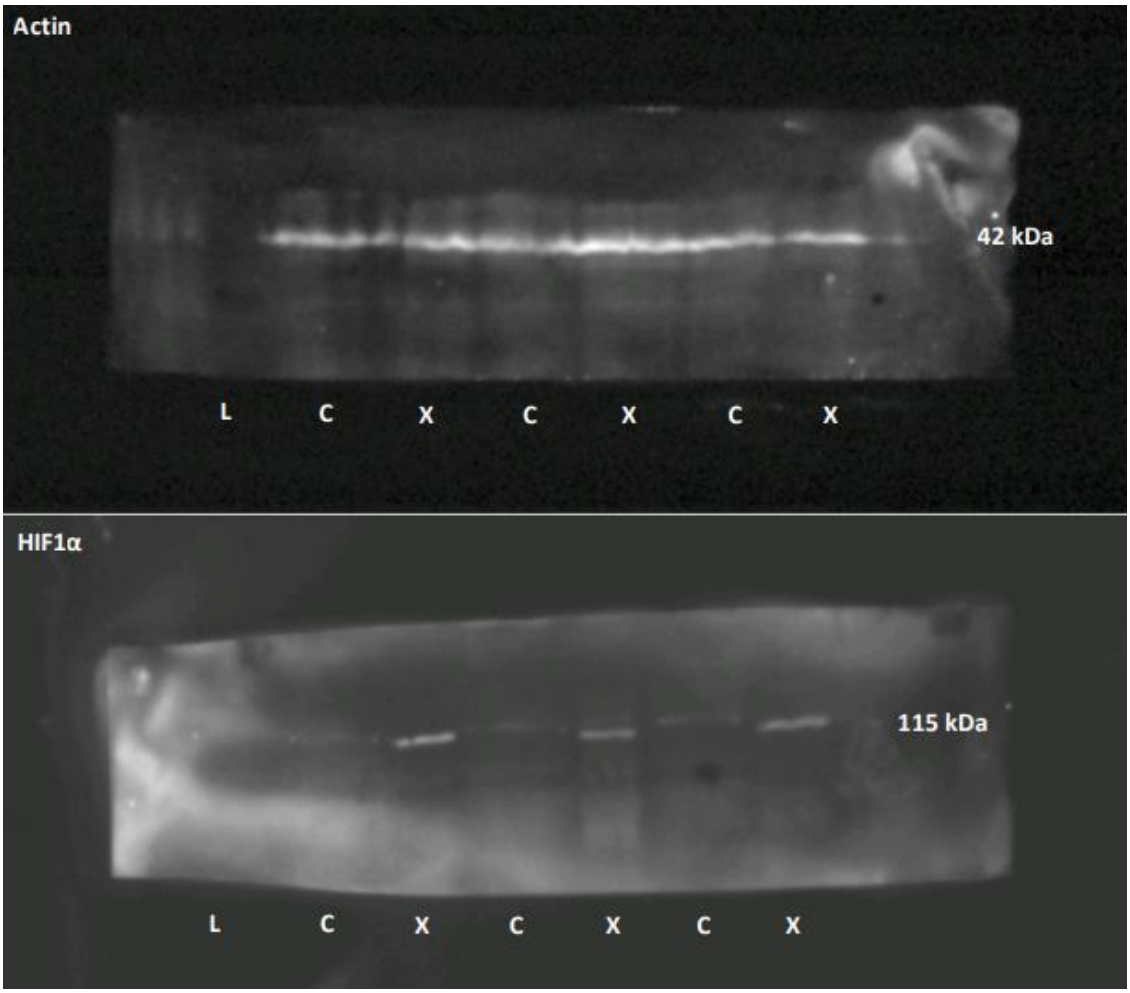


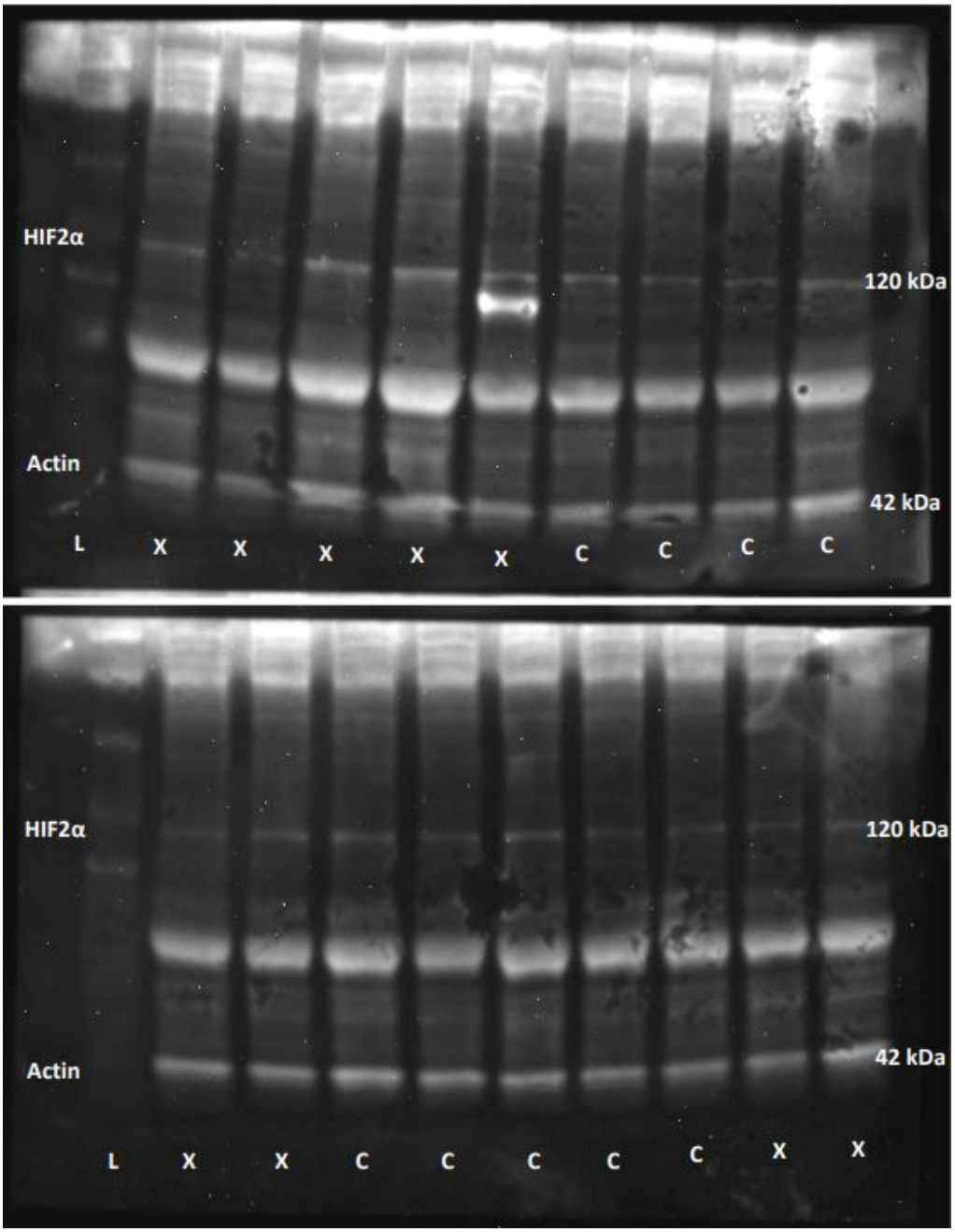
Figure 3: Effects of myoblast hypoxia on erythropoiesis. Effect of growth media conditioned with proliferating myoblasts exposed to hypoxia on bone marrow erythropoiesis. Bone marrow was treated for 24 and 72 h with conditioned media. Media from regular proliferating myoblasts served as a control. At 24 h, the experiment was performed with and without the use of an erythropoietin-neutralizing antibody. A. Hypoxic-conditioned media increased the number of cells in the erythroid lineage by 32% at 24 h ($P < 0.05$), and this increase was ablated with the neutralizing antibody; data are expressed as percent total Ter-119-positive cells. B. Hypoxic-conditioned media increased the number of cells in the erythroid lineage by 67% at 72 h ($P < 0.05$); data are expressed as percent total Ter-119-positive cells.

2.4 – Supplementary Figure 1



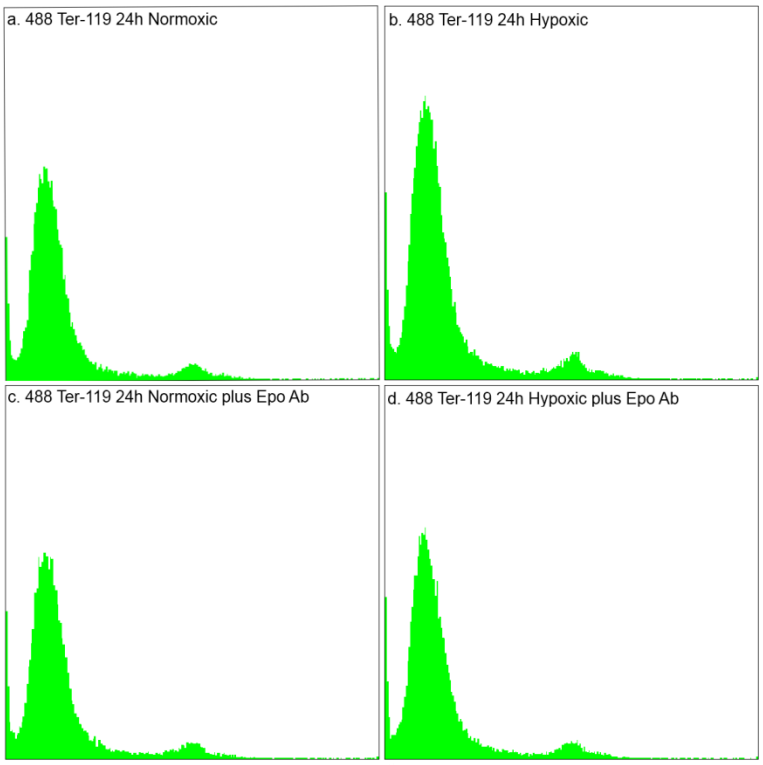
Supplementary Figure 1: Representative HIF1α blots. L=ladder, C=control, X=exercise.

2.5 – Supplementary Figure 2



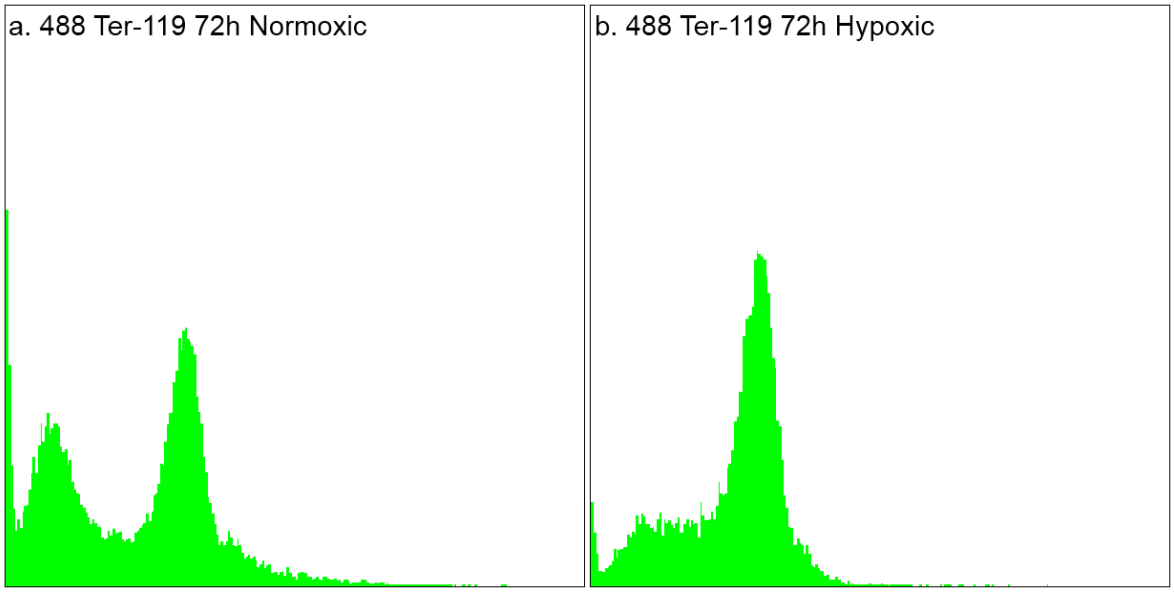
Supplementary Figure 2: Representative HIF2α blots. L=ladder, C=control, X=exercise.

2.6 – Supplementary Figure 3



Supplementary Figure 3: Representative flow plots for Ter119 positivity in bone marrow treated with media taken from cells in a normoxic or hypoxic atmosphere, with or without an added EPO neutralizing antibody.

2.7 – **Supplementary Figure 4**



Supplementary Figure 4: Representative flow plots for Ter119 positivity in bone marrow treated with media taken from cells in a normoxic or hypoxic atmosphere after 72 hours.

2.8 – References

1. Allsop P, Jorfeldt L, Rutberg H, Lennmarken C, Hall GM. Delayed recovery of muscle pH after short duration, high intensity exercise in malignant hyperthermia susceptible subjects. Br J Anaesth. 1991;66(5):541–5.

2. Ameln H, Gustafsson T, Sundberg CJ, et al. Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J. 2005;19(8):1009–11.

3. Baker JM, De Lisio M, Parise G. Endurance exercise training promotes medullary hematopoiesis. FASEB J. 2011;25(12):4348–57.

4. Berglund B, Birgegard G, Hemmingsson P. Serum erythropoietin in cross-country skiers. Med Sci Sports Exerc. 1988;20(2):208–9.

5. Blanco-Bose WE, Yao CC, Kramer RH, Blau HM. Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res. 2001;265(2):212–20.

6. De Lisio M, Baker JM, Parise G. Exercise promotes bone marrow cell survival and recipient reconstitution post-bone marrow transplantation, which is associated with increased survival. Exp Hematol. 2013;41(2):143–54.

7. Debeljak N, Sytkowski AJ. Erythropoietin and erythropoiesis stimulating agents. Drug Test Anal. 2012;4(11):805–12.

8. Elkins JM, Hewitson KS, McNeill LA, et al. Structure of factor-inhibiting hypoxia-inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1 alpha. J Biol Chem. 2003;278(3):1802–6.

9. Haase VH. Regulation of erythropoiesis by hypoxia-inducible factors. Blood Rev. 2013;27(1):41–53.

10. Johnston AP, Baker J, Bellamy LM, McKay BR, De Lisio M, Parise G. Regulation of muscle satellite cell activation and chemotaxis by angiotensin II. PLoS One. 2010;5(12):e15212.

11. Kina T, Ikuta K, Takayama E, et al. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. Br J Haematol. 2000;109(2):280–7.

12. Klausen T, Breum L, Fogh-Andersen N, Bennett P, Hippe E. The effect of short and long duration exercise on serum erythropoietin concentrations. Eur J Appl Physiol Occup Physiol. 1993;67(3):213–7.

13. Klausen T, Dela F, Hippe E, Galbo H. Diurnal variations of serum erythropoietin in trained and untrained subjects. Eur J Appl Physiol Occup Physiol. 1993;67(6):545–8.

14. Li W, Hu ZF, Chen B, Ni GX. Response of C2C12 myoblasts to hypoxia: the relative roles of glucose and oxygen in adaptive cellular metabolism. Biomed Res Int. 2013;2013:326346.

15. Liu CT, Brooks GA. Mild heat stress induces mitochondrial biogenesis in C2C12 myotubes. J Appl Physiol (1985). 2012;112(3):354–61.

16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25(4):402–8.

17. Mason S, Johnson RS. The role of HIF-1 in hypoxic response in the skeletal muscle. Adv Exp Med Biol. 2007;618:229–44.

18. Mason SD, Rundqvist H, Papandreou I, et al. HIF-1alpha in endurance training: suppression of oxidative metabolism. Am J Physiol Regul Integr Comp Physiol. 2007;293(5):R2059–69.

19. Morici G, Zangla D, Santoro A, et al. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. Am J Physiol Regul Integr Comp Physiol. 2005;289(5):R1496–503.

20. Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol. 1994;125(6):1275–87.

21. Ratcliffe PJ. HIF-1 and HIF-2: working alone or together in hypoxia? J Clin Invest. 2007;117(4):862–5.

22. Roberts D, Smith DJ. Erythropoietin concentration and arterial haemoglobin saturation with supramaximal exercise. J Sports Sci. 1999;17(6):485–93.

23. Rundqvist H, Rullman E, Sundberg CJ, et al. Activation of the erythropoietin receptor in human skeletal muscle. Eur J Endocrinol. 2009;161(3):427–34.

24. Schmidt W, Eckardt KU, Hilgendorf A, Strauch S, Bauer C. Effects of maximal and submaximal exercise under normoxic and hypoxic conditions on serum erythropoietin level. Int J Sports Med. 1991;12(5):457–61.

25. Schmidt W, Spielvogel H, Eckardt KU, Quintela A, Peñaloza R. Effects of chronic hypoxia and exercise on plasma erythropoietin in high-altitude residents. J Appl Physiol (1985). 1993;74(4):1874–8.

26. Schwandt HJ, Heyduck B, Gunga HC, Rocker L. Influence of prolonged physical exercise on the erythropoietin concentration in blood. Eur J Appl Physiol Occup Physiol. 1991;63(6):463–6.

27. Yusof A, Leithauser RM, Roth HJ, Finkernagel H, Wilson MT, Beneke R. Exercise-induced hemolysis is caused by protein modification and most evident during the early phase of an ultraendurance race. J Appl Physiol (1985). 2007;102(2):582–6.

Chapter 3 – Aerobic exercise in humans mobilizes HSCs in an intensity-dependent manner

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Abstract

Hematopoietic stem and progenitor cells are necessary to maintain, repair, and reconstitute the hematopoietic blood cell system. Mobilization of these cells from bone marrow to blood can be greatly increased under certain conditions, one such being exercise. The purpose of this study was to identify the importance of exercise intensity in hematopoietic mobilization, to better understand the mobilization kinetics post exercise, and to determine if exercise is capable of mobilizing several specific populations of hematopoietic cells that have clinical relevance in a transplant setting. Healthy individuals were exercised on a cycle ergometer at 70% of their peak work rate (WR_{peak}) until volitional fatigue and at 30% of their WR_{peak} work matched to the 70% WR_{peak} bout. Blood was collected before, immediately post, and 10, 30, and 60 min postexercise. Total blood cells, hematocrit, and mononuclear cells isolated by density gradient centrifugation were counted. Specific populations of hematopoietic stem cells were analyzed by flow cytometry. Mononuclear cells, $CD34^+$, $CD34^+/CD38^-$, $CD34^+/CD110^+$, $CD3^-/CD16^+/CD56^+$, $CD11c^+/CD123^-$, and $CD11c^-/CD123^+$cells per millilter of blood increased post exercise. Overall, the 70% WR_{peak} exercise group showed greater mobilization immediately post exercise, while there was no observable increase in mobilization in the work matched 30% WR_{peak} exercise group. Mobilization of specific populations of hematopoietic cells mirrored changes in the general mobilization of mononuclear cells, suggesting that exercise serves as a nonspecific mobilization stimulus. Evidently, higher intensity exercise is capable of mobilizing hematopoietic cells to a large extent and immediately post exercise is an ideal time point for their collection.

New & Noteworthy: Here we demonstrate for the first time that mobilization of *hematopoietic stem cells* (HSCs) through exercise is intensity dependent, with the greatest mobilization occurring immediately after high-intensity exercise. As well, we show that exercise is a general stimulus for mobilization: increases in specific HSC populations are reliant on general mononuclear cell mobilization. Finally, we demonstrate no differences in mobilization between groups with different aerobic fitness.

Introduction

The hematopoietic system consists of mature blood cells, found mostly in circulation, as well as multiple types of stem and progenitor cells (HSCs), found predominantly in the bone marrow. The roughly 10 billion new blood cells required per day to maintain homeostasis are generated through the self-renewal, proliferation, and differentiation of these specific hematopoietic stem and progenitor cells (1). Hematological malignancies that disrupt this system are cured through HSC transplantation. Nearly 20,000 HSC transplants are performed each year in the USA (44). In recent years it has become increasingly evident that the quality of a blood graft extracted from a donor is critical for the success of the transplant outcome (26). All hematopoietic progenitor cell types must be adequately represented in a donor graft to properly restore hematopoiesis in a recipient.

In humans, HSCs are generally identified as side population cells from the bone marrow (45) or as $CD34^+$ cells in peripheral blood (5). While HSCs in general can be detected with CD34, identifying specific subpopulations of $CD34^+$ HSCs requires additional surface markers. For example, while total $CD34^+$ content in a graft may be high, $CD34^+/CD110^+$ content, the cell population responsible for the development of platelets, may be low. The result of peripheral blood stem cell (PBSC) transplants with such grafts are recipient individuals who have an inability to properly regenerate platelets (34), resulting in bleeding, bruising, and susceptibility to infection, all of which are complications that can lead to death. Thus the development of mobilization regimes able to adequately mobilize both sufficient numbers and specific types of HSCs from bone marrow to blood is of incredible importance.

HSCs are retained in their niche through specific adhesion molecules and chemokine gradients (30). Although the majority of HSCs are found in the bone marrow, a small fraction of HSCs can be found in blood (41). In adult mice, it is estimated that ~1–5% of HSCs circulate between bone marrow and blood each day (4). However, depending on certain conditions, the number of mobilized cells can be greatly increased. In early development HSCs migrate from the fetal liver and spleen and populate the bone marrow (29). HSCs in adults migrate toward sites of injury or inflammation to aid in tissue repair (19). In

a clinical setting, in lieu of extracting cells directly from bone marrow, HSCs are mobilized into circulation with granulocyte-colony-stimulating factor (G-CSF) to provide sufficient cells for a graft (16). G-CSF is a colony-stimulating hormone released endogenously by various cells throughout the body to stimulate production of granulocytes and it also has potent mobilization properties. While G-CSF is mainly used alone, many interleukins, chemokines, or hematopoietic growth factors can also be used in conjunction with G-CSF to bring about mobilization with various efficiencies and kinetics. However, there may be important limitations to pharmacological interventions. Considering that there can be negative side-effects from long-term G-CSF dosing (18) or that some clinical populations, such as individuals with cancer (31) or diabetes (12), may not respond well to G-CSF substitute and/or synergistic strategies should be investigated.

In this capacity, a lesser studied stimulus for HSC mobilization is exercise. Very little data exist on the effects of acute exercise or exercise training on mobilization of human HSCs (10). Exercise seems to acutely increase peripheral blood HSC quantity; however, firm conclusions are difficult to make when comparing studies due to the wide range of subject characteristics, lack of proper markers used to define HSCs, and timing of analysis. Bonsignore and colleagues compared the quantity of HSCs in peripheral blood, identified as $CD34^+$ cells, between individuals competing in a 1.5-km field test (5). $CD34^+$ cells as well as burst-forming unit erythroid (BFU-E) and colony-forming unit-granulocyte macrophage (CFU-GM), erythrocyte and granulocyte macrophage colonies measured using cell culture assays, increased in circulation after the field test (5). Morici and colleagues evaluated the quantity of HSCs in peripheral blood immediately before and following an all-out rowing test in competitive rowers (28). Although HSCs, identified as $CD34^+$ cells, were increased in peripheral blood immediately following exercise in both males and females tested, the most primitive HSC population, $CD34^+/CD38^-$ cells, were unaffected by the acute exercise stimulus (28). Wardyn and colleagues compared HSC content in peripheral blood in previously sedentary individuals vs. habitual exercisers following an acute treadmill test (45). The authors concluded that HSC quantity in peripheral blood, identified there as side population cells, increased

irrespective of training status and gender immediately post exercise (45). Zaldivar and colleagues examined the response of early vs. late pubertal boys to 20 min of high-intensity cycling and observed equal increases in peripheral $CD34^+$ cells in both groups (50). Thijssen and colleagues observed a similar relative increase in circulating HSCs following an acute exercise stimulus in young and elderly individuals (40). When taken together, comparing the modalities of exercise used in these studies, it would seem that the acute increase in peripheral HSC quantity following exercise is intensity dependent.

Little knowledge exists, however, on the ability of exercise to mobilize specific populations of HSCs that are clinically relevant. We set out to address this. The purpose of this study was to further understand how exercise intensity effects HSC mobilization, to establish the kinetics of mobilization post exercise, to understand how HSC mobilization differs in individuals of differing aerobic capacity, and to determine the specificity of the stimulus by assaying several specific subpopulations of HSCs.

Methods

Participants

Eleven healthy young men (age 23.5 ± 2.9 yr) were recruited to participate in this study. Participants were asked to not strenuously exercise or remain recreationally active during their participation in the study. Exclusion criteria included smoking, diabetes, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or statins, and history of respiratory disease and/or any major orthopedic disability. The study was approved by the Hamilton Health Sciences Integrated Research Ethics Board and conformed to the guidelines outlined in the Declaration of Helsinki. Participants gave their informed written consent before their inclusion to the study. For subject characteristics, see Table 1.

Exercise Protocol

Each participant reported to the laboratory to perform a ramp incremental exercise test (50 W baseline for 3 min followed by a 30 W/min ramp) on an electronically braked cycle ergometer (Excalibur Sport V2.0; Lode, Groningen, The Netherlands) for determination of peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$) and peak work

rate (WR_{peak}). Expired gas and ventilatory parameters were collected for the determination of $\dot{V}O_{2peak}$ (Moxus Metabolic System; AEI Technologies, Pittsburgh, PA). Subjects were asked to maintain a cadence between 60 and 70 rpm during the test, and the test was terminated upon volitional fatigue. Following a 1-wk washout period, participants exercised at a workload pertaining to 70% of their WR_{peak} until volitional fatigue. Following another 1-wk washout period, participants performed a bout of cycling at 30% WR_{peak}, with the work output matched to the 70% WR_{peak} bout. These two intensities were chosen as they would likely fall within different very different exercise domains (46).

Blood collection and processing

Fifteen milliliters of blood were collected via venous catheter before (Pre), immediately after (Post), and 10, 30, and 60 min post exercise using heparinized vacutainers (0268795, Fisher Scientific Canada).Whole blood total cell counts were done using a countess automated cell counter (Invitrogen, Carlsbad, CA). Hematocrit was measured with microhematocrit tubes (22-274-913; Beckman Coulter). Mononuclear cells were then isolated from the remainder of the blood using Ficoll-Paque Plus (14) (17-1440-02; GE Healthcare Life Sciences), were resuspended in 1% bovine serum albumin in PBS, and were counted using a Countess automated cell counter.

Flow cytometry

Mononuclear cells were analyzed via flow cytometry (CyFlow Space, Partec) immediately after their isolation from blood. The following antibodies were used in the analysis: CD34 (FAB7227G; R&D Systems), CD110 (FAB1016A; R&D Systems), CD38 (FAB2404A; R&D Systems), CD41 (FAB7616A; R&D Systems), CD4 (FAB3791F; R&D Systems), CD8 (FAB1509A; R&D Systems), CD11c (FAB1777N; R&D Systems), CD123 (FAB301P; R&D Systems), and CD3/CD(15+56) cocktail (319101; BioLegend). Antibody concentrations used reflect manufacturer recommendations. At least 1×10^6 cells were stained for each antibody, with at least 5×10^5 events captured and analyzed. Unstained and single stain controls were used for compensation and gating. Final gates were based on FSC/SSC and two parameter plots.

Cell culture

Isolated mononuclear cells were also used for the long-term culture initiating cell (LTC-IC) assay, a limiting dilution assay described in more detail elsewhere (43). In brief, following collection, mononuclear cells were plated on a feeder layer of the FBMD-1 cell line, grown for 35 days with weekly media changes, switched to Human Methylcellulose Complete Medium (HSC003; R&D Systems), and then grown for a further 14 days. The presence of colonies was then scored and LTC-IC frequency was calculated using L-Calc (Stem Cell Technologies, British Colombia, Canada).

Statistical analysis

Statistical analysis was performed using Sigma Stat 11.0 analysis software (Systat Software, Chicago, IL). Data and graphs are expressed as means ± SE with $P \leq 0.05$ considered significant. Statistical differences between time points and groups were determined using one and two way repeated measures ANOVA. Tukey corrections were applied to account for multiple comparisons. LTC-IC data analyzed with a one-tailed *t*-test, justified as the culture experiments were performed after the majority of the flow cytometry had been analyzed. Statistical differences in $\dot{V}O_{2peak}$subgroups were analyzed by *t*-test. Differences in mononuclear mobilization between $\dot{V}O_{2peak}$subgroups were analyzed with a three way ANOVA.

Results

Exercise did not alter total blood cell counts or hematocrit.

Total blood cell counts and hematocrit were measured for each time point Pre and Post exercise. No significant differences were detected at any time point Pre or Post exercise at either intensity (data not shown).

Mononuclear cell numbers per milliliters of blood increased Post exercise in an intensity-dependent manner.

Although blood and hematocrit were not measurable different, mononuclear cell number was greatly impacted by exercise. Mononuclear cells were isolated via density gradient centrifugation. Peripheral blood mononuclear cells consist of monocytes, dendritic cells, lymphocytes, and a tiny fraction of multipotent and more differentiated HSCs. In the 70% WR_{peak} trial mononuclear cells increased by 139% from Pre to immediately Post exercise ($P < 0.05$) (**Fig. 1**). No significant differences in mononuclear cell number were observed in the 30% WR_{peak} trial, at any time point. Mononuclear cell number was increased by 96% at Post in the 70% WR_{peak} trial when compared with Post in the 30% WR_{peak} trial ($P < 0.05$).

Exercise acted as a general stimulus; many specific populations of HSCs and leukocytes are mobilized in the same magnitude as mononuclear cells.

Several specific populations of HSCs and white blood cells were measured with flow cytometry. Many of these populations were chosen as they represent specific populations needed in a blood cell graft to fully reconstitute the blood cell pool of a transplant recipient post myeloablation.

$CD34^+$ cells represent general hematopoietic progenitors and are used as the standard measure of graft quality. In the 70% WR_{peak} trial $CD34^+$ cells per milliliters of blood increased from Pre to immediately Post by 98.4% ($P < 0.05$) (**Fig. 2**). No differences were detected between Pre, Post, or any other time point post exercise following the 30% WR_{peak} trial. Finally, $CD34^+$ cells per milliliters of blood at Post in the 70% WR_{peak} trial were 170% higher than at Post in the 30% WR_{peak} trial ($P < 0.05$).

$CD34^+/CD38^-$ represent a more primitive and undifferentiated $CD34^+$ population. In the 70% WR_{peak} trial $CD34^+/CD38^-$ cells per milliliters of blood increased by 88% ($P < 0.05$) from Pre to immediately Post exercise (**Fig. 3***A*). Again, no differences were detected following the 30% WR_{peak}trial at any time point. Finally, $CD34^+/CD38^-$ cells per milliliters of blood at Post in the 70% WR_{peak}trial were 106% higher than at Post in the 30% WR_{peak} trial ($P < 0.05$).

$CD3^-/CD(16+56)^+$ characterize natural killer cells. $CD3^-/CD(16+56)^+$ cells per milliliters of blood increased by 291% ($P < 0.05$) from Pre to Post exercise following the 70% WR_{peak} trial (**Fig. 3***B*) and then returned to Pre exercise levels. No differences were detected at any other time point in the 70% WR_{peak} trial or at any time point in the 30% WR_{peak} trial. As well, $CD3^-/CD(16+56)^+$ cells per milliliters of blood at Post in the 70% WR_{peak} trial were 293% higher than at Post in the 30% WR_{peak} trial ($P < 0.05$).

The $CD4^+/CD8^+$ lymphocyte ratio is a general measure of immune function, and here, a predictor of graft quality. The calculated $CD4^+/CD8^+$ cell ratio was maintained around an ideal 2 at all measured time points (average 70% WR_{peak} trial: 2.36 ± 0.44; average 30% trial: 1.83 ± 0.65) and did not drop below that level (**Fig. 3***C*).

$CD34^+/CD41^+$ and $CD34^+/CD110^+$ represent megakaryocyte progenitors; cell types that work to reconstitute platelets in a recipient. While numbers of circulating $CD34^+/CD41^+$ cells did not change **(Fig. 3***D*), circulating $CD34^+/CD110^+$ cells increased. $CD34^+/CD110^+$ cells per milliliters of blood increased from Pre to Post in the 70% WR_{peak} trial by 169% ($P < 0.05$) (**Fig. 3***E*). No changes were detectable in the 30% WR_{peak} trial. As well, $CD34^+/CD110^+$ cells per milliliters of blood at Post in the 70% WR_{peak} trial were 129% higher than at Post in the 30% WR_{peak} trial ($P < 0.05$).

$CD11c^+/CD123^-$ and $CD11c^-/CD123^+$ demarcate dendritic type 1 and type 2 cells, respectively. $CD11c^+/CD123^-$ cells per milliliters of blood increased by 149% ($P < 0.05$) from Pre to Post exercise following the 70% WR_{peak} trial (**Fig. 4***A*). Following the established trend, no differences were detected following the 30% WR_{peak} trial at any time point. As well, $CD11c^+/CD123^-$ cells per milliliters of blood at Post in the 70% WR_{peak} trial were 77% higher than at Post in the 30% WR_{peak}trial ($P < 0.05$).

$CD11c^-/CD123^+$ cells per milliliters of blood increased by 165% ($P < 0.05$) from Pre to Post exercise following the 70% WR_{peak} trial (**Fig. 4***B*) and remained significantly higher than Pre until 30 min post exercise. Again, no differences were detected following the 30% WR_{peak} trial at any time point. Finally, $CD11c^-/CD123^+$ cells per milliliters of blood at Post in the 70% WR_{peak} trial were 168% higher than at

Post in the 30% WR_{peak} trial ($P < 0.05$) and were also significantly higher at 10 and 30 min following exercise between the 70% WR_{peak} and 30% WR_{peak} trials.

The same number of mononuclear cells from each exercise session and each collection time point were used for the flow cytometry analysis. In all cases, there were no detectable differences in the raw number of positive cells (data not shown). Only when expressing the populations as positive number of cells per milliliters of blood, taking into account the increased mononuclear cell numbers in blood, were any statistical differences detected. The differences are thus highly dependent on the general mobilization of mononuclear cells, rather than the mobilization of specific subpopulations of cells.

Long-term culture initiating cell frequency increased in blood in an intensity-dependent manner.

LTC-IC were grown from mononuclear input cells and enumerated following well established tissue culture protocols (43). These cells represent primitive HSCs and are quantified based on their ability to produce colonies in culture after 5 wk. Again, the results here follow a similar trend to the results mentioned previous to this point. LTC-IC number per milliliters of blood increased by 713% ($P < 0.05$) from Pre to immediately Post exercise following the 70% WR_{peak} trial (**Fig. 5**). No differences were detected between Pre and Post exercise following the 30% WR_{peak} trial.

Fitness level had no bearing on the ability to mobilize mononuclear cells to the blood cell pool.

Of the 11 total subjects, four had a significantly higher $\dot{V}O_{2peak}$ ($P > 0.05$) than the average of the entire group. These four subjects [average $\dot{V}O_{2peak}$ of 66.375 ± 4.35 ml/(kg·min)] were split from the remaining seven subjects [average $\dot{V}O_{2peak}$ of 49 ± 4.94 ml/(kg·min)] to determine if fitness level had an impact on the magnitude of mobilization. Though the subgroup with the higher fitness level performed more total work than the subgroup with the lower fitness level during both stages (70% WR_{peak} and 30% WR_{peak} work matched) of the test, no significant differences were detected between groups in total blood cell counts (data not shown), hematocrit (data not shown), mononuclear cell counts (**Fig. 6**), or specific

HSC populations at any time point (data not shown). The only detectable differences were as before; Pre and immediately Post exercise in the 70% WR_{peak} trial for each fitness subgroup.

Discussion

Here we demonstrate that high-intensity endurance exercise is capable of mobilizing hematopoietic cells from bone marrow to blood and that the ideal time point for their collection is immediately following exercise. Lower intensity exercise work matched to the higher intensity test failed to mobilize hematopoietic cells in all tested measures, suggesting that intensity of exercise is far more important than total work. As well, we demonstrate that exercise mediated mobilization seems to be a general process; increases in mobilization of specific populations of HSCs were dependent on overall mononuclear cell mobilization. Finally, we demonstrate that between two differing fitness levels no differences in the extent of exercise-mediated mobilization could be determined.

In general, the observed lack of mobilization in the 30% WR_{peak} work-matched trial, when compared with the 70% WR_{peak} until volitional fatigue trial, demonstrates that intensity may be a key factor in exercise induced mobilization. While the precise mechanism of exercise induced hematopoietic mobilization remains largely unknown, that intensity plays role is no surprise. Exercise can have dramatic effects on the body. For example, increases of ~8,000-fold in circulating levels of IL-6 have been observed in athletes after finishing a marathon race (24). Large increases in TNF-α, IL-1β, and IGF-binding protein 1, which stimulates inflammatory cytokine production, have also been observed (33). Increased production of cytokines and mobilizing factors both within the bone marrow niche and in circulation in response to exercise has been suggested as a mechanism for exercise-induced hematopoietic mobilization (11). Consequently, increased exercise intensity, leading to the subsequent release of greater amounts of these factors, followed by increased mobilization is no surprise. Increased blood flow within bone during high-intensity exercise (36, 37) may also lead to increase HSC efflux from marrow. As mobilization was seen to peak immediately post exercise, presumably when many of these factors are at their highest and bone marrow blood flow is most increased, the results make sense. However, while the

purpose of the present study was to observe how exercise intensity affects HSC mobilization, future studies should aim to address the notion that fatiguing exercise vs. non fatiguing exercise may impact mobilization, regardless of intensity.

Ensuring that clinically relevant HSC subpopulations are present in mobilized PBSC grafts is important for HSC transplant success. Exogenous treatment of clinical donors with G-CSF for several days before cell collection can raise circulating $CD34^+$ cells anywhere from two- to fourfold (21, 23) to as much as 20-fold (6), though the extent of mobilization varies greatly on an individual basis. Unfortunately, G-CSF treatment regimens often fail. When considering $CD34^+$ content alone, mobilization regimes have a 30% failure rate among healthy individuals and a greater than 60% failure rate in high-risk patients, such as those with lymphoid malignancies, prior exposure to chemotherapeutic agents, or radiation exposure (15, 27, 42). Poor $CD34^+$ G-CSF induced mobilization is also observed with increasing donor age (38). Furthermore, G-CSF treatment can also fail to mobilize other clinically relevant HSC populations, such as long-term $CD34^+/CD38^-$ cells (13) and B and T lymphocytes (21). An additional method for mobilizing HSCs, such as the high-intensity exercise intervention presented here, could potentially synergize with more traditional strategies. Here we demonstrate that high-intensity exercise induced a twofold increase in circulating $CD34^+$ cells (**Fig. 2**). However, when comparing the magnitude of this increase to the increase in circulating $CD34^+$ cells in healthy individuals mobilized with G-CSF [average 15-fold increase (39) or an average 23-fold increase (20)], exercise falls on the low end of the reported G-CSF spectrum.

Mobilization of clinically relevant HSC subpopulations in a donor can mean the difference between transplant success or transplant failure. The more cells that are mobilized, the easier it is for clinicians to harvest threshold doses necessary for transplant success. While $CD34^+$ cell dose is largely considered an important determining factor for transplant success (35), higher graft content of more specific HSC subpopulations has been shown to correlate with better PBSC transplant outcomes. PBSC grafts with a greater content of more primitive populations of HSCs, identified as $CD34^+/CD38^-$ cells, have been

shown to correlate to greater overall recipient survival (**17**). Here, we demonstrate that exercise is capable of mobilizing these cells to a large extent (**Fig. 3***A*). Immune reconstitution post transplant is critically important, helping myeloablated recipients to fight off infections and stay healthy. $CD3^{-}/CD(16+56)^{+}$ natural killer cell graft content also correlates with successful immune reconstitution (32). Again, high-intensity exercise appears to be capable of mobilizing this population (**Fig. 3***B*). Increased lymphocyte dosage during a transplant ($CD4^{+}$ and $CD8^{+}$ cells) correlates with increased recipient neutrophil recovery and decreased infection rates (25). A high CD4/CD8 ratio, however, can predict adverse immune-related outcomes post transplantation (49). Here we demonstrate exercise induced increased in numbers of $CD4^{+}$ and $CD8^{+}$ cells maintained at or near an ideal ratio of 2 (**Fig. 3***C*). Finally, increased graft content of another $CD34^{+}$ subset, $CD34^{+}/CD110^{+}$ platelet progenitors, correlates with increased platelet reconstitution post transplant (34). As well, increased $CD34^{+}/CD41^{+}$ graft content can predict increased platelet and neutrophil recovery post transplant (9, 26). In this capacity, high-intensity exercise appears to result in a non significant trend toward mobilization of more $CD34^{+}/CD41^{+}$ cells over time and a significant increase in mobilization of $CD34^{+}/CD110^{+}$ cells (**Fig. 3,** ***D*** **and** ***E***). As well, high-type one dendritic cell content ($CD11c^{+}/CD123^{-}$), high-type two dendritic cell content ($CD11c^{-}/CD123^{+}$), and total dendritic cell content have all been shown to be predictive of overall survival post transplantation (8); interestingly, we observe increases in both $CD11c^{+}/CD123^{-}$ and $CD11c^{-}/CD123^{+}$ cells following high-intensity exercise (**Fig. 4,** ***A*** **and** ***B***). In addition, several cell culture colony forming assays can be used to predict recipient survival. Increased numbers of LTC-ICs in a given graft has been correlated with increased recipient survival (3) and we have demonstrated the capacity of exercise to mobilize more cells of this type (**Fig. 5**).

Oftentimes hematopoietic donors are in poor health, have a relatively lower fitness level, and the exercise tolerance of such individuals is questionable. Determining how fitness level effects mobilization is therefore important and clinically relevant. To this end, subjects were split into a relatively higher fitness level [$\dot{V}O_{2peak}$: 66.375 ± 4.35 ml/(kg·min)] and relatively lower fitness level [$\dot{V}O_{2peak}$: 49 ± 4.94

ml/(kg·min)] in post analysis. Given previous findings that long-term endurance exercise can greatly increase the HSC pool (2, 7), it was expected that the higher fitness level group would show a larger magnitude of mobilization than the lower fitness level group. This was not the case (**Fig. 6**). As well, a correlation analysis between $\dot{V}O_{2peak}$ and circulating mononuclear cell number also revealed no relationship (data not shown). Differences in fitness levels appear to have little bearing on the magnitude of mononuclear cell mobilization. This result carried over to the analysis of the specific HSC populations (data not shown). Although the average $\dot{V}O_{2peak}$ of our subjects was higher than those of sedentary individuals, and individuals at a relatively lower fitness level may mobilize differently due to their health, what this suggests is that fitness level may be unimportant. Unhealthy individuals, with a fitness level lower than average, may mobilize HSCs to the same extent as normal individuals. Again, the fitness level of these two subgroups was higher than average; this may not necessarily translate to individuals with a lower than average $\dot{V}O_{2peak}$. However, populations of the elderly, sick, or otherwise impaired needing to donate blood for a PBSC graft may potentially be able to exercise at a high level of intensity, relative to their low $\dot{V}O_{2peak}$ and, in combination with traditional mobilization strategies, mobilize sufficient HSCs to collect a successful graft. Indeed, the safety and feasibility of exercise in patients undergoing HSC transplant have previously been demonstrated (47). As well, higher intensity exercise protocols have been well tolerated in a number of clinical populations (22, 48), suggesting that these protocols are viable in non healthy individuals.

In conclusion, high-intensity endurance exercise may serve as an aid to more traditional HSC mobilization strategies. It is often the case that some patients mobilize poorly; in such situations, additional chemokines and cytokines are used with traditional mobilizing agents (42). While these optimizations help in some situations, developing additional methods will always be useful for healthcare providers. In isolation, the magnitude of exercise-induced HSCs mobilization falls on the low end of G-CSF-based mobilization. Although leukapheresis post exercise, repeated over the course of several days, could in theory harvest enough HSCs for a suitable graft, combining exercise with traditional

mobilization strategies would be a better option. Based on our findings, although limited by a relatively low sample size, exercise could be considered as a means to enhance HSC content in a donor's blood graft for patients undergoing PBSC transplantation.

3.1 – Table 1

Subject characteristics

Peak work rate (WR_{peak},)

Subject Characteristics	**Means ± SD**
Age	23.5 ± 2.9 yr
Height	178 ± 4 cm
Weight	77.3 ± 2.2 kg
$\dot{V}O_{2peak}$	54.3 ± 9.5 ml/(kg·min)
Average time to exhaustion (70% WR_{peak} trial)	17.8 ± 4 min
Worked-matched time (30% WR_{peak} trial)	41.4 ± 3 min

3.2 – Figure 1

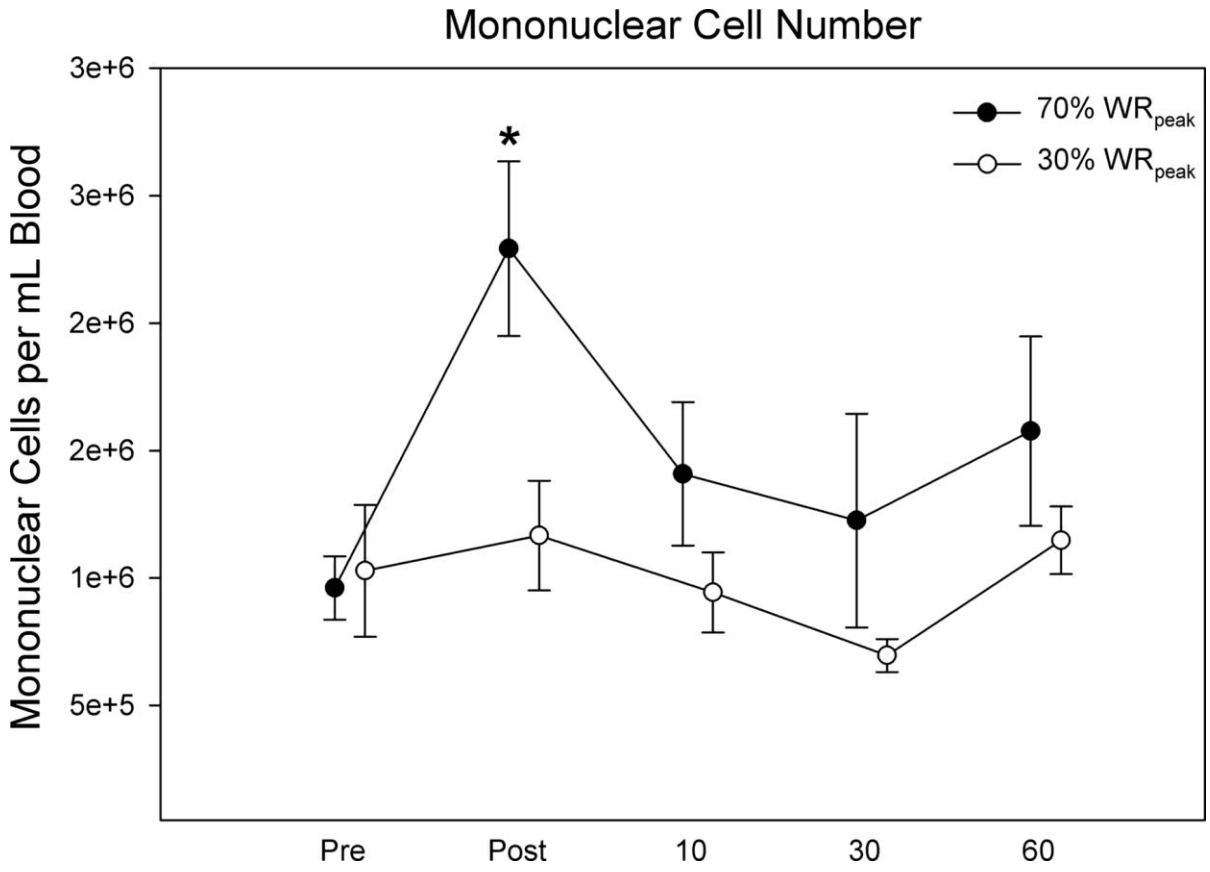

Figure 1: Mononuclear cells counts. Mononuclear cell counts, as isolated through density gradient centrifugation, expressed per milliliters of blood for the 70% WR_{peak} and 30% WR_{peak} exercise sessions across the time course (where WR_{peak} is peak work rate). *$P < 0.05$, between groups and from before (Pre) exercise with respect to time.

3.3 – Figure 2

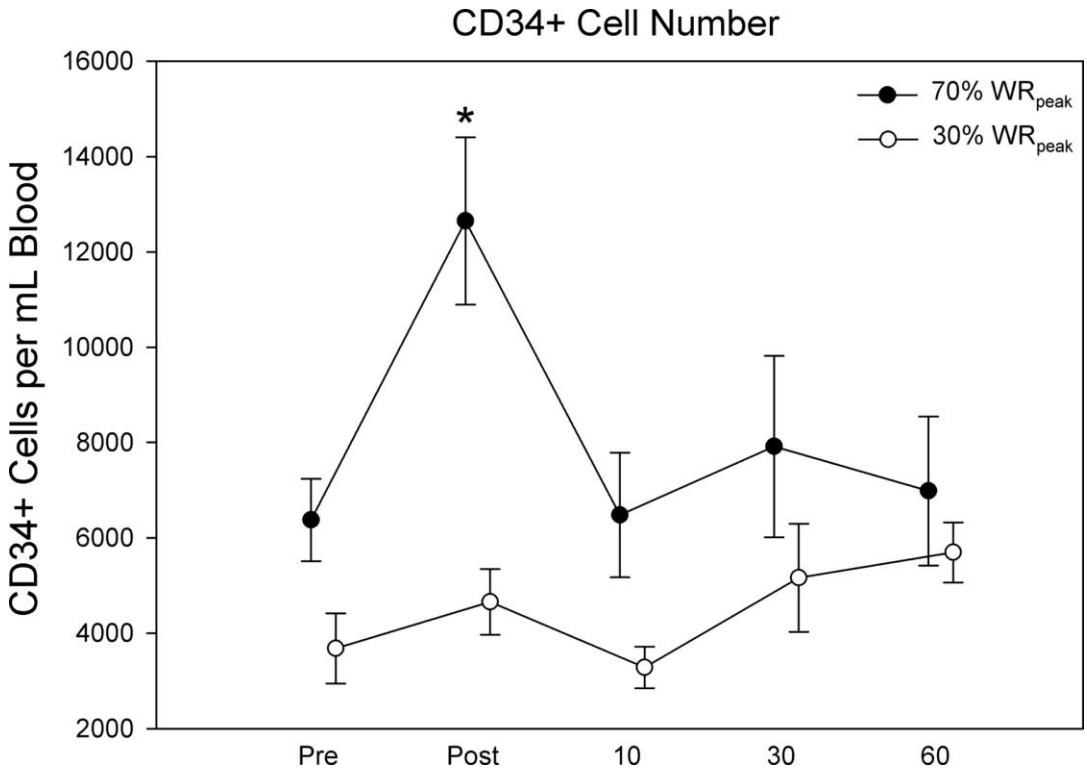

Figure 2: CD34$^{\pm}$ cells per milliliters blood. CD34^{+} cell counts, analyzed with flow cytometry, expressed per milliliters of blood for the 70% WR_{peak} and 30% WR_{peak} exercise sessions across the time course. *P < 0.05, between groups and from Pre exercise with respect to time.

3.4 – Figure 3

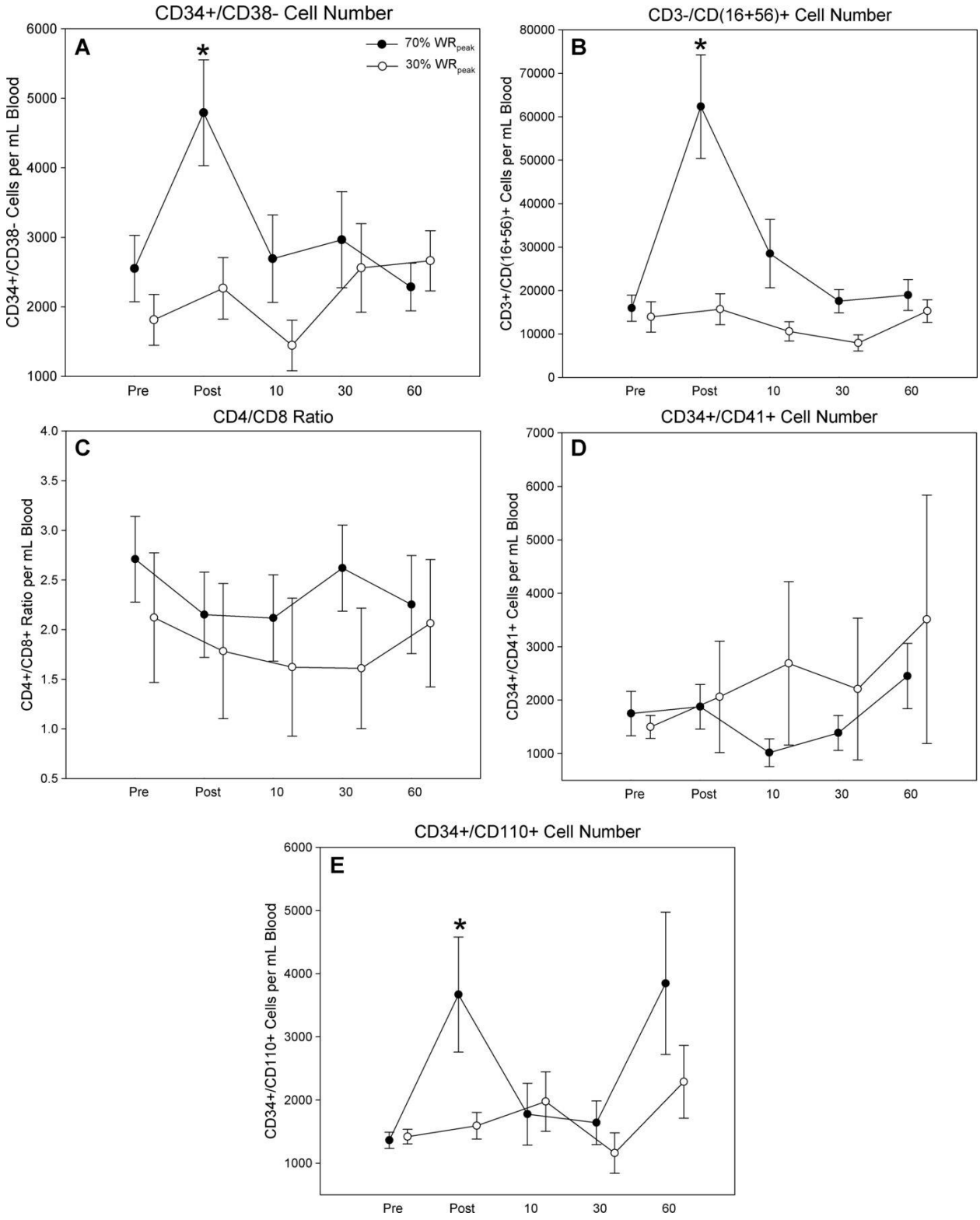
CD34+/CD38- Cell Number
A
*
70% WR$_{peak}$
30% WR$_{peak}$
CD34+/CD38- Cells per mL Blood
6000
5000
4000
3000
2000
1000
Pre
Post
10
30
60
CD3-/CD(16+56)+ Cell Number
B
*
CD3+/CD(16+56)+ Cells per mL Blood
80000
70000
60000
50000
40000
30000
20000
10000
0
Pre
Post
10
30
60
CD4/CD8 Ratio
C
CD4+/CD8+ Ratio per mL Blood
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Pre
Post
10
30
60
CD34+/CD41+ Cell Number
D
CD34+/CD41+ Cells per mL Blood
7000
6000
5000
4000
3000
2000
1000
Pre
Post
10
30
60
CD34+/CD110+ Cell Number
E
*
CD34+/CD110+ Cells per mL Blood
6000
5000
4000
3000
2000
1000
Pre
Post
10
30
60

Figure 3: Specific hematopoietic stem cell (HSC) populations. Five different blood cell populations, analyzed by flow cytometry, known to positively correlate with successful outcomes post blood cell graft. *A*: $CD34^{+}/CD38^{-}$. *B*: $CD3^{-}/CD(16+56)^{+}$. *C*: $CD4^{+}/CD8^{+}$ ratio. *D*: $CD34^{+}/CD41^{+}$. *E*: $CD34^{+}/CD110^{+}$. Each expressed as positive cells per milliliters of blood for the 70% WR_{peak} and 30% WR_{peak} exercise sessions, across the time course. $*P < 0.05$,between groups and from Pre exercise with respect to time.

3.5 – Figure 4

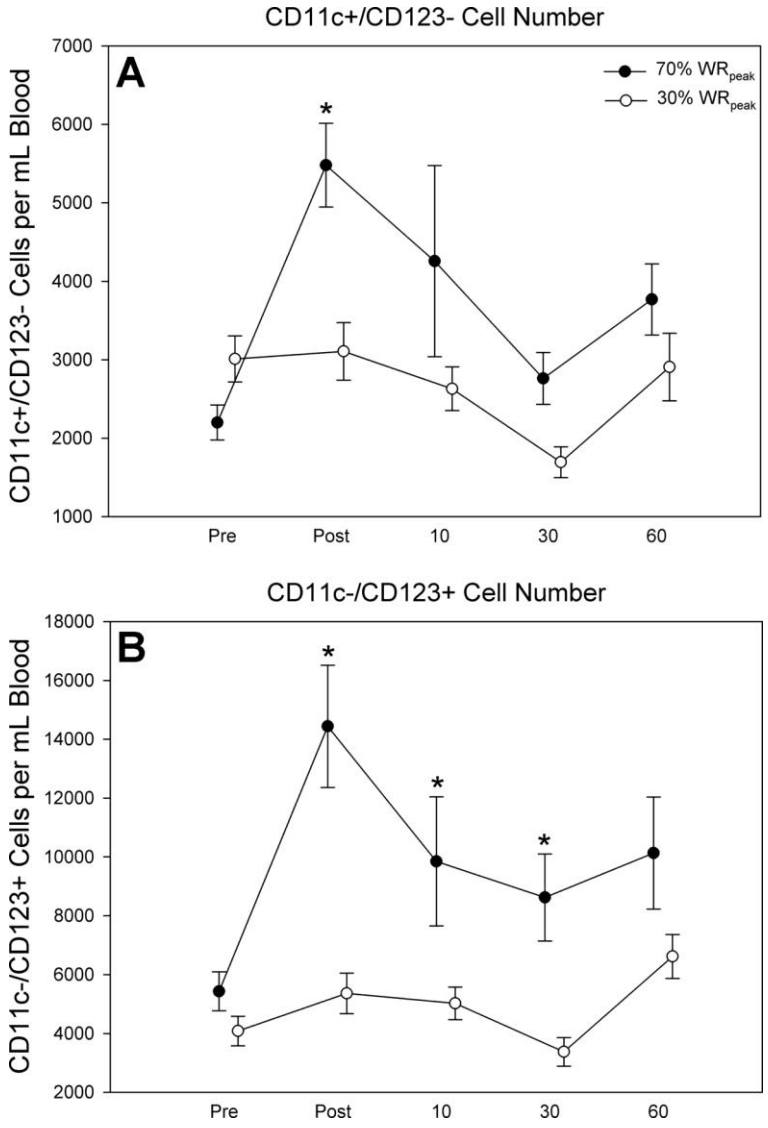

Figure 4: Dendritic cell populations. Two dendritic cell populations, analyzed by flow cytometry, known to positively correlate with successful outcomes post blood cell graft. *A*: $CD11c^{+}/CD123^{-}$. *B*: $CD11c^{-}/CD123^{+}$. Each expressed as positive cells per milliliters of blood for the 70% WR_{peak} and 30% WR_{peak} exercise sessions, across the time course. *$P < 0.05$, between groups and from Pre exercise with respect to time.

3.6 – Figure 5

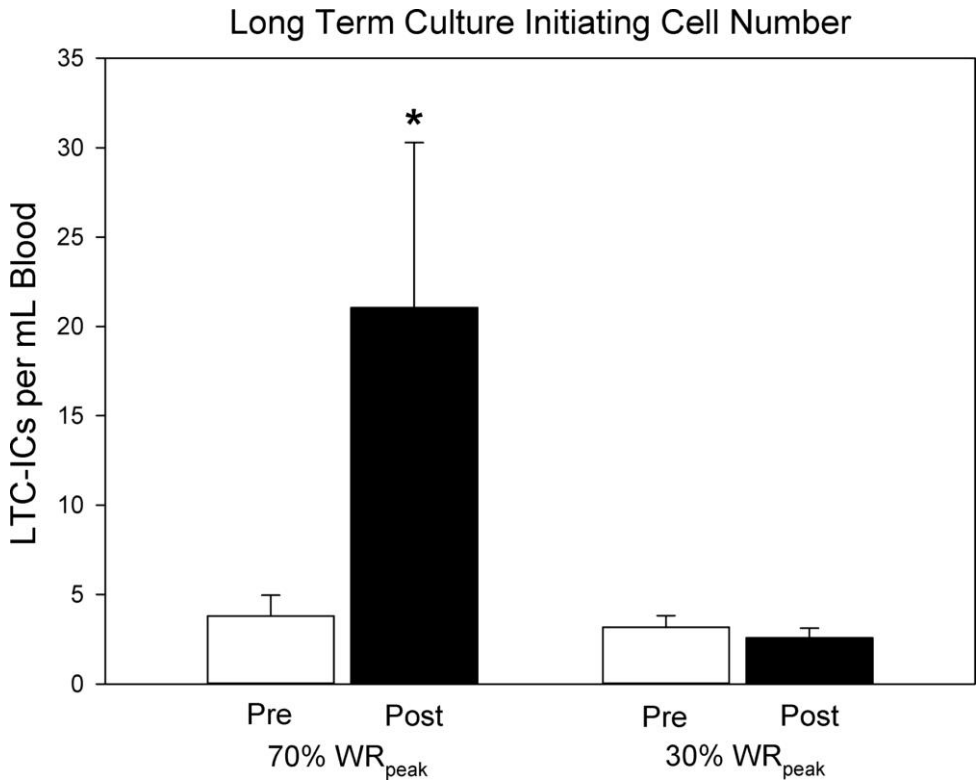

Figure 5: Long-term culture initiating cell frequency (LTC-IC). LTC-IC, expressed as number of cells per milliliters blood, Pre and immediately Post exercise for the 70% WR_{peak} and 30% WR_{peak} exercise sessions. *$P < 0.05$, between groups and between Pre and immediately after (Post) exercise with respect to time.

3.7 – Figure 6

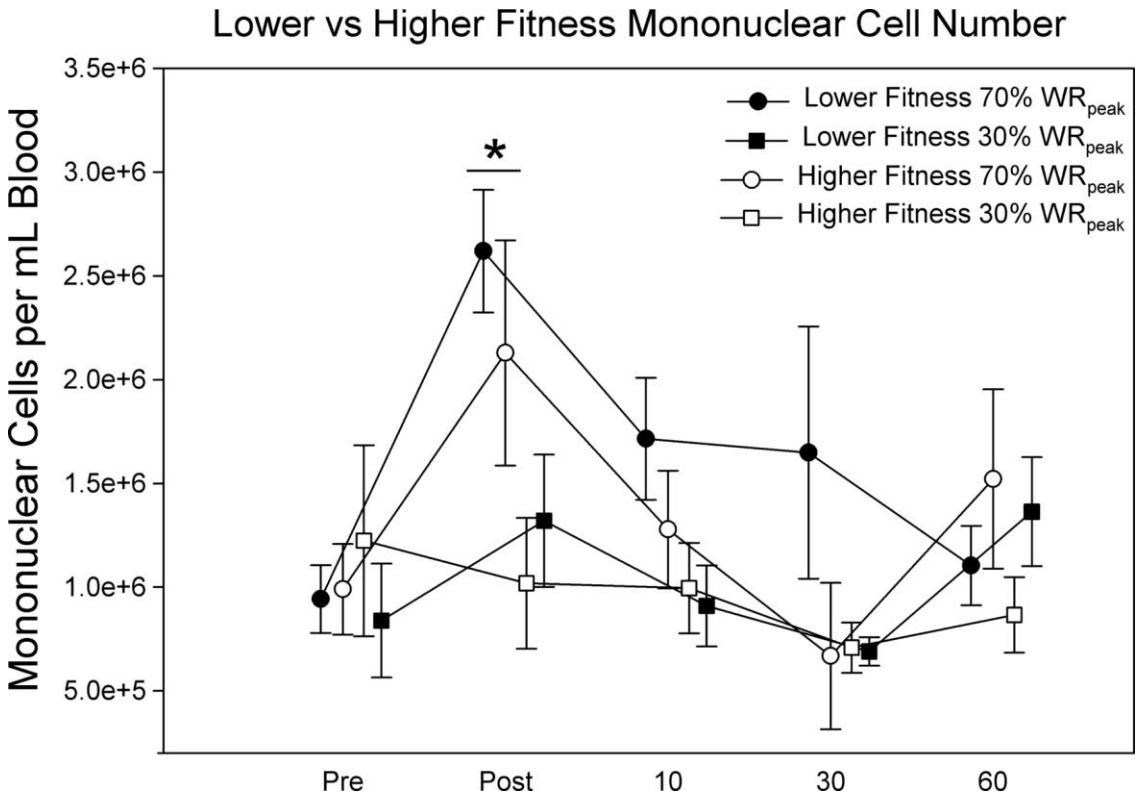

Figure 6: Fitness comparison. A comparison between mononuclear cell counts, as isolated by density gradient centrifugation, expressed per milliliters of blood for the 70% WR_{peak} and 30% WR_{peak} exercise sessions, including the two fitness subgroups, across the time course. *P < 0.05, within fitness level subgroups for intensity, but no differences were detected between the two fitness subgroups.

3.8 – References

1. Attar EC, Scadden DT. Regulation of hematopoietic stem cell growth. Leukemia 18: 1760–1768, 2004. doi:10.1038/sj.leu.2403515.

2. Baker JM, De Lisio M, Parise G. Endurance exercise training promotes medullary hematopoiesis. FASEB J 25: 4348–4357, 2011. doi:10.1096/fj.11-189043.

3. Benboubker L, Binet C, Cartron G, Bernard MC, Clement N, Delain M, Degenne M, Desbois I, Colombat P, Domenech J. Frequency and differentiation capacity of circulating LTC-IC mobilized by G-CSF or GM-CSF following chemotherapy: a comparison with steady-state bone marrow and peripheral blood. Exp Hematol 30: 74–81, 2002. doi:10.1016/S0301-472X(01)00762-7.

4. Bhattacharya D, Czechowicz A, Ooi AG, Rossi DJ, Bryder D, Weissman IL. Niche recycling through division-independent egress of hematopoietic stem cells. J Exp Med 206: 2837–2850, 2009. doi:10.1084/jem.20090778.

5. Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U, Hogg JC. Circulating hematopoietic progenitor cells in runners. J Appl Physiol (1985) 93: 1691–1697, 2002. doi:10.1152/japplphysiol.00376.2002.

6. Dale DC, Bonilla MA, Davis MW, Nakanishi AM, Hammond WP, Kurtzberg J, Wang W, Jakubowski A, Winton E, Lalezari P. A randomized controlled phase III trial of recombinant human granulocyte colony-stimulating factor (filgrastim) for treatment of severe chronic neutropenia. Blood 81: 2496–2502, 1993.

7. De Lisio M, Baker JM, Parise G. Exercise promotes bone marrow cell survival and recipient reconstitution post-bone marrow transplantation, which is associated with increased survival. Exp Hematol 41: 143–154, 2013. doi:10.1016/j.exphem.2012.10.003.

8. Dean R, Masci P, Pohlman B, Andresen S, Serafino S, Sobecks R, Kuczkowski E, Curtis J, Maciejewski J, Rybicki L, Kalaycio M, Hsi E, Theil K, Bolwell BJ. Dendritic cells in autologous hematopoietic stem cell transplantation for diffuse large B-cell lymphoma: graft content and post transplant recovery predict survival. Bone Marrow Transplant 36: 1049–1052, 2005. doi:10.1038/sj.bmt.1705183.

9. Dercksen MW, Rodenhuis S, Dirkson MK, Schaasberg WP, Baars JW, van der Wall E, Slaper-Cortenbach IC, Pinedo HM, Von dem Borne AE, van der Schoot CE. Subsets of CD34+ cells and rapid hematopoietic recovery after peripheral-blood stem-cell transplantation. J Clin Oncol 13: 1922–1932, 1995.

10. Emmons R, Niemiro GM, De Lisio M. Exercise as an adjuvant therapy for hematopoietic stem cell mobilization. Stem Cells Int 2016: 7131359, 2016. doi:10.1155/2016/7131359.

11. Emmons R, Niemiro GM, Owolabi O, De Lisio M. Acute exercise mobilizes hematopoietic stem and progenitor cells and alters the mesenchymal stromal cell secretome. J Appl Physiol (1985) 120: 624–632, 2016. doi:10.1152/japplphysiol.00925.2015.

12. Ferraro F, Lymperi S, Méndez-Ferrer S, Saez B, Spencer JA, Yeap BY, Masselli E, Graiani G, Prezioso L, Rizzini EL, Mangoni M, Rizzoli V, Sykes SM, Lin CP, Frenette PS, Quaini F, Scadden DT. Diabetes impairs hematopoietic stem cell mobilization by altering niche function. Sci Transl Med 3: 104ra101, 2011. doi:10.1126/scitranslmed.3002191.

13. Fruehauf S, Veldwijk MR, Seeger T, Schubert M, Laufs S, Topaly J, Wuchter P, Dillmann F, Eckstein V, Wenz F, Goldschmidt H, Ho AD, Calandra G. A combination of granulocyte-colony-stimulating factor (G-CSF) and plerixafor mobilizes more primitive peripheral blood progenitor cells than G-CSF alone: results of a European phase II study. Cytotherapy 11: 992–1001, 2009. doi:10.3109/14653240903121245.

14. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. In: Current Protocols in Immunology. New York: Wiley, 2009, chapt. 7, unit 71.

15. Gertz MA. Current status of stem cell mobilization. Br J Haematol 150: 647–662, 2010. doi:10.1111/j.1365-2141.2010.08313.x.

16. Guardiola P, Runde V, Bacigalupo A, Ruutu T, Locatelli F, Boogaerts MA, Pagliuca A, Cornelissen JJ, Schouten HC, Carreras E, Finke J, van Biezen A, Brand R, Niederwieser D, Gluckman E, de Witte TM; Subcommittee for Myelodysplastic Syndromes of the Chronic Leukaemia Working Group of the European Blood and Marrow Transplantation Group. Retrospective comparison of bone marrow and granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells for allogeneic stem cell transplantation using HLA identical sibling donors in myelodysplastic syndromes. Blood 99: 4370–4378, 2002. doi:10.1182/blood.V99.12.4370.

17. Hénon PH, Sovalat H, Bourderont D. Importance of CD34+ cell subsets in autologous PBSC transplantation: the mulhouse experience using CD34+CD38− cells as predictive tool for hematopoietic engraftment. J Biol Regul Homeost Agents 15: 62–67, 2001.

18. Hilbe W, Nussbaumer W, Bonatti H, Thaler J, Niederwieser D, Nachbaur D. Unusual adverse events following peripheral blood stem cell (PBSC) mobilisation using granulocyte colony stimulating factor (G-CSF) in healthy donors. Bone Marrow Transplant 26: 811–813, 2000. doi:10.1038/sj.bmt.1702581.

19. Lapid K, Glait-Santar C, Gur-Cohen S, Canaani J, Kollet O, Lapidot T. Egress and mobilization of hematopoietic stem and progenitor cells: a dynamic multi-facet process. In: StemBook. Cambridge, MA: Wiley, 2008.Google Scholar

20. Lee S, Im SA, Yoo ES, Nam EM, Lee MA, Ahn JY, Huh JW, Kim DY, Lee SN, Kim MJ, Lee SJ, Chung WS, Seong CM. Mobilization kinetics of CD34(+) cells in association with modulation of CD44 and CD31 expression during continuous intravenous administration of G-CSF in normal donors. Stem Cells 18: 281–286, 2000. doi:10.1634/stemcells.18-4-281.

21. Liles WC, Rodger E, Broxmeyer HE, Dehner C, Badel K, Calandra G, Christensen J, Wood B, Price TH, Dale DC. Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. Transfusion 45: 295–300, 2005. doi:10.1111/j.1537-2995.2005.04222.x.

22. Little JP, Gillen JB, Percival ME, Safdar A, Tarnopolsky MA, Punthakee Z, Jung ME, Gibala MJ. Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. J Appl Physiol (1985) 111: 1554–1560, 2011. doi:10.1152/japplphysiol.00921.2011.

23. Lubenau H, Bias P, Maly AK, Siegler KE, Mehltretter K. Pharmacokinetic and pharmacodynamic profile of new biosimilar filgrastim XM02 equivalent to marketed filgrastim Neupogen: single-blind, randomized, crossover trial. BioDrugs 23: 43–51, 2009. doi:10.2165/00063030-200923010-00005.

24. Margeli A, Skenderi K, Tsironi M, Hantzi E, Matalas AL, Vrettou C, Kanavakis E, Chrousos G, Papassotiriou I. Dramatic elevations of interleukin-6 and acute-phase reactants in athletes participating in the ultradistance foot race spartathlon: severe systemic inflammation and lipid and lipoprotein changes in protracted exercise. J Clin Endocrinol Metab 90: 3914–3918, 2005. doi:10.1210/jc.2004-2346.

25. Menéndez P, Caballero MD, Prosper F, Del Cañizo MC, Pérez-Simón JA, Mateos MV, Nieto MJ, Corral M, Romero M, García-Conde J, Montalbán MA, San Miguel JF, Orfao A. The composition of leukapheresis products impacts on the hematopoietic recovery after autologous transplantation independently of the mobilization regimen. Transfusion 42: 1159–1172, 2002. doi:10.1046/j.1537-2995.2002.00190.x.

26. Millar BC, Millar JL, Shepherd V, Blackwell P, Porter H, Cunningham D, Judson I, Treleaven J, Powles RL, Catovsky D. The importance of CD34+/CD33- cells in platelet engraftment after intensive

therapy for cancer patients given peripheral blood stem cell rescue. Bone Marrow Transplant 22: 469–475, 1998. doi:10.1038/sj.bmt.1701368.

27. Morgan SJ, Seymour JF, Grigg A, Matthews JP, Prince HM, Wolf MM, Januszewicz EH. Predictive factors for successful stem cell mobilization in patients with indolent lymphoproliferative disorders previously treated with fludarabine. Leukemia 18: 1034–1038, 2004. doi:10.1038/sj.leu.2403326.

28. Morici G, Zangla D, Santoro A, Pelosi E, Petrucci E, Gioia M, Bonanno A, Profita M, Bellia V, Testa U, Bonsignore MR. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. Am J Physiol Regul Integr Comp Physiol 289: R1496–R1503, 2005. doi:10.1152/ajpregu.00338.2005.

29. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132: 631–644, 2008. doi:10.1016/j.cell.2008.01.025.

30. Papayannopoulou T, Scadden DT. Stem-cell ecology and stem cells in motion. Blood 111: 3923–3930, 2008. doi:10.1182/blood-2007-08-078147.

31. Perseghin P, Terruzzi E, Dassi M, Baldini V, Parma M, Coluccia P, Accorsi P, Confalonieri G, Tavecchia L, Verga L, Ravagnani F, Iacone A, Pogliani EM, Pioltelli P. Management of poor peripheral blood stem cell mobilization: incidence, predictive factors, alternative strategies and outcome. A retrospective analysis on 2177 patients from three major Italian institutions. Transfus Apheresis Sci 41: 33–37, 2009. doi:10.1016/j.transci.2009.05.011.

32. Porrata LF, Gastineau DA, Padley D, Bundy K, Markovic SN. Re-infused autologous graft natural killer cells correlates with absolute lymphocyte count recovery after autologous stem cell transplantation. Leuk Lymphoma 44: 997–1000, 2003. doi:10.1080/1042819031000077089.

33. Ransom JI, Powers JG, Thompson Hobbs N, Baker DL. Ecological feedbacks can reduce population-level efficacy of wildlife fertility control. J Appl Ecol 51: 259–269, 2014. doi:10.1111/1365-2664.12166.

34. Sartor MM, Garvin F, Antonenas V, Bradstock KF, Gottlieb DJ. Failure to achieve a threshold dose of CD34+ CD110+ progenitor cells in the graft predicts delayed platelet engraftment after autologous stem cell transplantation. Bone Marrow Transplant 40: 851–857, 2007. doi:10.1038/sj.bmt.1705818.

35. Solá C, Maroto P, Salazar R, Mesía R, Mendoza L, Brunet J, López-Pousa A, Tabernero JM, Montesinos J, Pericay C, Martínez C, Cancelas JA, López-López JJ. Bone marrow transplantation: prognostic factors of peripheral blood stem cell mobilization with cyclophosphamide and filgrastim (r-metHuG-CSF): the CD34+ cell dose positively affects the time to hematopoietic recovery and supportive requirements after high-dose chemotherapy. Hematology 4: 195–209, 1999. doi:10.1080/10245332.1999.11746443.

36. Spodaryk K, Dabrowski Z. Blood flow in different regions of bone marrow after short-term execise. Acta Physiol Hung 77: 13–17, 1991.

37. Stabley JN, Moningka NC, Behnke BJ, Delp MD. Exercise training augments regional bone and marrow blood flow during exercise. Med Sci Sports Exerc 46: 2107–2112, 2014. doi:10.1249/MSS.0000000000000342.

38. Stiff PJ. Management strategies for the hard-to-mobilize patient. Bone Marrow Transplant 23, Suppl 2: S29–S33, 1999. doi:10.1038/sj.bmt.1701671.

39. Stroncek DF, Clay ME, Herr G, Smith J, Jaszcz WB, Ilstrup S, McCullough J. The kinetics of G-CSF mobilization of CD34+ cells in healthy people. Transfus Med 7: 19–24, 1997. doi:10.1046/j.1365-3148.1997.d01-75.x.

40. Thijssen DH, Vos JB, Verseyden C, van Zonneveld AJ, Smits P, Sweep FC, Hopman MT, de Boer HC. Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. Aging Cell 5: 495–503, 2006. doi:10.1111/j.1474-9726.2006.00242.x.

41. Thomas J, Liu F, Link DC. Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr Opin Hematol 9: 183–189, 2002. doi:10.1097/00062752-200205000-00002.

42. To LB, Levesque JP, Herbert KE. How I treat patients who mobilize hematopoietic stem cells poorly. Blood 118: 4530–4540, 2011. doi:10.1182/blood-2011-06-318220.

43. van Os RP, Dethmers-Ausema B, de Haan G. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods Mol Biol 430: 143–157, 2008. doi:10.1007/978-1-59745-182-6_10.

44. Wang MC. Current Uses and Outcomes of Hematopoietic Stem Cell Transplantation: CIBMTR Summary Slides (Online). Center for International, Blood & Marrow Transplant Research. 2015.

45. Wardyn GG, Rennard SI, Brusnahan SK, McGuire TR, Carlson ML, Smith LM, McGranaghan S, Sharp JG. Effects of exercise on hematological parameters, circulating side population cells, and cytokines. Exp Hematol 36: 216–223, 2008. doi:10.1016/j.exphem.2007.10.003.

46. Whipp BJ, Ward SA, Lamarra N, Davis JA, Wasserman K. Parameters of ventilatory and gas exchange dynamics during exercise. J Appl Physiol Respir Environ Exerc Physiol 52: 1506–1513, 1982.

47. Wiskemann J, Huber G. Physical exercise as adjuvant therapy for patients undergoing hematopoietic stem cell transplantation. Bone Marrow Transplant 41: 321–329, 2008. doi:10.1038/sj.bmt.1705917.

48. Wisløff U, Støylen A, Loennechen JP, Bruvold M, Rognmo Ø, Haram PM, Tjønna AE, Helgerud J, Slørdahl SA, Lee SJ, Videm V, Bye A, Smith GL, Najjar SM, Ellingsen Ø, Skjaerpe T. Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study. Circulation 115: 3086–3094, 2007. doi:10.1161/CIRCULATIONAHA.106.675041.

49. Xu LP, Luo XH, Chang YJ, Liu DH, Liu KY, Chen YH, Huang XJ. High CD4/CD8 ratio in allografts predicts adverse outcomes in unmanipulated HLA-mismatched/haploidentical hematopoietic stem cell

transplantation for chronic myeloid leukemia. Ann Hematol 88: 1015–1024, 2009. doi:10.1007/s00277-009-0728-4.

50. Zaldivar F, Eliakim A, Radom-Aizik S, Leu SY, Cooper DM. The effect of brief exercise on circulating CD34+ stem cells in early and late pubertal boys. Pediatr Res 61: 491–495, 2007. doi:10.1203/pdr.0b013e3180332d36.

Chapter 4 – Exercise training promotes extramedullary hematopoiesis

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Abstract

Hematopoietic stem cell development is highly dependent on specialized niche environments that provide appropriate signalling and adhesion molecules for these cells. Exercise training has been shown to increase numbers of hematopoietic stem cells in bone marrow and increase hematopoietic cell mobilization to blood, through changes in niche structure and release of hematopoietic growth factors. Exercise affects spleen, an extramedullary source of hematopoiesis, in a similar manner. Exercise increased the number of hematopoietic stem and progenitor cells in the spleen; LSK was increased by 3.5 fold, CFU-GEMM by increased by 4 fold, and increased BFU-E by 4.5 fold. Exercise also increased the number of lineage positive cells in the spleen by 10 to 30%. Furthermore, expression of genes associated with hematopoietic homing, adhesion, quiescence, and growth were all increased in the spleen as a result of exercise training. Similar to previous reports focused on bone marrow and the medullary hematopoietic niche, exercise greatly increases hematopoietic activity in the spleen.

Introduction

The hematopoietic system consists of terminally differentiated blood cells in circulation that are replenished by hematopoietic stem and progenitors cells (HSPC) found in bone marrow. These cells self-renew, proliferate, and differentiate in a highly controlled fashion. Bone marrow is the primary site of hematopoiesis. Within bone, HSPC are supported by specialized niche environments; the endosteal niche, where HSPC interact with osteoblast cell types, and the vascular niche, where HSPC interact with endothelial cell types. Other cell types within bone, such as adipocytes, negatively influence HSPC function. Bone marrow and HSPC health are critical; roughly 10 billion new blood cells are required per day to maintain blood homeostasis (4).

Exercise has been shown to impact the hematopoietic system in a number of ways. In bone marrow, exercise increases osteoblast number and decreases adipocyte number, changing the structure of the bone marrow niche to better support HSPC. It does this by altering the differentiation potential of mesenchymal stem cells within bone marrow, the progenitor cell for adipocytes and osteoblasts (5). In skeletal muscle, exercise changes expression of several hematopoietic cytokines; GM-CSF, M-CSF, IL3, and EPO expression are all increased as a result of exercise (5). The result of these changes are increased numbers of HSPC in the bone marrow of exercise trained animals.

Exercise also increases HSPC mobilization from bone marrow to blood. In humans, acute exercise increases numbers of circulating mononuclear and HSPC in an intensity dependent manner (6). In mice, acute exercise does the same (21). Though the precise mechanisms of action by which acute exercise triggers HSPC mobilization are still unknown, increases in production of chemoattractants from extramedullary tissues, such as skeletal muscle and the spleen, and release of local factors from stromal cells in bone marrow are probable (21). Exercise training also increases the basal level of HSPC found in blood (5). This is likely due to increased numbers of marrow HSPC that result from exercise training (5), baseline levels of mobilization from bone marrow to blood (10), and exercise mediated increases in production and release of hematopoietically relevant growth factors (7, 51).

Another site of hematopoiesis, outside the bone marrow medullary cavity, is the spleen. The classical role the spleen plays is the filtering and removal of old or damaged erythrocytes, reclaiming their iron content, as well as removal of blood borne microorganisms and cellular debris (43). The white pulp of the spleen plays a role in the immune system, containing a diverse number of lymphocytes that trap and remove blood borne antigens and initiate innate and adaptive immune responses (12). Spleen also acts as a reserve of red blood cells; horse spleen stores a red blood cell volume equal to a third of the circulating red blood cell mass (40), though in humans and other animals this number is much lower. Exercise also increases the amount of reserve cells stored (22). As well, spleen stores and is the site of rapid deployment for monocytes, releasing them into circulation in response to injury (61).

Hematopoiesis in the spleen is more prevalent early in life. During the first few months *in utero* the human spleen is hematopoietically active, slowing down and shifting primarily to bone marrow after five fetal months (16). Splenic EMH (extramedullary hematopoiesis) in mice follows a similar pattern but retains a higher level of activity into development than in humans. In mice, bone marrow, spleen, and to a lesser extent the liver, all act cooperatively to contribute to hematopoiesis homeostasis (66). In both humans and mice when regular hematopoiesis is impaired, due to disease or injury, extramedullary hematopoiesis is often upregulated. In cases of osteopetrosis, where the size of the medullary cavity is reduced, or in cases of myelofibrosis, where hematopoietic stem and progenitor cells in the bone marrow cavity are supplanted by fibrosis and increasing collagen deposition, splenic EMH is upregulated in an attempt to maintain blood homeostasis (31, 49).

Given the role exercise plays in increasing production and release of pro hematopoietic cytokines and growth factors, the ability of exercise to increase the number of circulating HSPC available to the spleen, and the role spleen plays in filtering and sequestering reserve blood, it is reasonable to assume that exercise may also impact EMH in the spleen. Here, we set out to determine the effects of an endurance exercise training protocol on murine spleen EMH.

Methods

Animals

Adult male C57Bl/6J mice (n=30) (Jackson Laboratories), aged 4 weeks, were used for the experiments. Sedentary and exercised mice were kept in separate cages. No more than 5 mice were housed per cage and mice were provided food and water *ad libitum*. Mice were maintained on a 12 hour light-dark cycle at 22 ± 2°C. Ethics approval was granted by the McMaster University Animal Research Ethics Board and conformed to standards established by the Canadian Council on Animal Care.

Exercise Training Protocol

Mice were exercise trained (n=15) on an Exer 6M Treadmill (Columbus Instruments) 3 days per week for 10 weeks. The mice were allowed to acclimatize to the treadmill a week before training. For the 10 week training period, mice were subjected to a progressive exercise protocol; the training portion of the protocol began at 14 m/min for 45 min (week 1) and increased to 24 m/min for 45 min (week 10). Training was always preceded by a 10 min warm-up at 10 m/min and followed by a 5 min cool-down at 10 m/min. Mice were encouraged to run using a mild electric shock or hindlimb stimulation. A matching set of mice (n=15) served as sedentary controls. These mice were exposed to the treadmill and similar encouragements, on the same days as exercised mice, but were not exercised.

Tissue Collection

To avoid acute exercise effects, mice were euthanized 2 days following their last training session. Mice were briefly anaesthetized with isoflurane (Abraxis Bioscience) and blood was collected via facial vein puncture into heparinized tubes (Sigma-Aldrich). Mice were then euthanized via cervical dislocation. Spleens were removed and then either flash frozen or minced into a cell suspension. Mononuclear cells were isolated from spleen cell suspensions and blood using density gradient centrifugation (Ficoll-Paque; GE Healthcare).

Hematopoietic Colony Forming Assays

2×10^5 exercised and sedentary spleen and blood cells were plated separately in triplicate into Mouse Methylcellulose Complete Medium (R&D Systems). Burst forming unit erythroid (BFU-E), colony forming unit (CFU) granulocyte monocyte (CFU-GM), and CFU granulocyte erythrocyte monocyte megakaryocyte (CFU-GEMM) colonies were scored 12 d later on a Nikon Eclipse TS100 microscope.

Flow Cytometry

The number of lineage negative (lineage panel; BD Pharmingen), c-kit positive (anti-mouse c-Kit 2B8; eBiosciences), and Sca-1 positive (anti-mouse Sca-1 E13–161.7; eBiosciences) cells in each exercised and sedentary spleen mononuclear cell isolate was measured. The numbers of single lineage panel positive cells were also quantified. Analysis was done with a CyFlow Space flow cytometer (Partec). Gating and compensation were established based on single-stained and unstained controls. Cells were quantified as a percentage of total input.

RNA Isolation and qPCR

Total RNA was isolated from exercised and sedentary spleens using a combination TRIzol (Life Technologies Inc.) and total RNA kit (Omega Bio-Tek) method (42). RNA was reverse transcribed using a commercially available kit (high-capacity cDNA reverse transcription kit; Life Technologies Inc.) with a Mastercycler epGradient Thermal Cycler (Eppendorf Canada). Quantitative reverse transcriptase polymerase chain reaction (qPCR) reactions were conducted using a Mastercycler epRealplex 2S qPCR (Eppendorf Canada) and prepared using an epMotion 5075 (Eppendorf Canada) pipetting robot. All samples were normalized to GAPDH, and fold changes in gene expression were calculated using the $\Delta\Delta$Ct method (37). Primer sequences can be found in Supplementary Table 1.

Statistics

Statistical analysis was performed using Sigma Stat 12.0 analysis software (Systat Software). Data and graphs are expressed as mean ± standard error (SE) with $p \leq 0.05$ considered significant. Statistical

differences between groups were determined using Student's t tests or one way ANOVA. Tukey corrections were applied to account for multiple comparisons.

Results

Spleen contained higher total CFU than blood

Total blood and spleen CFU were measured for both exercised and sedentary mice. CFU are measured as a single hematopoietic progenitor cell capable of proliferating and giving rise to a larger colony of more differentiated hematopoietic cells. At baseline, total CFU in sedentary blood numbered 10 per input of 10^6 cells (**Figure 1**). Exercise increased the number of total blood CFU to 35 per 10^6 cells. Sedentary spleen contained 40 total CFU per 10^6 cells; significantly different than sedentary blood ($p<0.05$), though this number was not significantly different than exercised blood. Exercised spleen contained 63 total CFU per 10^6 cells, significantly different than all other total CFU measurements ($p<0.05$).

Exercise increased the number of lineage positive cell types in spleen

The number of lineage (Lin) positive mononuclear cell types were measured with flow cytometry in exercised and sedentary spleen (**Figure 2**). Lineage positive (Ly6C, Ter119, CD3, CD11b, and CD45) cell types represent lineage committed hematopoietic progenitors and/or partially differentiated hematopoietic cell types. Ly6C is expressed on monocyte/macrophages (30); exercise increased the number $Ly6C^+$ cells in spleen by 18.6% ($p<0.05$). CD11b marks various monocytes, macrophages, and granulocytes (60); exercise increased $CD11b^+$ cells in spleen by 18.3% ($p<0.05$). The number of $CD45^+$ cells in exercised spleen, a pan leukocyte marker expressed on all hematopoietic cells except erythrocytes and platelets (35), was increased by 11.8% ($p<0.05$). The number of $CD3e^+$ cells in exercised spleen, a T cell marker (53), were increased by 29.6% ($p<0.05$). Finally, $Ter119^+$ cells taken from exercised spleen, a marker of the late stages of the erythroid lineage (32), were increased by 28.2% ($p<0.05$). All above comparisons were made against mononuclear cells taken from sedentary spleens.

Exercise increased the number of hematopoietic progenitor cells in spleen

As opposed to Lin^+ cell types, $Lin^- Sca1^+ c\text{-}Kit^+$ (LSK), CFU-GEMM, BFU-E, and CFU-GM represent true hematopoietic stem and progenitor cells. LSK cells, multipotent HSPC capable of entirely reconstituting an irradiated recipient (46), were increased in exercised spleen by nearly 3.5 fold ($p<0.05$) (**Figure 3A**). CFU-GEMM, a somewhat more differentiated but still multipotent HSPC (13), were also increased in exercised spleen by more than 4 fold ($p<0.05$) (**Figure 3B**). The number of BFU-E in exercised spleen, a committed erythroid progenitor (52), increased by 4.5 fold ($p<0.05$) (**Figure 3C**). All above comparisons made against mononuclear cells isolated from sedentary spleen. Finally, no differences in the number of CFU-GM, a committed granulocyte monocyte progenitor (52), were detectable between exercised and sedentary spleens (**Figure 3D**).

HSC homing, adhesion, and quiescence gene expression was increased in exercised spleen

The expression of genes associated with HSPC homing and adhesion were measured with qPCR. Angiopoietin-1 (Ang1) and Tie2 facilitate the ability of HSC in bone marrow to become quiescent and adhere to the endosteal niche (3). In exercised spleen, expression of Ang1 was increased by 6 fold ($p<0.05$) and expression of Tie2 was increased by almost 4 fold ($p<0.05$), when compared to sedentary controls (**Figure 4**). Similarly, SDF-1 and CXCR4 coordinate HSC homing to bone marrow and help to facilitate engraftment of HSC in their niche (65). In exercised spleen, expression of SDF-1 was increased by more than 5 fold ($p<0.05$) and expression of CXCR4 was increased by more than 3 fold ($p<0.05$), when compared to sedentary controls (**Figure 4**).

Angiogenic associated growth factor expression was increased in exercised spleen

Finally, expression of genes associated with angiogenesis was also analyzed with qPCR (**Figure 5**). VEGF and VEGFR2 signalling leads to endothelial cell proliferation, migration, and vessel formation (2); exercise increased spleen expression of these genes by 11 fold ($p<0.05$) and almost 3 fold ($p<0.5$), respectively, when compared to sedentary controls. Expression of MMP2, another facilitator of

angiogenesis (58), also increased in exercised spleen by more than 8 fold ($p<0.05$), when compared to sedentary controls. Expression of PECAM and VCAM1, adhesion molecules expressed on endothelial cells that facilitate adherence and migration of blood cells through the endothelium of blood vessels (54), also increased by more than 2 fold ($p<0.05$) and 8 fold ($p<0.05$), respectively, in exercised spleens when compared to sedentary controls.

Hematopoietic growth factor expression was increased in exercised spleen

qPCR was also used to analyze changes in hematopoietic growth factor expression in spleen. GM-CSF and IL3, cytokines that support the development of many hematopoietic cell types, increased in expression by more than 2 and almost 3 fold, respectively, in exercised spleens when compared to sedentary controls ($p<0.05$) (**Figure 6**). SCF, another hematopoietic cytokine, was also found to be expressed in spleen but no significant differences between exercised and sedentary mice could be detected. PDGF and HGF, two multi-functional growth factors also able to influence hematopoiesis, were found to be expressed in spleen and their expression trended higher in exercised samples than in sedentary controls ($p=0.08$ and $p=0.09$, respectively) (**Figure 6**).

Discussion

Blood contains a baseline level of circulating HSPC at all times. It is estimated in humans that this number is 1-2% of the total HSPC pool (10). Here we demonstrate that there are more total CFU in spleen than there are in blood, in both sedentary and exercised states (**Figure 1**). EMH in mice is always active at some level, this is no surprise. Per previous reports (5), exercise training was also able to increase the number of circulating CFU in blood (**Figure 1**). As the mice in this study were sacrificed two days after their last exercise session this measure represents a baseline level of circulating HSPC, not HSPC mobilized temporarily by exercise (6). The novel observation, however, is that the number of total CFU in spleen taken from exercised animals was higher than that of sedentary spleen (**Figure 1**). As the primary role of spleen is blood filtration, the ability of exercise to increase numbers of circulating HSPC through training (**Figure 1**) and temporarily after acute exercise may have contributed to this. That is, with a

greater number of cells in circulation presented to the spleen there is a greater likelihood of seeding the splenic hematopoietic niche.

Supporting the idea that exercise increases splenic EMH, the number of lineage positive cell populations isolated from the spleens of exercised animals was higher than that of sedentary animals (**Figure 2**). Lineage positive populations represent terminally differentiated blood cell populations or HSPC committed to a particular blood cell lineage (15). The presence of these cells demonstrates that the spleen is hematopoietically active; HSPC within the spleen, proliferating and differentiating, giving rise to blood cell progeny. As well, exercise increased the number of all lineage positive cells rather than a specific subtype. Unlike EMH associated with various myeloproliferative disorders (50), the increase in lineage positive cells was not on the order of several magnitudes and specific lineages of blood were not over represented.

The presence of several multipotent, undifferentiated cell types in spleen also indicates that it is hematopoietically active. As well, exercise increased the number of these cells: LSK, CFU-GEMM, and BFU-E in the spleen all increased after exercise training (**Figure 3**). LSK are cells capable of giving rise to both myeloid and lymphoid progenitors, CFU-GEMM are capable of giving rise to all myeloid cell types, and BFU-E are a committed erythroid progenitors. The exercise mediated increase in all types of committed lineage positive cells is explained by the presence of these multipotent HSPC. Interestingly, despite the fact that the number of white blood lineage committed cells was increased by exercise, the immediate progenitor for granulocytes and monocytes, CFU-GM, did not increase (**Figure 3**). Unlike pathogenic myeloproliferative disorders, multipotent progenitors for all blood cell types were equally represented. Lymphoma and leukemia, for example, typically present higher numbers of splenic CFU-GM progenitors with small increases in other progenitor cell populations (26).

The presence of functional HSPC in the spleen, and the ability of exercise to increase the numbers of these cells, implies that a splenic hematopoietic niche exists and that exercise is able to influence it. Without a niche microenvironment providing appropriate signalling, HSPC do not self renew, proliferate,

or differentiate (11). Unfortunately, unlike the medullary hematopoietic niche, the splenic hematopoietic niche is poorly understood. Only a handful of studies have focused on the topic. *In vitro*, several spleen cell types have been shown to support hematopoiesis in a co-culture environment. Endothelial cell types in particular have been shown to support maturation of erythrocytes (67) and, when allowed to develop into more mature tube like structures, support the development of other HSPC (18). Spleen stromal cells have also been shown to support several types of hematopoiesis (48). *In vivo*, endothelial cells, macrophages, dendritic cells, reticular cells, and fibroblasts all have potential to contribute to a splenic hematopoietic niche (69). Vascular endothelial cells and *Tcf21*$^{+}$ stromal cells in particular have been associated with a potential splenic hematopoietic niche; conditional deletion of SCF and SDF1 from these cell types severely reduces EMH (28). Spleen macrophages, through adhesion with VCAM1, have also been shown to retain HSPC in the spleen (20).

Using qPCR, the expression of several genes associated with function of the medullary and splenic niche were measured. CXCR4 and its ligand SDF1 are critical for regulating hematopoietic homing; SDF1 is released by niche cells and HSPC expressing CXCR4 migrate to its source (36). In the medullary hematopoietic niche, most HSPC are found in close proximity to SDF1 expressing sinusoidal endothelial and endosteal cells (59). Exercise was able to increase spleen expression of both SDF1 and CXCR4 (**Figure 4**). Similar to the liver, where EMH is increased when the liver is made to express SDF1 (44), increased spleen expression of SDF1 may mobilize HSPC to the spleen and lead to EMH. In a similar fashion, the Tie2 receptor and its ligand Ang1 are expressed on both HSPC and cells that make up the bone marrow niche. Signalling through this axis is responsible for maintaining HSPC quiescence and maintaining the vascular endothelial niche (68). When either of these genes are conditionally deleted, HSPC number in bone marrow quickly decreases (3). Here, exercise increased spleen expression of both Ang1 and Tie2 (**Figure 4**). Increased Ang1 and Tie2 expression in spleen may play a role in building up the splenic hematopoietic niche and promoting quiescence of HSPC localized to the spleen through SDF1/CXCR4 homing.

Given that the medullary hematopoietic niche is built in part on vascular endothelial cells, and that endothelial cells have been implicated in forming a splenic hematopoietic niche, expression of genes associated with endothelial cell function and angiogenesis were measured. VEGF and VEGFR2 play important roles in angiogenesis, stimulating proliferation and migration of endothelial cells (33). As well, these genes can also aid in HSPC proliferation and survival (25) and stimulate release of SDF1 from vascular endothelial cells (62). Here, exercise increased spleen expression of both VEGF and VEGFR2 (**Figure 5**). Exercise may alter the structure of the splenic hematopoietic niche through increased expression of these genes. Similarly, MMP2 expression was increased in exercised spleens (**Figure 5**). MMPs are proteases that facilitate angiogenesis through cleavage of extracellular matrix proteins (70). Again, exercise increased expression of genes associated with promoting angiogenesis may work towards developing a more suitable splenic hematopoietic niche. Expression of PECAM was also increased in exercised spleens (**Figure 5**). PECAM is expressed on most endothelial cells. PECAM expression on vascular endothelial cells in bone marrow allows for efficient transition of HSPC between blood and bone marrow (56). As well, HSPC in the bone marrow vascular niche are found in close association with $PECAM^+$ endothelial cells (34). Similar to VEGF, VEGFR2, and MMP2, exercise induced expression of spleen PECAM may indicate the build-up, or help facilitate development, of a more functional splenic vascular hematopoietic niche. Finally, expression of VCAM1 was also increased in exercised spleens (**Figure 5**). When VCAM1 is ablated in mice, HSPC are released from bone marrow and circulating HSPC are increased (64). Here, exercise may increase retention of HSPC in spleen by way of increased numbers of $VCAM1^+$ macrophages. VCAM1 is also an endothelial cell marker and, similar to the endothelial cell associated genes mentioned previously, may indicate a build-up of a splenic endothelial hematopoietic niche.

Just as HSPC in their niche need appropriate signalling and adhesion molecules, they also require the presence of many different hematopoietic growth factors. Granulocyte macrophage colony stimulating factor (GM-CSF) supports the maturation of HSPC leading to white blood cell development (27). Here,

we show that exercise increases spleen expression of GM-CSF (**Figure 6**). Spleen expression of IL3, a cytokine needed to stimulate myeloid lineage development in HSPC (27), is also increased with exercise. Previous reports demonstrate that certain niche cells must express SCF in order to maintain EMH (28). While this study did not detect differences in SCF expression between sedentary and exercised mice, spleen was found to express SCF at detectable levels. HGF has roles in HSPC proliferation (45); exercise was able to increase its expression in spleen (**Figure 6**). Expression of PDGF, a growth factor able to stimulate endothelial cell development and angiogenesis (71), as well as causing stromal cells to release other hematopoietic growth factors (72), was also increased as a result of exercise. Aside from the hematopoietic growth factors mentioned, spleen is known to express many others. The observed increases in spleen HSPC content following exercise, as well as the increases in lineage positive hematopoietic progenitors, could only have occurred if the spleen were able to express certain hematopoietic growth factors to support their development. It is likely that exercise increased expression of these factors also facilitated the higher numbers of HSPC observed in exercised spleens.

Given that exercise increases splenic EMH in mice, several questions remain: why and for what purpose. Generally, EMH is triggered by hematopoietic stresses or disorders. A host of issues, including myelofibrosis (1), osteopetrosis (49), anemia (9), pregnancy (23), infection (8), myeloablation (47), blood loss (28), and myocardial infarction (19), all lead to increase splenic EMH in mice. However, describing a ten week exercise training program as equivalent to a hematopoietic disorder seems inaccurate. It could be that the stress of exercise leads to increased splenic EMH as a compensatory mechanism to maintain blood homeostasis. Certainly, exercise can induce intravascular hemolysis through increased sheer stress (63), leading to increased erythropoiesis (38), resulting in a pool of younger, more flexible erythrocytes (39). Yet the magnitude of intravascular hemolysis induced by exercise is in no way equivalent to the level of bloodletting required to stimulate EMH. One study in humans reports erythrocyte destruction at 2% of the population per day at the start of an exercise training period, quickly decreasing as training

continued (57). Meanwhile, in mice, blood loss across a two week period cumulatively equivalent to the total blood volume of the animal is used to stimulate EMH (28).

The perception that EMH is only associated with pathological processes does make sense. When major blood disorders are present is when EMH is most noticeable. Certainly, splenic EMH can be the source of a problem rather than a symptom, cases of splenomegaly following myelofibrosis being one example (55). Yet when EMH is triggered by physiological issues, following blood loss (28) or during pregnancy (23), it is neither a symptom to be treated nor the root of the issue. As well, the EMH observed in the current study, where by all common knowledge the exercised animals should have been healthier than their sedentary counterparts, wasn't pathological in nature. In these cases EMH might be more accurately described as an adaptive response; a backup for regular hematopoiesis, helping to alleviate strain on the hematopoietic system.

Exercise mediated increases in splenic EMH may instead represent a new equilibrium, the carrying capacity of the hematopoietic system increased as a result of the changes induced by exercise. Certainly, hematopoietic equilibrium can be depressed, in the example of nephrectomy (29), or stimulated, through regular hematopoietic growth factor administration (14). Even base physiological variation can result in individuals with higher or lower red blood cell counts (41). Exercise, resulting in increased hematopoietic growth factor production, a buildup of medullary and extramedullary hematopoietic niches, and increased hematopoietic mobilization, may lead to a more robust level of hematopoiesis better able to deal with hematopoietic stress. Indeed, survival of animals following a grievous hematopoietic insult, lethal irradiation followed by bone marrow transplantation, is increased by more than three fold in recipients that were exercise trained (17). In this particular case, whether or not splenic EMH contributed significantly to increased hematopoietic recovery is unknown. In situations where the spleen is shielded during irradiation, however, HSPC from the spleen are able to increase the rate of hematopoietic recovery (24).

Aside from its traditional role in immunity and blood filtration, the spleen acts as a hematopoietic organ. Here we show that exercise increases splenic EMH in mice. This occurs devoid of any pathological blood disorders or extreme hematopoietic stresses. The findings herein may increase understanding of EMH as an extension of the hematopoietic system rather than a symptom of another issue. Whether or not exercise may stimulate EMH in human spleen, where baseline levels are much lower than in mice, is questionable. Using exercise to strengthen the ability of spleen to deal with hematopoietic stress may potentially be beneficial. The build-up of a splenic hematopoietic niche preceding myeloablation and bone marrow transplantation, for example, could give donor HSPC additional microenvironments to engraft into, facilitating blood reconstitution. Further study is required to answer these questions.

4.1 – Figure 1

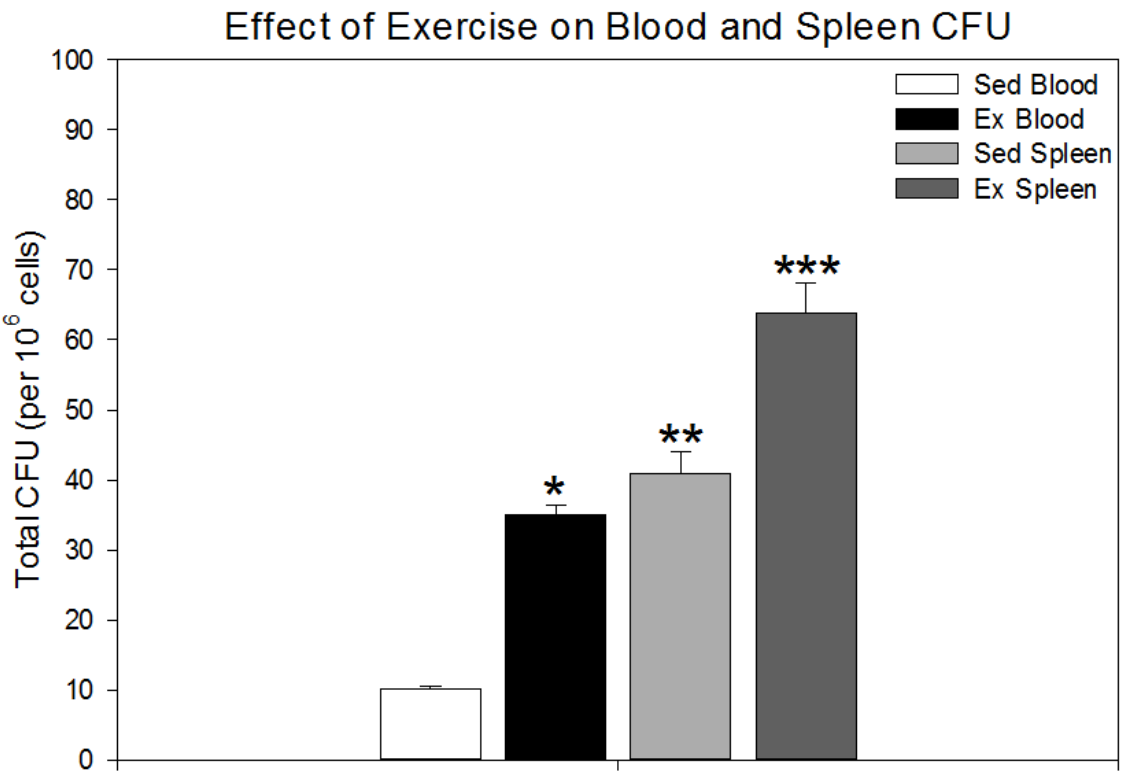

Figure 1: Total CFU in blood and spleen, from exercised and sedentary mice. Data is presented as CFU per 10^6 mononuclear cells input. * indicates $p<0.05$ within blood, ** indicates $p<0.05$ between spleen and blood, and *** indicates $p<0.05$ from all other measures.

4.2 – Figure 2

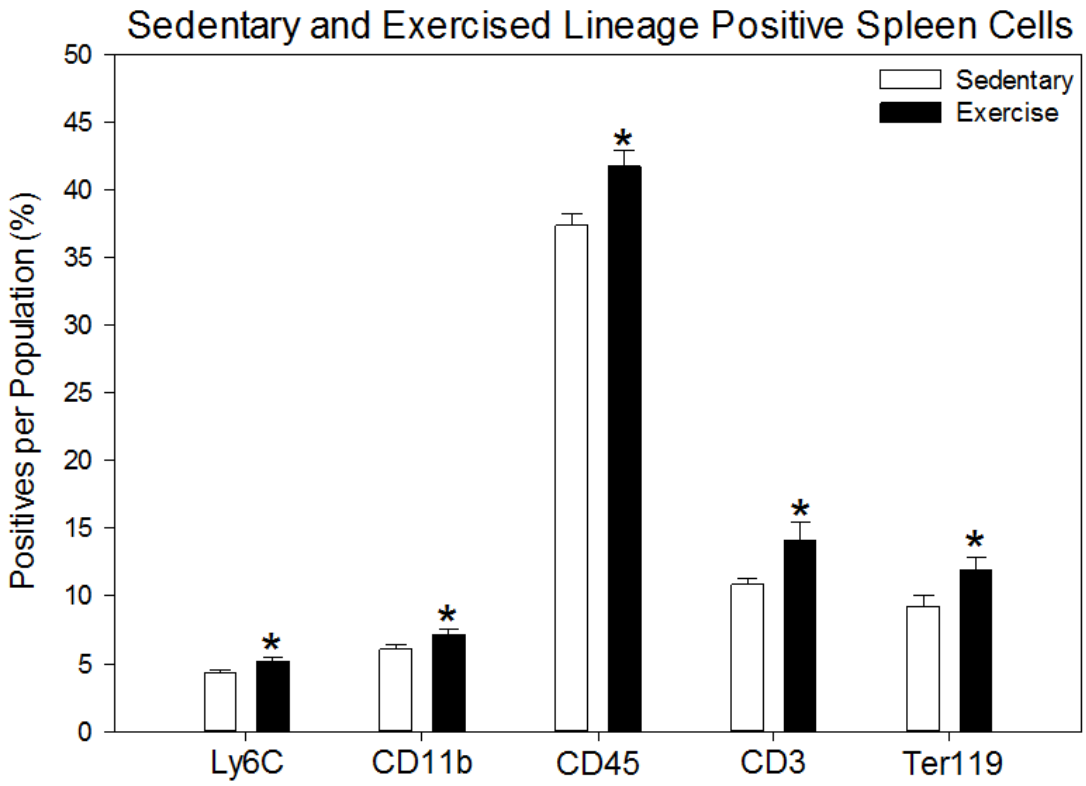

Figure 2: Percentage of lineage positive cell types in exercised and sedentary spleen. Mononuclear cells were taken from spleen and quantified with flow cytometry for each lineage marker. Left to right: $Ly6C^+$, $CD11b^+$, $CD45^+$, $CD3e^+$, $Ter119^+$. * indicates $p<0.05$.

4.3 – Figure 3

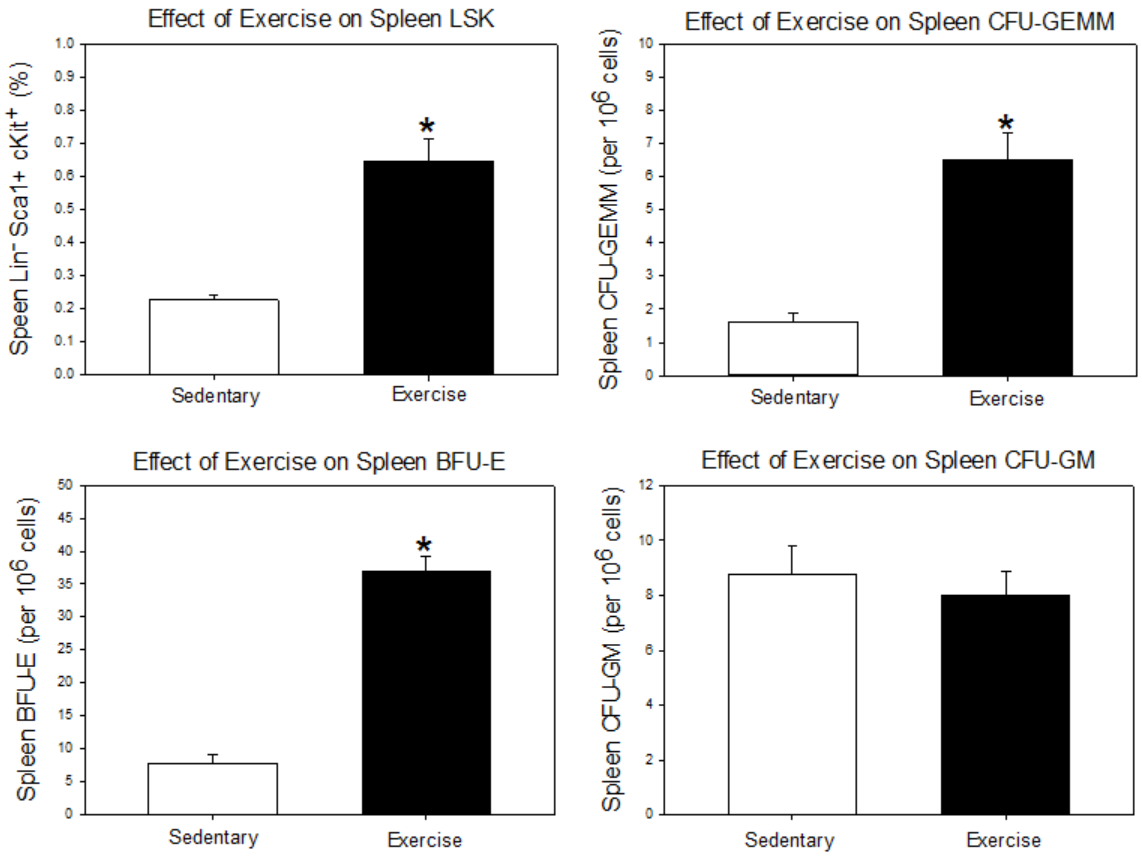

Figure 3: Number of hematopoietic stem and progenitor cells in exercised and sedentary spleen. **A)** Percentage of LSK from spleen, analyzed with flow cytometry. **B)** Number of CFU-GEMM colonies per 10^6 spleen mononuclear cells. **C)** Number of BFU-E colonies per 106 spleen mononuclear cells. **D)** Number of CFU-GM colonies per 106 spleen mononuclear cells. * indicates $p<0.05$.

4.4 – Figure 4

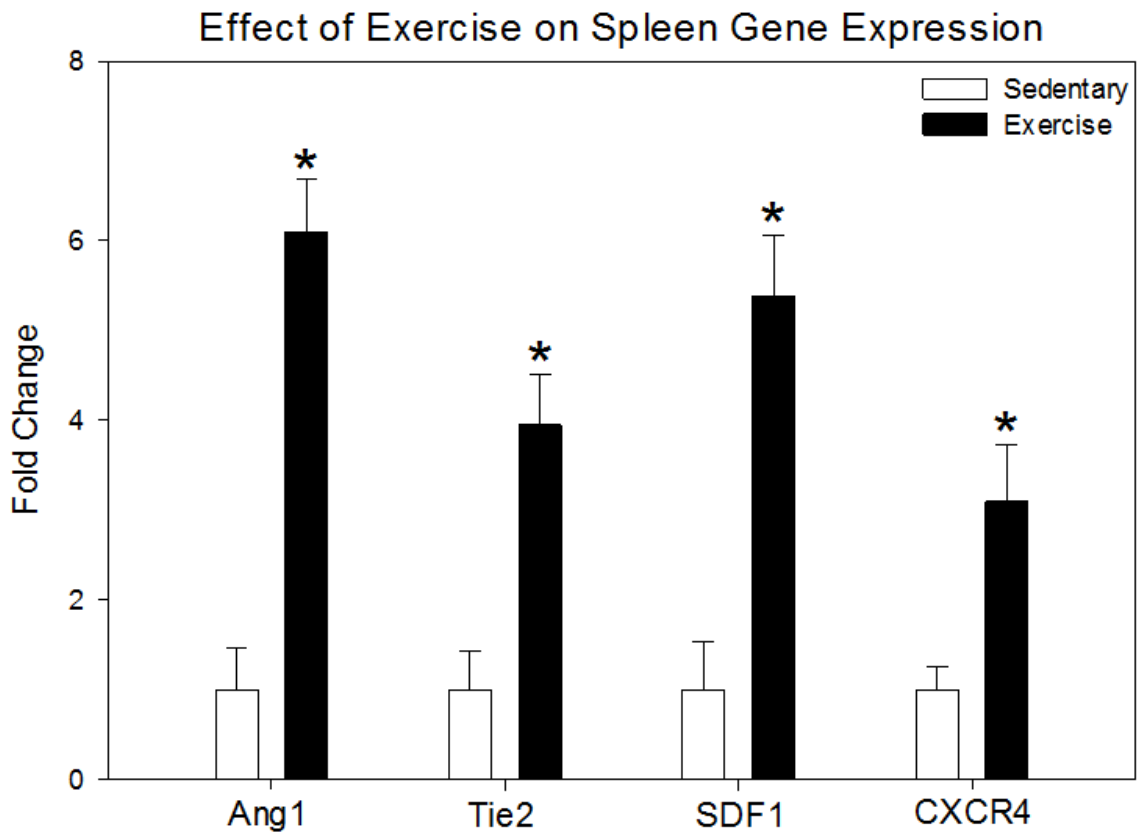

Figure 4: Expression analysis of genes associated with hematopoietic stem and progenitor cell homing, adhesion, and quiescence. Angiopoietin-1, Tie2, SDF1, and CXCR4 expression was measured in exercised and sedentary spleens. * indicates $p<0.05$.

4.5 – Figure 5

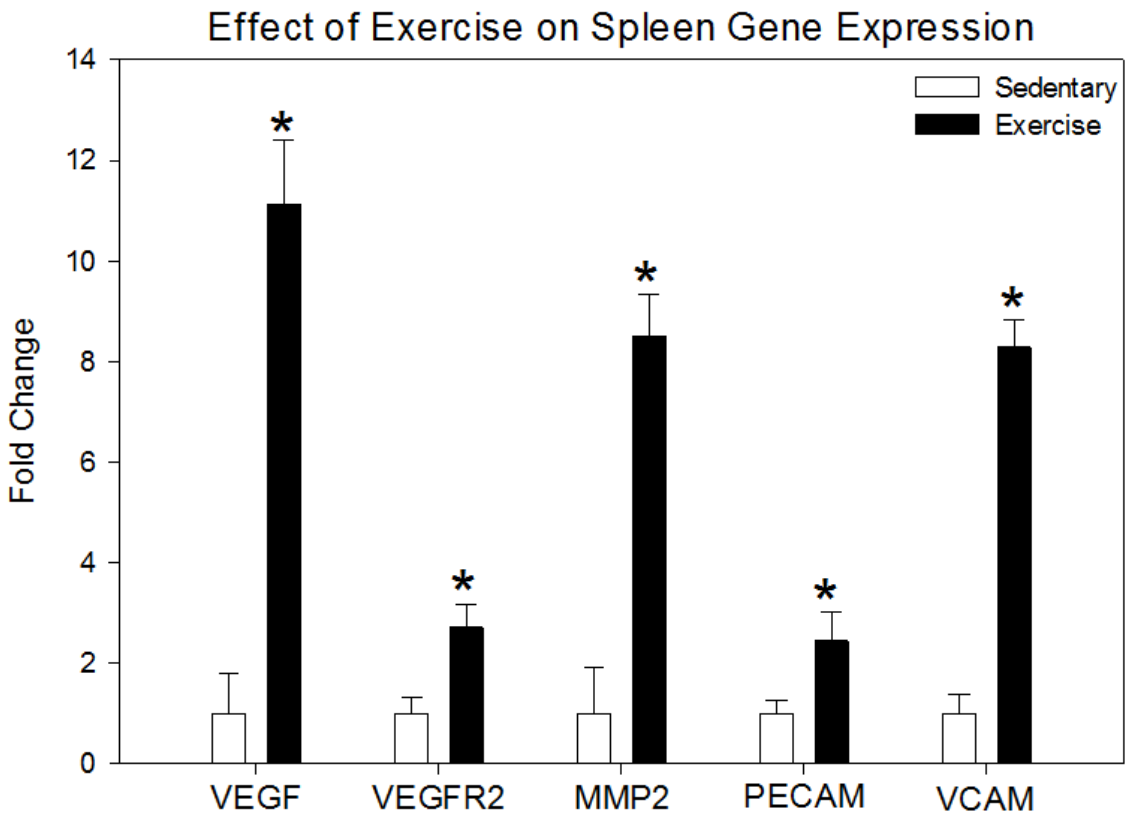

Figure 5: Expression analysis of genes associated with angiogenesis and endothelial cell function. VEGF, VEGFR2, MMP2, PECAM, and VCAM1 expression was measured in exercised and sedentary spleens. * indicates $p<0.05$.

4.6 – Figure 6

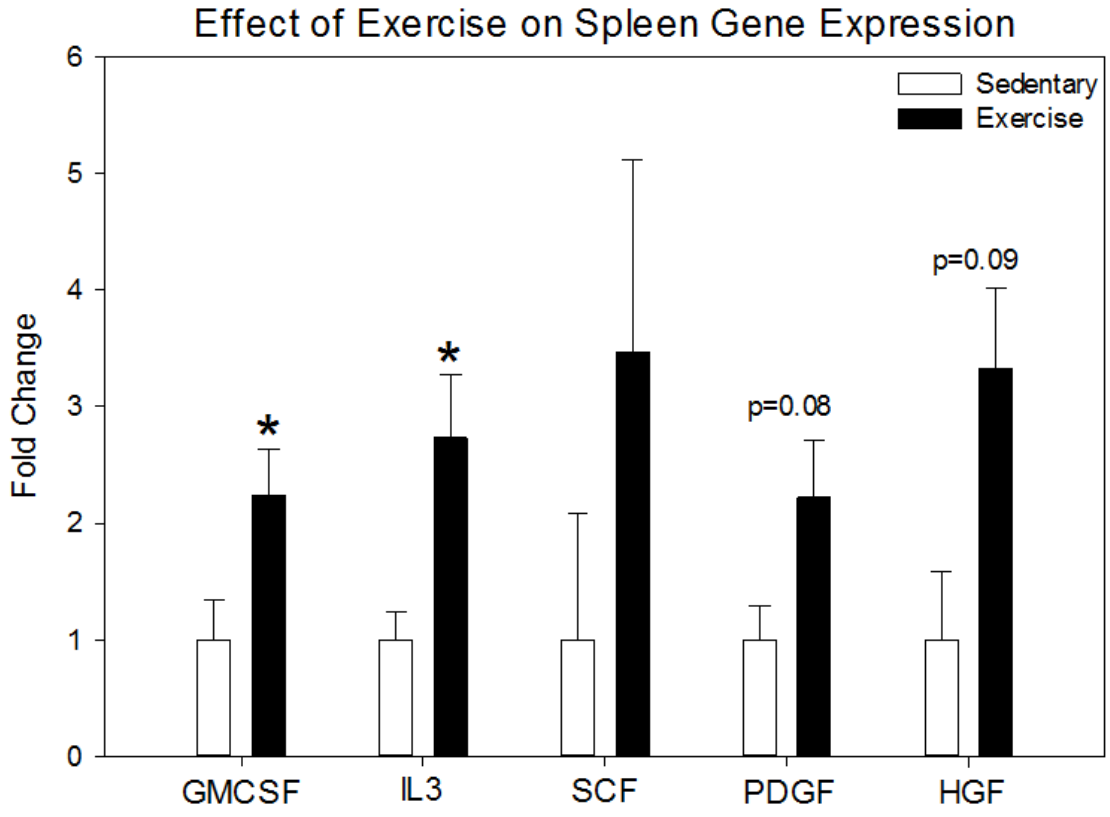

Figure 6: Expression analysis of hematopoietic growth factors and other growth factors known to influence hematopoiesis. GM-CSF, IL3, SCF, PDGF, and HGF expression was measured in exercised and sedentary spleens. * indicates $p<0.05$, larger p values are otherwise indicated.

4.7 – Primers

Primer		Sequence
Ang1	Forward	CTCAGTGGCTGCAAAAACTTG
Ang1	Reverse	CAGAATTTCATTTGTCTGTTGGA
Tie2	Forward	GTGCTTATTTCTGTGAAGGTCGAG
Tie2	Reverse	TCCGTATCCTTATAGCCTGTCCTC
SDF1	Forward	CGTGAGGCCAGGGAAGAGT
SDF1	Reverse	TGATGAGCATGGTGGGTTGA
CXCR4	Forward	CTCGCTATTGTCCACGCCAC
CXCR4	Reverse	CCCTGACTGATGTCCCCCTG
GM-CSF	Forward	ACCACCTATGCGGATTTCAT
GM-CSF	Reverse	TCATTACGCAGGGACAAAAG
IL3	Forward	TACATCTGCGAATGACTCTGC
IL3	Reverse	GGCTGAGGTGGTCTAGAGGTT
SCF	Forward	CTCTCTCTTTCTGTTGCAAC
SCF	Reverse	GCTTGACTACTCTTCTGGAC
PDGF	Forward	GGCCACACACCTTCTCTGAT
PDGF	Reverse	GTGGAGGAGCAGACTGAAGG
HGF	Forward	CGGGGTAAAGACCTACAGG
HGF	Reverse	CCCATTGCAGGTCATGC

VEGF	Forward	TTACTGCTGTACCTCCACCA
VEGF	Reverse	ACAGGACGGCTTGAAGATGTA
VEGFR2	Forward	GGCGGTGGTGACAGTATCTT
VEGFR2	Reverse	TCTCCGGCAAGCTCAAT
MMP2	Forward	CTAAGCTCATCGCAGACTCCTGGAATG
MMP2	Reverse	GGTTCTCCAGCTTCAGGTAATAAGCAC
PECAM	Forward	CTGCCAGTCCGAAAATGGAAC
PECAM	Reverse	CTTCATCCACCGGGGCTATC
VCAM	Forward	AGTTGGGGATTCGGTTGTTCT
VCAM	Reverse	CCCCTCATTCCTTACCACCC
GAPDH	Forward	CATCACCATCTTCCAGGAGC
GAPDH	Reverse	ATGCCAGTGAGCTTCCCGTC

4.8 - References

1. Abdel-Wahab OI, Levine RL. Primary myelofibrosis: update on definition, pathogenesis, and treatment. *Annu Rev Med* 60: 233–245, 2009.

2. Abhinand CS, Raju R, Soumya SJ, Arya PS, Sudhakaran PR. VEGF-A/VEGFR2 signalling network in endothelial cells relevant to angiogenesis. *J Cell Commun Signal* 10: 347–354, 2016.

3. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 118: 149–161, 2004.

4. Attar EC, Scadden DT. Regulation of hematopoietic stem cell growth. *Leukemia* 18: 1760–1768, 2004.

5. Baker JM, De Lisio M, Parise G. Endurance exercise training promotes medullary hematopoiesis. *FASEB J Off Publ Fed Am Soc Exp Biol* 25: 4348–4357, 2011.

6. Baker JM, Nederveen JP, Parise G. Aerobic exercise in humans mobilizes HSCs in an intensity-dependent manner. *J Appl Physiol Bethesda Md 1985* 122: 182–190, 2017.

7. Baker JM, Parise G. Skeletal Muscle Erythropoietin Expression Is Responsive to Hypoxia and Exercise. *Med Sci Sports Exerc* 48: 1294–1301, 2016.

8. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* 465: 793–797, 2010.

9. Bennett M, Pinkerton PH, Cudkowicz G, Bannerman RM. Hemopoietic progenitor cells in marrow and spleen of mice with hereditary iron deficiency anemia. *Blood* 32: 908–921, 1968.

10. Bhattacharya D, Czechowicz A, Ooi AGL, Rossi DJ, Bryder D, Weissman IL. Niche recycling through division-independent egress of hematopoietic stem cells. *J Exp Med* 206: 2837–2850, 2009.

11. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. *Blood* 125: 2621–2629, 2015.

12. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity* 39: 806–818, 2013.

13. Carow CE, Hangoc G, Broxmeyer HE. Human multipotential progenitor cells (CFU-GEMM) have extensive replating capacity for secondary CFU-GEMM: an effect enhanced by cord blood plasma. *Blood* 81: 942–949, 1993.

14. Carrington PA, Hill RJ, Stenberg PE, Levin J, Corash L, Schreurs J, Baker G, Levin FC. Multiple in vivo effects of interleukin-3 and interleukin-6 on murine megakaryocytopoiesis. *Blood* 77: 34–41, 1991.

15. Challen GA, Boles N, Lin KK, Goodell MA. Mouse Hematopoietic Stem Cell Identification And Analysis. *Cytom Part J Int Soc Anal Cytol* 75: 14–24, 2009.

16. Crosby WH. Hematopoiesis in the Human Spleen. *Arch Intern Med* 143: 1321–1322, 1983.

17. De Lisio M, Baker JM, Parise G. Exercise promotes bone marrow cell survival and recipient reconstitution post-bone marrow transplantation, which is associated with increased survival. *Exp Hematol* 41: 143–154, 2013.

18. Despars G, O'Neill HC. Splenic endothelial cell lines support development of dendritic cells from bone marrow. *Stem Cells Dayt Ohio* 24: 1496–1504, 2006.

19. Dutta P, Courties G, Wei Y, Leuschner F, Gorbatov R, Robbins CS, Iwamoto Y, Thompson B, Carlson AL, Heidt T, Majmudar MD, Lasitschka F, Etzrodt M, Waterman P, Waring MT, Chicoine AT, van der Laan AM, Niessen HWM, Piek JJ, Rubin BB, Butany J, Stone JR, Katus HA, Murphy SA, Morrow DA, Sabatine MS, Vinegoni C, Moskowitz MA, Pittet MJ, Libby P, Lin CP, Swirski FK, Weissleder R, Nahrendorf M. Myocardial infarction accelerates atherosclerosis. *Nature* 487: 325–329, 2012.

20. Dutta P, Hoyer FF, Grigoryeva LS, Sager HB, Leuschner F, Courties G, Borodovsky A, Novobrantseva T, Ruda VM, Fitzgerald K, Iwamoto Y, Wojtkiewicz G, Sun Y, Silva ND, Libby P, Anderson DG, Swirski FK, Weissleder R, Nahrendorf M. Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. *J Exp Med* 212: 497–512, 2015.

21. Emmons R, Niemiro GM, Owolabi O, Lisio MD. Acute exercise mobilizes hematopoietic stem and progenitor cells and alters the mesenchymal stromal cell secretome. *J Appl Physiol* 120: 624–632, 2016.

22. von Engelhardt W. Cardiovascular effects of exercise and training in horses. *Adv Vet Sci Comp Med* 21: 173–205, 1977.

23. Fowler JH, Nash DJ. Erythropoiesis in the spleen and bone marrow of the pregnant mouse. *Dev Biol* 18: 331–353, 1968.

24. Fujioka S, Hirashima K, Kumatori T, Takaku F, Nakao K. Mechanism of Hematopoietic Recovery in the X-Irradiated Mouse with Spleen or One Leg Shielded. *Radiat Res* 31: 826–839, 1967.

25. Gerber H-P, Ferrara N. The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med Berl Ger* 81: 20–31, 2003.

26. Greenberg PL, Steed SM. Splenic granulocytopoiesis and production of colony-stimulating activity in lymphoma and leukemia. *Blood* 57: 119–129, 1981.

27. Hara T, Miyajima A. Function of the IL-3 receptor system in hematopoiesis. In: *Gene Technology*. Springer, Berlin, Heidelberg, p. 295–307.

28. Inra CN, Zhou BO, Acar M, Murphy MM, Richardson J, Zhao Z, Morrison SJ. A perisinusoidal niche for extramedullary haematopoiesis in the spleen. *Nature* 527: 466–471, 2015.

29. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. *Nature* 179: 633–634, 1957.

30. Jutila MA, Kroese FG, Jutila KL, Stall AM, Fiering S, Herzenberg LA, Berg EL, Butcher EC. Ly-6C is a monocyte/macrophage and endothelial cell differentiation antigen regulated by interferon-gamma. *Eur J Immunol* 18: 1819–1826, 1988.

31. Kim CH. Homeostatic and pathogenic extramedullary hematopoiesis. *J Blood Med* 1: 13–19, 2010.

32. Kina T, Ikuta K, Takayama E, Wada K, Majumdar AS, Weissman IL, Katsura Y. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *Br J Haematol* 109: 280–287, 2000.

33. Koch S, Claesson-Welsh L. Signal Transduction by Vascular Endothelial Growth Factor Receptors. *Cold Spring Harb Perspect Med* 2, 2012.

34. Kusumbe AP, Ramasamy SK, Itkin T, Mäe MA, Langen UH, Betsholtz C, Lapidot T, Adams RH. Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* 532: 380–384, 2016.

35. Lai L, Alaverdi N, Maltais L, Morse HC. Mouse cell surface antigens: nomenclature and immunophenotyping. *J Immunol Baltim Md 1950* 160: 3861–3868, 1998.

36. Lau TT, Wang D-A. Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine. *Expert Opin Biol Ther* 11: 189–197, 2011.

37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif* 25: 402–408, 2001.

38. Mairbäurl H. Red blood cells in sports: Effects of exercise and training on oxygen supply by red blood cells. *Front Physiol* 4, 2013.

39. Mairbaurl H, Humpeler E, Schwaberger G, Pessenhofer H. Training-dependent changes of red cell density and erythrocytic oxygen transport. *J Appl Physiol* 55: 1403–1407, 1983.

40. Mangseth GR, Hornof WJ. A review of knowledge regarding blood volume and splenic reserve in the horse. *J Equine Vet Sci* 3: 94–98, 1983.

41. McDunough JR, Hames CG, Garrison GE, Stulb SC, Lichtman MA, Hefelfinger DC. The relationship of hematocrit to cardiovascular states of health in the Negro and white population of Evans County, Georgia. *J Chronic Dis* 18: 243–257, 1965.

42. McKay BR, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G. Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans. *J Physiol* 586: 5549–5560, 2008.

43. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol* 5: 606–616, 2005.

44. Mendt M, Cardier JE. Role of SDF-1 (CXCL12) in regulating hematopoietic stem and progenitor cells traffic into the liver during extramedullary hematopoiesis induced by G-CSF, AMD3100 and PHZ. *Cytokine* 76: 214–221, 2015.

45. Mizuno K, Higuchi O, Ihle JN, Nakamura T. Hepatocyte growth factor stimulates growth of hematopoietic progenitor cells. *Biochem Biophys Res Commun* 194: 178–186, 1993.

46. Morcos MNF, Schoedel KB, Hoppe A, Behrendt R, Basak O, Clevers HC, Roers A, Gerbaulet A. SCA-1 Expression Level Identifies Quiescent Hematopoietic Stem and Progenitor Cells. *Stem Cell Rep* 8: 1472–1478, 2017.

47. Morrison SJ, Wright DE, Weissman IL. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci* 94: 1908–1913, 1997.

48. Ni K, O'Neill H. Spleen stromal cells support haemopoiesis and in vitro growth of dendritic cells from bone marrow. *Br J Haematol* 105: 58–67, 1999.

49. Nilsson SK, Bertoncello I. Age-related changes in extramedullary hematopoiesis in the spleen of normal and perturbed osteopetrotic (op/op) mice. *Exp Hematol* 22: 377–383, 1994.

50. O'Malley DP, Kim YS, Perkins SL, Baldridge L, Juliar BE, Orazi A. Morphologic and immunohistochemical evaluation of splenic hematopoietic proliferations in neoplastic and benign disorders. *Mod Pathol* 18: 1550–1561, 2005.

51. Pedersen BK. Muscles and their myokines. *J Exp Biol* 214: 337–346, 2011.

52. Pereira C, Clarke E, Damen J. Hematopoietic colony-forming cell assays. *Methods Mol Biol Clifton NJ* 407: 177–208, 2007.

53. Pinchuk LM, Filipov NM. Differential effects of age on circulating and splenic leukocyte populations in C57BL/6 and BALB/c male mice. *Immun Ageing A* 5: 1, 2008.

54. Quarmby S, Hunter RD, Kumar S. Irradiation induced expression of CD31, ICAM-1 and VCAM-1 in human microvascular endothelial cells. *Anticancer Res* 20: 3375–3381, 2000.

55. Randhawa J, Ostojic A, Vrhovac R, Atallah E, Verstovsek S. Splenomegaly in myelofibrosis—new options for therapy and the therapeutic potential of Janus kinase 2 inhibitors. *J Hematol OncolJ Hematol Oncol* 5: 43, 2012.

56. Ross EA, Freeman S, Zhao Y, Dhanjal TS, Ross EJ, Lax S, Ahmed Z, Hou TZ, Kalia N, Egginton S, Nash G, Watson SP, Frampton J, Buckley CD. A Novel Role for PECAM-1 (CD31) in Regulating Haematopoietic Progenitor Cell Compartmentalization between the Peripheral Blood and Bone Marrow. *PLOS ONE* 3: e2338, 2008.

57. Schmidt W, Maassen N, Trost F, Böning D. Training induced effects on blood volume, erythrocyte turnover and haemoglobin oxygen binding properties. *Eur J Appl Physiol* 57: 490–498, 1988.

58. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 103: 1237–1241, 1999.

59. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25: 977–988, 2006.

60. Sunderkötter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJM. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol Baltim Md 1950* 172: 4410–4417, 2004.

61. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo J-L, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science* 325: 612–616, 2009.

62. Tamma R, Ribatti D. Bone Niches, Hematopoietic Stem Cells, and Vessel Formation. *Int J Mol Sci* 18, 2017.

63. Telford RD, Sly GJ, Hahn AG, Cunningham RB, Bryant C, Smith JA. Footstrike is the major cause of hemolysis during running. *J Appl Physiol* 94: 38–42, 2003.

64. Ulyanova T, Scott LM, Priestley GV, Jiang Y, Nakamoto B, Koni PA, Papayannopoulou T. VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. *Blood* 106: 86–94, 2005.

65. Vagima Y, Lapid K, Kollet O, Goichberg P, Alon R, Lapidot T. Pathways implicated in stem cell migration: the SDF-1/CXCR4 axis. *Methods Mol Biol Clifton NJ* 750: 277–289, 2011.

66. Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF. Roles of spleen and liver in development of the murine hematopoietic system. *Exp Hematol* 30: 1010–1019, 2002.

67. Yanai N, Satoh T, Obinata M. Endothelial cells create a hematopoietic inductive microenvironment preferential to erythropoiesis in the mouse spleen. *Cell Struct Funct* 16: 87–93, 1991.

68. Zhou BO, Ding L, Morrison SJ. Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. *eLife* 4: e05521, 2015.

69. Mueller SN, Germain RN. Stromal cell contributions to the homeostasis and functionality of the immune system. Nat Rev Immunol. 2009 Sep;9(9):618–29.

70. Sang QX. Complex role of matrix metalloproteinases in angiogenesis. Cell Res. 1998 Sep;8(3):171-7.

71. Sato N, Beitz JG, Kato J, Yamamoto M, Clark JW, Calabresi P, et al. Platelet-derived growth factor indirectly stimulates angiogenesis in vitro. Am J Pathol. 1993 Apr;142(4):1119–30.

72. Xue Y, Lim S, Yang Y, Wang Z, Jensen LDE, Hedlund E-M, et al. PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells. Nat Med. 2011 Dec 4;18(1):100–10.

Chapter 5 – Mechanical loading during exercise training, but not humoral factors, increases marrow cellularity in old mice

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Abstract

Aging gradually renders bone marrow hematopoietically inactive. Endurance exercise reverses this phenotype in young mice; here, we determine the effects in aged animals. Twenty-two month old mice (n=6) underwent a progressive exercise training protocol. Marrow cellularity increased by 51% in exercised animals ($p<0.05$) and marrow CFU, CFU-GM, and CAFC increased by 12%, 71%, and 86%, respectively ($p<0.05$). Vertebral cellularity remained unchanged. The mechanical forces associated with treadmill exercise training may be responsible for these observations.

Key Words: Exercise, marrow, hematopoiesis, bone, mice, cell culture.

Introduction

Hematopoietic stem and progenitor cells found in marrow proliferate and differentiate in a highly controlled manner to maintain blood homeostasis. Early in life, marrow is hematopoietically active. With advancing age, first in the appendicular skeleton and later in the axial skeleton, the marrow cavity fills with adipocytes and becomes hematopoietically inactive (Małkiewicz and Dziedzic 2012). When blood cell homeostasis can no longer be maintained, the body is able to reconvert inactive marrow back to hematopoietically active tissue. In cases of advanced age, damage from radiation or chemotherapy, or in individuals with conditions such as aplastic anemia, this reconversion is unsuccessful and hematopoiesis cannot be properly restored (Daldrup-Link et al. 2007).

Osteoblasts, osteogenic cells that line the endosteum and make up the endosteal hematopoietic niche, positively regulate hematopoiesis. Ablation of osteoblasts leads to a direct decline in bone marrow cellularity and hematopoietic stem cell (HSC) quantity (Visnjic et al. 2001). Artificially increasing osteoblast number in the niche leads to direct increases in HSC number (Calvi et al. 2003). Conversely, adipocytes negatively regulate hematopoiesis. Mice with artificially impaired adipogenesis demonstrate increased hematopoietic recovery after bone marrow ablation (Naveiras et al. 2009). Adipocytes are unable to maintain HSC survival and self renewal (Corre et al. 2006). An age-associated shift from osteogenesis to adipogenesis in marrow thus parallels age associated marrow conversion and loss of marrow hematopoiesis (Takeshita et al. 2014).

In young animals, exercise training increases marrow cellularity, increases marrow HSC content, decreases marrow adipogenesis, increases marrow osteogenesis, and increases muscle expression of hematopoietic cytokines (Baker et al. 2011). However, the direct mechanism(s) by which exercise can influence hematopoiesis and the hematopoietic niche remain unknown. Skeletal muscle endocrine signalling may be one possibility. Exercise training has been shown to increase muscle expression of IL-3, M-CSF, and GM-CSF (Baker et al. 2011). Furthermore, skeletal muscle has been shown to produce and release EPO following exercise (Baker and Parise 2016). Mechanical forces applied to bones during

exercise may be another possible mechanism. Mesenchymal stem cells (MSC) in bone marrow can differentiate into osteoblasts and adipocytes; niche supporting or niche repressing cells. Mechanical forces both *in vitro* and *in vivo* has been shown to inhibit MSC adipogenesis (Li et al. 2015) and enhance MSC osteogenesis (Yourek et al. 2010), shifting the niche towards a phenotype more supporting of hematopoiesis.

Here we examined whether exercise training alters bone marrow cellularity in aged animals. Aged animals were used to determine if exercise can reverse the aging marrow phenotype in appendicular bones and to assess marrow changes in the axial skeleton, which only occur later in life (Ou-yang and Lu 2015).

Materials and Methods

Animals and Exercise

22 month old male C57Bl/6J mice (n=6, Charles River, Framingham, MA, USA; Jackson Laboratory, Bar Harbor, ME, USA) were exercised for 40 min/d, 3d/week, for 8 weeks on an Exer 6M treadmill (Columbus Instruments, Columbus, OH, USA) with gradually increasing speeds (Joanisse et al. 2016). Post-training, mice were euthanized three days after their last bout of exercise to avoid observing acute exercise effects. A matching set of mice were used as sedentary controls. Mice were housed 3 animals per cage, provided food and water *ad libitum*, and kept on a 12 hour light/dark cycle. Ethics approval was granted by the McMaster University Animal Research Ethics Board and conformed to standards established by the Canadian Council on Animal Care.

Histology

Humeri and ulna, together, and lumbar vertebrae were fixed (4% formaldehyde in PBS, 3-5 days, at 6.9- 7.2 pH), decalcified in a 10% HCl chelating solution (Richard-Allan Scientific, San Diego, CA, USA), paraffin embedded, longitudinally sectioned at 4 μm, and stained with hematoxylin and eosin (Sigma-Aldrich, Oakville, ON, Canada). Images were taken with a Nikon 90i using Nikon Elements AR

4.4 and analyzed with ImageJ using the Fiji package. For analysis, manually counted cell number was inversely correlated to green pixel value (RSQ>0.95, $p<0.05$). Using this correlation, overall cellularity for the entire marrow area was calculated per sample.

Hematopoietic Colony-forming Assays

Femurs and tibiae, together, were flushed with IMDM containing 2% FBS (Sigma-Aldrich, Oakville, ON, Canada) to collect bone marrow. Mononuclear cells were isolated using Ficoll Paque PLUS (GE Healthcare, Burlington, ON, Canada) and were grown in Mouse Methylcellulose Complete Medium (R&D Systems, Minneapolis, MN, USA). Erythroid (BFU-E), Granulocyte Monocyte (CFU-GM), and Granulocyte Monocyte Erythroid Megakaryocyte (CFU-GEMM) colonies were scored 12 days later. Mononuclear cells were also used to measure cobblestone area forming cells (CAFC), scored at 35 days post plating (van Os et al. 2008).

Statistics

Student's *t*-tests and Pearson correlations using SigmaStat 12.0 analysis software (Systat Software, San Jose, CA, USA) were used to determine differences between groups. CAFC frequency was calculated using L-Calc (Stem Cell Technologies, Vancouver, BC, Canada). Results are presented as means ± SEM with $p<0.05$ considered significant.

Results

Exercise increased femur and tibiae marrow colony forming capacity

Previously we demonstrated that in young animals exercise is able to restore bone marrow cellularity and HSC content (Baker et al. 2011). Here we observe that in aged animals exercise is able to do the same. In marrow taken from femur and tibiae exercise increased total CFU by 12% **(Figure 1A)**, CFU-GM by 71% **(Figure 1B)**, and CAFC frequency by 86% **(Figure 1E)** ($p<0.05$ for all). Interestingly, erythrocyte progenitors, BFU-E and CFU-GEMM, did not increase, only white blood cell progenitors.

Exercise increases marrow cellularity in humeri and ulnae, but not vertebrae

In humeri and ulnae marrow, overall cellularity increased by 51% **(Figure 2A)** ($p<0.05$). Representative images of these bones demonstrate the change in phenotype clearly; a reduction in adiposity concurrent with an increase in cellularity **(Figure 2B and 2C)**. However, this observation could only be made in weight bearing bones. There was no increase in cellularity in the vertebrae from aged exercised animals **(Figure 2D)**. Groups also had similar adipocyte distribution **(Figure 2E and 2F)**.

Discussion

Here we demonstrate that exercise is able to partially ameliorate the phenotype associated with aged bone marrow, reducing adiposity and increasing cellularity. As well, we demonstrate that these changes occur only in load bearing bones and occur independently of hematopoietic related endocrine signalling from skeletal muscle.

The aging marrow phenotype is well documented. In newborns almost all marrow is hematopoietically active (Muschler et al. 2001). With advancing age, marrow undergoes gradual increases in adiposity, decreases in cellularity, and decreases in the capacity of HSC to proliferate (Thiele et al. 2005). Endurance exercise training appears to reverse this trend. Increases in total CFU, CFU-GM, and CAFC were observed in marrow taken from appendicular bones post training **(Figure 1)**. CAFC represent a multipotent HSC population (Breems et al. 1994) able to reconstitute the hematopoietic system. As well, we show decreases in marrow adiposity and concomitant increases in marrow cellularity following endurance training **(Figure 2)**. In these mice, sedentary marrow appears older than the exercise trained marrow.

These findings are contrasted by no observed changes in vertebral cellularity **(Figure 2D)**. Loss of axial marrow cellularity should have been present in these animals as the mice utilized in this study were near the end of their typical lifespan (Rowlatt et al. 1976), well after vertebral marrow begins losing cellularity and gaining adiposity. That exercise did not restore vertebral cellularity is curious. This suggests that the mechanism by which exercise training is able to effect marrow changes is specific in nature and not a general, body wide stimuli.

Supporting this idea is the observation that these changes occurred independently of any potential endocrine signalling from skeletal muscle. Previously, we have demonstrated the ability of skeletal muscle in young male mice to both express (Baker et al. 2011) and produce (Baker and Parise 2016) hematopoietic cytokines. Increases in hematopoietic endocrine signalling should affect hematopoietic tissues throughout the body, including vertebral marrow. When hematopoietic cytokines are added endogenously, this is certainly the case (Ghielmini et al. 1996). In aged animals, however, it appears that exercise is not able to upregulate muscle derived hematopoietic endocrine signalling **(Figure S1)**. No changes in EPO expression could also explain the observation that erythrocyte containing progenitors did not increase as a result of exercise training **(Figure 1C and 1D)**.

One particular stimulus associated with our model, applying to appendicular but not axial bones are mechanical forces present during exercise. Mechanical forces are important to the development and maintenance of the hematopoietic niche as they have a direct impact on the predisposition of stromal cells to undergo osteogenesis or adipogenesis. Mechanical stress in bone promotes osteogenesis (Burger et al. 1992), regulates osteoblast proliferation (Guo et al. 2016), and induces MSC osteogenic differentiation (Yourek et al. 2010). Conversely, mechanical stretch inhibits MSC adipogenesis (Li et al. 2015) and reduces signalling associated with adipogenesis in many cell types (Turner et al. 2008). Limb bone stresses in running rodents decrease distally to proximally (Biewener 1983) and may not impact the vertebrae (Bott et al. 2016). If running exercise is unable to generate mechanical stress in the spine, this could explain why axial marrow cellularity does not increase **(Figure 2D)**.

In conclusion, exercise works towards reversing the bone marrow aging phenotype in old male mice. Adiposity is reduced and cellularity is increased; the opposite of aging. These changes occur only in load bearing bones and, as a result, may be due to the mechanical forces associated with exercise. Though this hypothesis is supported by the literature, more specific experiments are needed in order to directly test this claim.

5.1 – Figure 1

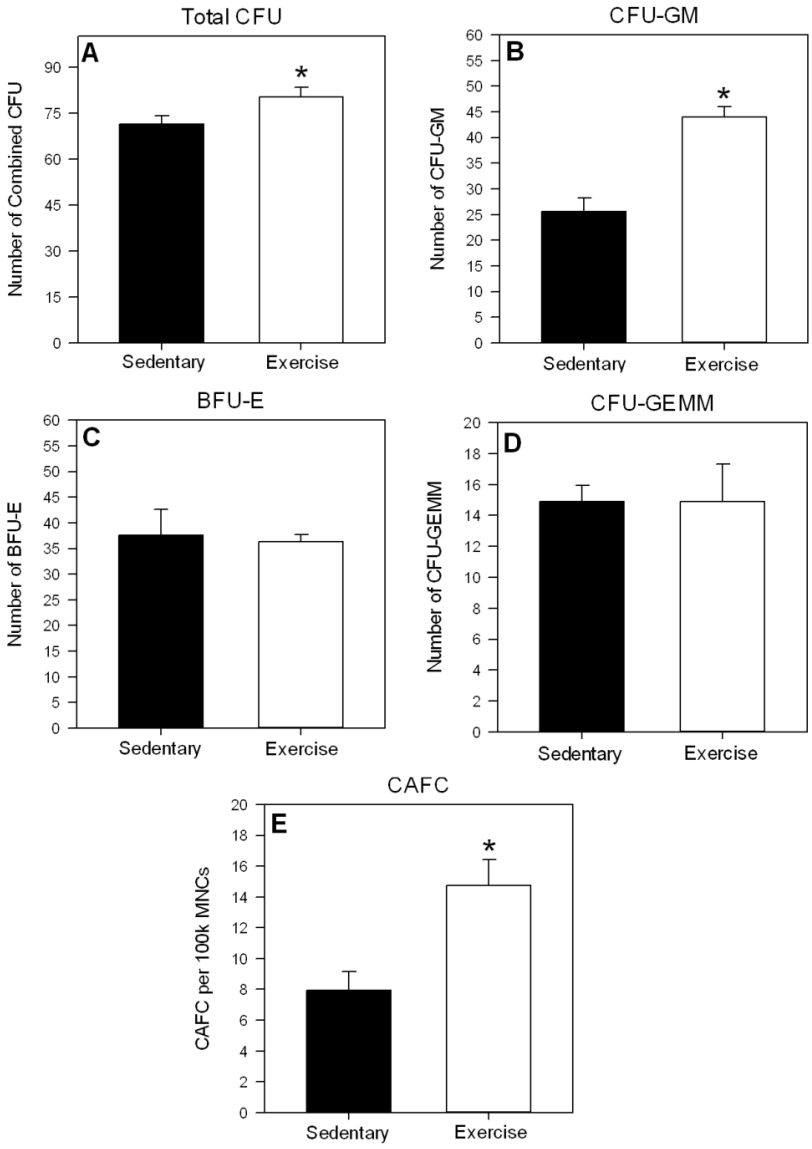

Figure 1: Femur and Tibia Colony Forming Capacity. Colony forming capacity (CFU) and cobblestone area forming cell (CAFC) frequency was measured for marrow isolated from the femur and tibia of exercise trained and sedentary aged mice. **(A)** Total CFU, **(B)** Granulocyte monocyte CFU, **(C)** Burst forming unit erythroid CFU, **(D)** Granulocyte erythroid monocyte megakaryocyte CFU, and **(E)** CAFC frequency. ***** indicates $p < 0.05$.

5.2 – Figure 2

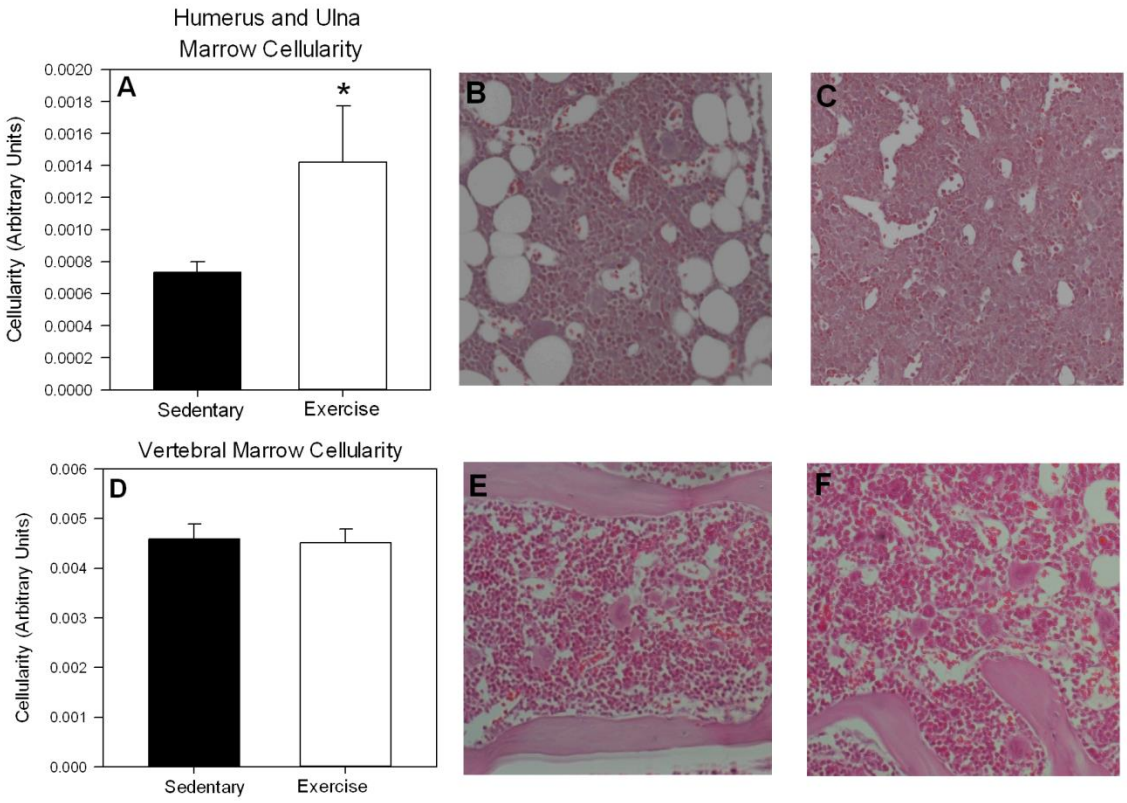

Figure 2: Marrow Cellularity in Load Bearing and Non-Load Bearing Bones. Comparison in the marrow cellularity of humerus and ulna (load bearing bones) and vertebrae (non load bearing bones) between exercise trained and sedentary aged mice. **(A)** Quantification of marrow cellularity in the humerus and ulna based on the inverse correlation established between green pixel value and manual cell counts, **(B, C)** Representative images of sedentary and exercise trained humerus and ulna marrow, respectively, **(D)** Quantification of marrow cellularity in the vertebrae based on the aforementioned correlation, **(E, F)** Representative images of sedentary and exercise trained vertebral marrow, respectively. ***** indicates $p < 0.05$.

5.3 – Supplementary Figure 1

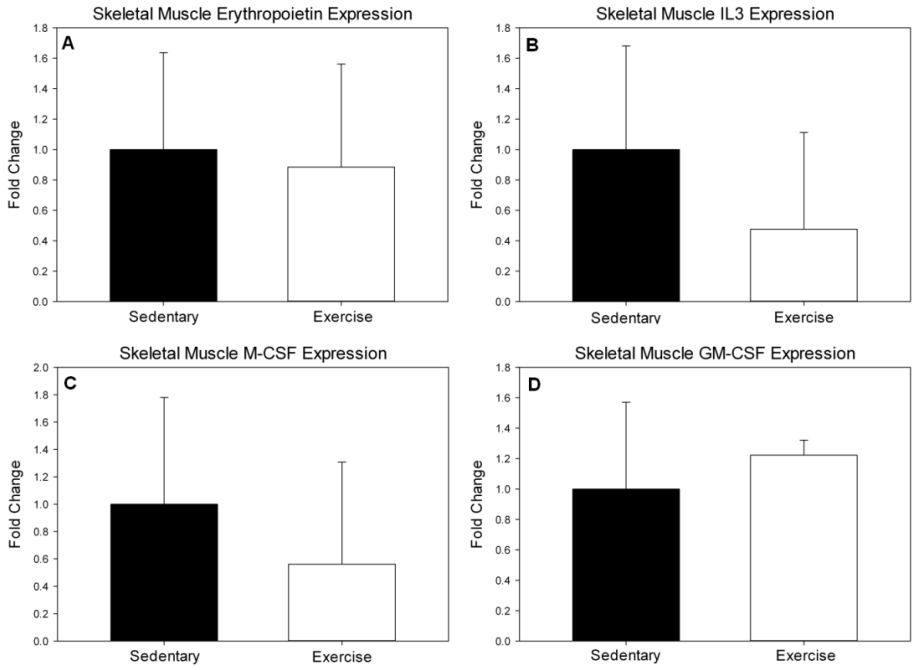

Figure S1: Muscle Hematopoietic Cytokine Gene Expression Profile. Gastrocnemius expression of hematopoietic cytokines between sedentary and exercise aged mice. **(A)** Erythropoietin, **(B)** IL3, **(C)** M-CSF, and **(D)** GM-CSF. No significant differences in expression were detected between sedentary and exercised groups.

Supplementary Methods: Quantitative PCR

RNA was isolated from gastrocnemius muscle (n=3) using TRIzol (Thermo Fisher, Waltham, MA, USA) and reverse transcribed using a high capacity cDNA reverse transcription kit (AB, Foster City, CA, USA) with an Eppendorf Mastercycler EP gradient thermal cycler (Eppendorf, Mississauga, ON, Canada). Reactions were run using SYBR Green qPCR master mix (SABioscience, Toronto, ON, Canada) in an Eppendorf Realplex2 Master Cycler EP GradientS (Eppendorf, Mississauga, ON, Canada). All samples were normalized to L32 expression. Relative expression was calculated using the ΔCT method.

5.4 – References

Baker, J.M., De Lisio, M., and Parise, G. 2011. Endurance exercise training promotes medullary hematopoiesis. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. **25**(12): 4348–4357. doi:10.1096/fj.11-189043.

Baker, J.M., and Parise, G. 2016. Skeletal Muscle Erythropoietin Expression Is Responsive to Hypoxia and Exercise. Med. Sci. Sports Exerc. **48**(7): 1294–1301. doi:10.1249/MSS.0000000000000899.

Biewener, A.A. 1983. Locomotory stresses in the limb bones of two small mammals: the ground squirrel and chipmunk. J. Exp. Biol. **103**: 131–154.

Bott, K.N., Sacco, S.M., Turnbull, P.C., Longo, A.B., Ward, W.E., and Peters, S.J. 2016. Skeletal site-specific effects of endurance running on structure and strength of tibia, lumbar vertebrae, and mandible in male Sprague-Dawley rats. Appl. Physiol. Nutr. Metab. Physiol. Appl. Nutr. Metab. **41**(6): 597–604. doi:10.1139/apnm-2015-0404.

Breems, D.A., Blokland, E.A., Neben, S., and Ploemacher, R.E. 1994. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia **8**(7): 1095–1104.

Burger, E.H., Klein-Nulend, J., and Veldhuijzen, J.P. 1992. Mechanical stress and osteogenesis in vitro. J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. **7 Suppl 2**: S397-401. doi:10.1002/jbmr.5650071406.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M., and Scadden, D.T. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature **425**(6960): 841–846. doi:10.1038/nature02040.

Corre, J., Barreau, C., Cousin, B., Chavoin, J.-P., Caton, D., Fournial, G., Penicaud, L., Casteilla, L., and Laharrague, P. 2006. Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. J. Cell. Physiol. **208**(2): 282–288. doi:10.1002/jcp.20655.

Daldrup-Link, H.E., Henning, T., and Link, T.M. 2007. MR imaging of therapy-induced changes of bone marrow. Eur. Radiol. **17**(3): 743–761. doi:10.1007/s00330-006-0404-1.

Ghielmini, M., Pettengell, R., Coutinho, L.H., Testa, N., and Crowther, D. 1996. The effect of the GM-CSF/IL-3 fusion protein PIXY321 on bone marrow and circulating haemopoietic cells of previously untreated patients with cancer. Br. J. Haematol. **93**(1): 6–12. doi:10.1046/j.1365-2141.1996.4731015.x.

Guo, Y., Lv, Q., Zou, X.-Q., Yan, Z.-X., and Yan, Y.-X. 2016. Mechanical Strain Regulates Osteoblast Proliferation Through Ca(2+)-CaMK-CREB Signal Pathway. Chin. Med. Sci. J. Chung-Kuo Hsueh Ko Hsueh Tsa Chih **31**(2): 100–106.

Joanisse, S., Nederveen, J.P., Baker, J.M., Snijders, T., Iacono, C., and Parise, G. 2016. Exercise conditioning in old mice improves skeletal muscle regeneration. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. **30**(9): 3256–3268. doi:10.1096/fj.201600143RR.

Li, R., Liang, L., Dou, Y., Huang, Z., Mo, H., Wang, Y., and Yu, B. 2015. Mechanical stretch inhibits mesenchymal stem cell adipogenic differentiation through TGFβ1/Smad2 signaling. J. Biomech. **48**(13): 3665–3671. doi:10.1016/j.jbiomech.2015.08.013.

Małkiewicz, A., and Dziedzic, M. 2012. Bone marrow reconversion – imaging of physiological changes in bone marrow. Pol. J. Radiol. **77**(4): 45–50.

Muschler, G.F., Nitto, H., Boehm, C.A., and Easley, K.A. 2001. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. J. Orthop. Res. **19**: 117–125. doi:10.1016/S0736-0266(00)00010-3.

Naveiras, O., Nardi, V., Wenzel, P.L., Hauschka, P.V., Fahey, F., and Daley, G.Q. 2009. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature **460**(7252): 259–263. doi:10.1038/nature08099.

van Os, R.P., Dethmers-Ausema, B., and de Haan, G. 2008. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods Mol. Biol. Clifton NJ **430**: 143–157. doi:10.1007/978-1-59745-182-6_10.

Ou-yang, L., and Lu, G. 2015. Decrease with aging of the microcirculatory function of the lumbar vertebral marrow preceding the loss of bone material density and the onset of intervertebral discal degeneration: A study about the potential cause. Chronic Dis. Transl. Med. **1**(2): 96–104. doi:10.1016/j.cdtm.2015.02.007.

Rowlatt, C., Chesterman, F.C., and Sheriff, M.U. 1976. Lifespan, age changes and tumour incidence in an ageing C57BL mouse colony. Lab. Anim. **10**(10): 419–442.

Takeshita, S., Fumoto, T., Naoe, Y., and Ikeda, K. 2014. Age-related Marrow Adipogenesis Is Linked to Increased Expression of RANKL. J. Biol. Chem. **289**(24): 16699–16710. doi:10.1074/jbc.M114.547919.

Thiele, J., Kvasnicka, H.M., Facchetti, F., Franco, V., Van Der Walt, J., and Orazi, A. 2005. European consensus on grading bone marrow fibrosis and assessment of cellularity. Haematologica **90**: 1128–1132.

Turner, N.J., Jones, H.S., Davies, J.E., and Canfield, A.E. 2008. Cyclic stretch-induced TGFbeta1/Smad signaling inhibits adipogenesis in umbilical cord progenitor cells. Biochem. Biophys. Res. Commun. **377**(4): 1147–1151. doi:10.1016/j.bbrc.2008.10.131.

Visnjic, D., Kalajzic, I., Gronowicz, G., Aguila, H.L., Clark, S.H., Lichtler, A.C., and Rowe, D.W. 2001. Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice. J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. **16**(12): 2222–2231. doi:10.1359/jbmr.2001.16.12.2222.

Yourek, G., McCormick, S.M., Mao, J.J., and Reilly, G.C. 2010. Shear stress induces osteogenic differentiation of human mesenchymal stem cells. Regen. Med. **5**(5): 713–724. doi:10.2217/rme.10.60.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.-G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y., and Li, L. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. Nature **425**(6960): 836–841. doi:10.1038/nature02041.

Chapter 6 – Expanded Discussion

The data presented herein provide additional support for the idea that exercise and skeletal muscle can play a role in regulating hematopoiesis. Chapter 2 demonstrates that mouse skeletal muscle can produce EPO and affect changes to erythropoiesis, mediated by exercise induced hypoxia. Chapter 3 shows that acute exercise in humans can mobilize HSC into circulation, that this process is intensity dependent, that these changes quickly subside following cessation of exercise, and that fitness has no bearing on level of mobilization. Chapter 4 establishes that exercise training can increase splenic EMH in mice, greatly increasing spleen HSC content and increasing spleen expression of a myriad of genes associated with HSC growth, homing, and engraftment. Finally, Chapter 5 provides some evidence for the hypothesis that mechanical forces transmitted through bones during exercise are responsible for the changes observed in marrow cellularity following an exercise training regime in mice. Taken together, these studies suggest that the ability of exercise to influence hematopoiesis is predicated on exercise mediated changes to hematopoietic growth factor expression and exercise mediated changes to the hematopoietic niche. As well, that acute exercise induced mobilization of HSC into circulation better allows HSC to efficiently benefit from these changes.

6.1 – Exercise Secretome

One of the means by which hematopoiesis is regulated is through the availability of hematopoietic factors. Addition of exogenous hematopoietic factors leads directly to changes in the hematopoietic system. For example, EPO doping is used by athletes in an attempt to increase red blood cell content (1), G-CSF can mobilize hematopoietic cells into circulation (2), and IL3 injections can lead to the expansion of certain blood cell populations (3). Conversely, removal of hematopoietic factors can have dire consequences for the hematopoietic system. A reduction in erythropoiesis and blood hemoglobin content when erythropoietin signalling is ablated by nephrectomy is an example of this (4,5).

Exercise may provide a physiological means for altering hematopoietic factor expression levels in tissues throughout the body. Exercise training has been shown to increase skeletal muscle expression of

EPO, IL3, M-CSF, and GM-CSF (6). Exercise training has also been shown, here, to increase spleen expression of a variety of genes associated with hematopoietic proliferation, differentiation, and homing. Acute exercise has been shown, here, to increase EPO expression in skeletal muscle. Acute exercise has also been shown to increase spleen expression of SCF and SDF1 (7). As well, many studies have demonstrated that exercise can increase circulating levels of other factors that influence hematopoiesis. A dramatic example of this is the 8000 fold increase in circulating IL6 following a marathon run (8). Skeletal muscle production of myokines other than the ones mentioned above, and the ability of differentiating skeletal muscle cells to release as many as 188 factors (9), may also play a role in hematopoietic regulation.

Many of the changes observed in the hematopoietic compartment following exercise training are likely a result of the changes observed to hematopoietic factor expression following exercise training. Increased expression of these factors would likely allow for increased HSC proliferation. An unpublished piece of data provides an example of this, where cell number per CFU in marrow samples was increased in exercise trained animals when compared to their sedentary counterparts (**Figure 1**) despite there being more total CFU in exercised animals (6). Increased IL3 and GM-CSF following exercise training could be the cause of this, both factors know to increase HSC proliferation (10). As well, increased availability of these factors could likely lead to some of the other observed changes. Increased HSC content in the spleen may result from increased HSC homing to the spleen, caused by increase spleen expression of SDF1. Increased spleen HSC content could also be due to increased spleen expression of factors such as Ang1. Increased spleen content of terminally differentiated blood cells may result from increased HSC differentiation in the spleen, caused by increases in spleen expression of hematopoietic growth factors such as GM-CSF. Thus, exercise leads to changes in expression of hematopoietic factors, leading to changes in the regulation of hematopoiesis.

6.2 – Exercise, Mechanical Stress, and the Endosteal Niche

Another means by which hematopoiesis is regulated is through the state of the hematopoietic niche microenvironment. Two niche types exist in bone marrow, the endosteal niche and the vascular niche. In Chapter 5, some evidence is provided for the hypothesis that the mechanical forces associated with treadmill running in mice are responsible for the observed increases in bone marrow cellularity. In such a scenario, increases in cellularity would occur as a result of development in the endosteal hematopoietic niche. Mechanical forces present during exercise would stimulate MSC proliferation and increase MSC potential for osteogenic differentiation, improving niche status. Some additional pilot data lends credibility to this argument. CFU-F, colony forming unit fibroblast, is used to enumerate the number of MSC within a mixed population of marrow cells (11). Mechanical forces of all kinds have been demonstrated to increase MSC number in culture (12–14). Climbing type exercise has also been shown to increase number of CFU-F in mice (15,16). Here, we demonstrate that exercise training increases the number of CFU-F in femur and tibia taken from trained animals when compared to their sedentary counterparts (**Figure 2**). Increased CFU-F is suggestive of the presence of mechanical forces in the bones of trained animals. Unfortunately, no data is available for MSC or CFU-F number in marrow taken from the vertebrae of trained animals.

In Chapter 5, mechanical forces were not directly measured and were only assumed to exist. That CFU-F number increased following exercise training, and that vertebral marrow was unaffected by exercise training, is certainly suggestive of the presence or lack of mechanical forces but is not direct evidence. To truly prove this argument, mechanical forces would need to be applied to bones *in vivo* in lieu of exercise. Models for this type of stimuli are available, vibrating platforms or direct application of mechanical force are two possibilities. Interestingly, “vibration therapy” in postmenopausal women with osteoporosis increases bone mineral density (17) and high frequency vibration of bone marrow cultures increases stromal cell osteogenic potential (18).

6.3 – Exercise, Hypoxia, Angiogenesis, and the Vascular Niche

Development of the vascular hematopoietic niche would also lead to increases in bone marrow and spleen HSC content and cellularity. This may occur in a similar manner to capillarization in skeletal muscle. Exercise training increases skeletal muscle capillarization, increasing muscle capillary density and capillary to fiber ratio (19,20). This occurs through increases in VEGF (21), generation of nitric oxide (22), and PGC-1α signalling (23). One hypothesis that explains how this process occurs is that the stimulus for capillarization is mediated through decreases in muscle oxygen content during exercise (24,25). Decreases in muscle oxygen content activates HIF1 signalling and increases muscle VEGF expression, leading to angiogenesis (25). In a manner similar to skeletal muscle, exercise could temporarily lower oxygen content in bone marrow and spleen. This would provide a stimulus for increased expression of pro angiogenic factors, leading to endothelial cell proliferation and potential expansion of the vascular niche. Unfortunately, no studies have measured bone marrow and spleen oxygen content following exercise. However, intense exercise can decrease blood oxygen saturation (26). This would imply that the amount of oxygen reaching bone marrow and spleen during exercise would be decreased. Whether or not exercise mediated decreases in blood oxygen content could lead to development of hypoxia in bone marrow and spleen significant enough to trigger VEGF expression is unknown. However, Chapter 4 demonstrates that VEGF expression and the expression of other pro angiogenic factors in the spleen are increased as a result of exercise training. Determining whether pro angiogenic factor expression in bone marrow and spleen is increased immediately following acute exercise would not be difficult to determine.

6.4 – Mobilization Fills the Expanded Niche

Aside from the collection of PBMC grafts for transplantation, the idea that exercise can increase circulating HSC has other implications. It might be logical to assume that HSC transplant recipients must first undergo myeloablative conditioning in order to successfully accept and engraft donor HSC. The reasoning for this is simple: if hematopoietic niche sites are occupied by native HSC, donor HSC will be

afforded no unoccupied spaces in which to engraft. They will then remain in circulation where they will eventually differentiate and die. This is because circulating HSC are typically characterized as non-quiescent cells that are actively engaged in proliferation, only when HSC engraft to a niche can they reach a state of quiescence (27). However, unconditioned HSC transplant recipients can accept and engraft donor HSC (28,29). This has been demonstrated across many studies and in a variety of conditions. The quantity of engraftment remains the debateable issue, not that engraftment occurs. What this means is that unoccupied niche microenvironments exist naturally (30). These sites are continually emptied through basal HSC mobilization and then refilled through natural HSC homing.

In this context, the ability of exercise to greatly albeit temporarily increase HSC mobilization is interesting to consider. As well, the ability of exercise to alter the niche such that it can support more HSC. Exercise leads to mechanical stress in bone and reduced blood oxygen content. These signals then lead to changes in MSC and endothelial cells that increase osteogenesis, decrease adipogenesis, and increase angiogenesis. As discussed previously, changes in marrow osteoblast (31) and endothelial cell content (32) are directly tied to marrow HSC content. Increasing osteoblast and endothelial cell content should then lead to increases in available hematopoietic niche space. Simultaneous to these happenings, acute bouts of exercise temporarily mobilize HSC into circulation. Mobilized out of bone marrow, or at least out of the endosteal niche, HSC have an increasing likelihood of undergoing self-renewal and proliferation. After receiving homing signals to return to a niche microenvironment, either as they were or having had divided, HSC would then find more vacant niche spaces available to fill. This cycle would continue across the duration of an exercise training program; continual mobilization and reseeding of an ever expanding niche microenvironment.

Certainly, a cycle such as this could explain increased splenic EMH and HSC content following exercise training. Especially considering increases in spleen SDF1 expression following exercise training. HSC previously found in bone marrow would be continuously mobilized by exercise, endlessly surveying for and eventually locating putative niche structures in the spleen that had been built up by exercise

training. An additional piece of data left out of Chapter 4, the ability of exercise training to increase total CFU in the liver of exercised trained animals when compared to their sedentary counterparts (**Figure 3**), could also be explained by this. More cells in circulation, concurrent with a stimulus for angiogenesis, potentially in the liver, through increased muscle and spleen expression of pro angiogenic factors, eventually leading to homing of HSC to a liver niche site.

6.5 – Exercise, Age, and Hematopoiesis

Hematopoiesis is typically impaired with aging. Early in life, bone marrow is almost entirely devoid of adipocytes and is hematopoietically active. With advancing age, adipocyte infiltration continues until 50-70% of the bone marrow volume in healthy adults is fat (33). This trend continues, elderly marrow contains even more adipose tissue than adult marrow (34). Specifically, the number of osteoblasts in marrow decrease with aging (35). As well, aging is associated with decreased adaptive immune function and increased incidence of anemia and other hematological disorders (36). Aging HSC are also skewed towards myeloid development, are less likely to maintain quiescence (37), and exhibit less regenerative capacity during transplantation (38). Decreased expression of several hematopoietic factors as a result of aging, such as SDF1 and SCF, are thought to account for some of these changes (35). As well, aging is associated with a shift towards a proinflammatory cytokine expression profile (39).

Some of the differences between young and old hematopoiesis mirror the differences observed between sedentary and exercise trained hematopoiesis. Specifically, when considering the state of the hematopoietic niche. In young trained animals, MSC differentiation potential shifts towards osteogenesis and away from adipogenesis (6). As well, marrow CFU-F increases in young exercised animals (15,16). This is the opposite of aging; decreased marrow CFU-F (40), a decrease in osteoblasts, an increase in marrow adipogenesis, and an overall increase in marrow adiposity (35,41). Chapter 5 demonstrates that exercise may reverse the aging marrow phenotype. An unpublished piece of pilot data further supports this claim, marrow CFU-F in aged sedentary animals is below that of young sedentary animals – as has been demonstrated in other studies. After exercise training, however, CFU-F in old exercised animals is

demonstrated to increase when compared to old sedentary animals (**Figure 2**). This suggests that exercise training benefits the state of the aged endosteal hematopoietic niche. Training increases the number of MSC available, and if the trend from young animals holds true, training would increase MSC osteogenesis, decrease MSC adipogenesis, and reverse the aging marrow phenotype.

Some as yet unpublished findings also demonstrate that acute exercise is able to mobilize hematopoietic cells into circulation in older individuals just as well as in younger individuals (**Figure 4**). Just as in young animals, exercise mediated mobilization of HSC in aged individuals could be beneficial. Exercise training in aged animals or individuals may increase niche development, leading to more available niche space, while simultaneously mobilizing HSC into circulation, leading to seeding of newly formed hematopoietic niche microenvironments. Ultimately, this would lead to more functional hematopoiesis. That said, mobilization is naturally increased by the process of aging in mice (42) and, as mentioned above, HSC quality in aged individual does decrease.

6.6 – Is more necessarily better?

Exercise training increases both bone marrow and spleen HSC content. As well, exercise training increases the basal level of HSC found in blood. However, an exercise trained individual having more HSC doesn't necessarily mean that they have better hematopoietic health and function. Twice as many HSC doesn't translate into twice as many terminally differentiated blood cells in circulation. Even if it did, high hematocrit is a predictor of heart failure (43), can be a risk factor for stroke (44), and a risk factor for venous thromboembolism (45). Certainly, individuals suffering from leukemia or other myeloproliferative disorders would say that more is not better. Having more is the problem that needs to be solved by myeloablation and PBMC transplant in those unfortunate individuals.

Yet myeloproliferative disorders and exercise training are fundamentally dissimilar. Obvious differences aside, one is a direct change to hematopoietic cell function, usually as a result of genetic mutation, while the other appears to be a change to both the HSC niche and the release of hematopoietic growth factors throughout the body. While the hematopoietic niche can play a role in sheltering and

supporting metastasized cancer cells (46,47), descriptions of myeloproliferative disorders resulting solely from deregulation and massive expansion of the hematopoietic niche have not been made. Though niche space has been expanded as a result of exercise training, allowing more HSC to fill that space, regulation of hematopoiesis has not been compromised.

For collection of HSC, more is better. In certain situations, more could also be better. Following bleeding in mice, HSC in bone marrow and the spleen begin to proliferate and self-renew (48) and EPO concentrations in circulation rise dramatically in order to begin the process of regenerating lost blood (49). In an event of massive blood loss, having more HSC available to begin the process of blood regeneration might be beneficial and serve to increase the rate of recovery. Unfortunately, no data from such a situation is available. Data correlating HSC number to hematopoietic rate *in vivo* is also unavailable, or difficult to find. In terms of the hematopoietic niche, is more necessarily better? In an event where the HSC niche is damaged, by radiation or chemical exposure, having a larger or more resilient niche structure in place could serve to protect hematopoiesis. Luckily, data from just such a situation is available. When exercise trained animals are lethally irradiated and given a bone marrow transplant, their rate of survival is nearly three times that of their sedentary counterparts (50). Though this study did not directly assess the state of the bone marrow niche prior to or following irradiation, the exercise training methodology used in this study was identical to a study that demonstrated improvements in the bone marrow niche following exercise (6). As well, exercise training has been observed to confer radioprotective effects to bone marrow cells in mice (51)

6.7 – Acute Exercise vs Exercise Training

Acute exercise temporarily increases skeletal muscle expression of EPO. As well, acute exercise temporarily increases numbers of circulating HSC. Exercise training, on the other hand, permanently increases skeletal muscle expression of EPO – at least until time of sacrifice (6). As well, exercise training permanently increases numbers of circulating HSC – again, at least until time of sacrifice. It would seem that while one bout of exercise can exert a temporary change, that change becomes

permanent following multiple bouts of exercise across the course of several months. At least for the two observations mentioned and so long as exercise is continued. It could be that epigenetic modifications of the tissues involved in these processes are responsible for this phenomenon. After all, despite their vastly different capabilities and functions, HSC and terminally differentiated blood cells have the same genome. The only differences between these cell types are regulation of gene expression through epigenetic modification (52). Skeletal muscle is also regulated by epigenetic mechanisms (53). As well, exercise training leads to epigenetic modification of skeletal muscle (54). Acute exercise is also able to remodel promoter methylation, inducing hypomethylation in several genes known to increase in expression post exercise (55). In regards to EPO expression, repeated bouts of exercise may slowly but surely decrease EPO promoter methylation in skeletal muscle. This could eventually lead to the constitutively increased EPO expression observed following exercise training. Supporting this idea is the observation that EPO expression is known to be regulated through epigenetic modification (56).

6.8 – Loss of Exercise Adaptations

Mobilization following acute exercise is transient and decreases within minutes following cessation of activity. As well, skeletal muscle HIF1α content, HIF2α content, and level of EPO expression quickly falls in the minutes following acute exercise (author's unpublished observation). In terms of exercise training, no data is available on the length of time needed for the changes observed in the hematopoietic compartment to return to untrained levels. All measurements made in the included exercise training studies were taken two or three days following the last bout of exercise. This means that the observations recorded do not reflect temporary effects of exercise. Given enough time, however, it should be expected that exercise training induced changes to the hematopoietic compartment would return to a natural state. How long this would take is unknown.

In a loosely similar situation, marrow quickly returns to its initial state. Following evacuation of marrow cavities entirely filled with fat through surgical means, marrow regeneration quickly begins (57). Four weeks after evacuation, MSC and endothelial cells develop and regenerate a niche structure.

Hematopoiesis can be observed at this stage. By six weeks, development of adipocytes can be observed. By six months, the marrow cavity is restored to its initial adipose filled state (57). In a similar study, the results are the same. A niche like structure is regenerated and hematopoiesis can be observed, but soon after the tissue is restored to its pre evacuation state (58). What this suggests is that there is a natural baseline that hematopoiesis will return to following cessation of stimuli.

How this state is decided upon, and what signals are required for it to be established, is entirely unknown. In the case of exercise training, ceasing activity would result in a decrease in hematopoietic factor expression, a decrease in mechanical stress felt in bone, a normalization of blood oxygen content, and a return to baseline levels of HSC mobilization. If the presence of these stimuli causes a change in one direction, the loss of these stimuli could certainly mean a change in the opposite direction. Again, the amount of time this would take is unknown. Fortunately, determining this would not be very difficult.

6.9 – Study Limitations

Both animals and humans were used as tissue sources in the included studies. This is a direct result of tissue availability. Measuring the effect of acute exercise on mobilization of HSC in mice is exceedingly difficult, given that only a small amount of blood can be collected. Collecting vertebral marrow aspirates from elderly humans is also very challenging. This leads to results being compared between mice and humans; in some situations, this may not necessarily be appropriate. The spleen, for example, is always hematopoietically active in mice (59). In humans, this is not the case – unless hematopoiesis in the marrow cavity becomes impaired (60). Still, having both animal and human data is important. With a precedent established, human studies will be easier to justify. For example, a study examining bone marrow content in humans following exercise, through use of MR imaging, will be much easier to develop.

As well, when referring to hematopoietic growth factors, the word ‘expression’ is used here interchangeably to mean transcription, translation, and release of these factors from the cell. In some cases, as with the EPO ELISA demonstrating that skeletal muscle cells subjected to hypoxia release EPO,

this is not an issue. In many other cases, however, no evidence is available to show that increased expression of a hematopoietic factor leads to increased translation of that same factor, let alone release of that factor from the tissue it is expressed in. Even so, increased levels of transcription are suggestive of increased levels of translation. As well, having the expression data available gives good reason to continue on and use ELISAs and antibodies to profile tissue concentration of these hematopoietic associated factors.

Finally, it could be considered a limitation that the included studies did not directly build on one another. However, as the ability of exercise to influence hematopoiesis is a largely unstudied concept, working towards a better understanding of the subject requires a broad approach rather than a focused one. Little background knowledge exists to provide specific direction. Broader reaching observations, including basic physiological measures, are needed before specific questions can be delicately answered. Here, that goal is achieved. With a broader understanding of the fundamentals, future studies examining specifics, such as the ability of mechanical forces to change the shape of the endosteal niche, are now more easily justifiable.

6.10 – Conclusions

This thesis provides further support to the idea that exercise can alter hematopoiesis. Previous to this, it was known that exercise training can affect changes in the bone marrow cavity that result in increased HSC content. As well, that acute exercise could mobilize HSC. Now, we have expanded our understanding. Firstly, we demonstrate that exercise can directly lead to skeletal muscle production of EPO through hypoxia. In addition, cells from skeletal muscle can directly stimulate erythropoiesis. Secondly, we demonstrate that mobilization of HSC following acute exercise is intensity dependent. Too light of an intensity does not increase mobilization; as well, demonstrated from a yet unpublished piece of data, too heavy of an intensity does not increase mobilization (**Figure 5**). In addition, we demonstrate that prior fitness levels have no impact on mobilization and that numbers of circulating HSC quickly fall post exercise. Thirdly, we demonstrate that exercise training increases splenic EMH. This change is likely due

to increased spleen expression of hematopoietic factors and the development of vascular like endothelial cell niche in spleen. Finally, we provide further evidence for the hypothesis that mechanical forces felt during exercise are strongly implicated in the training induced changes to the bone marrow niche. Yes, many ifs, ands, and maybes still remain when examining the results of the included studies. In general though, a better understanding of the means by which exercise is able to induce these changes has been achieved.

6.11 – Figure 1

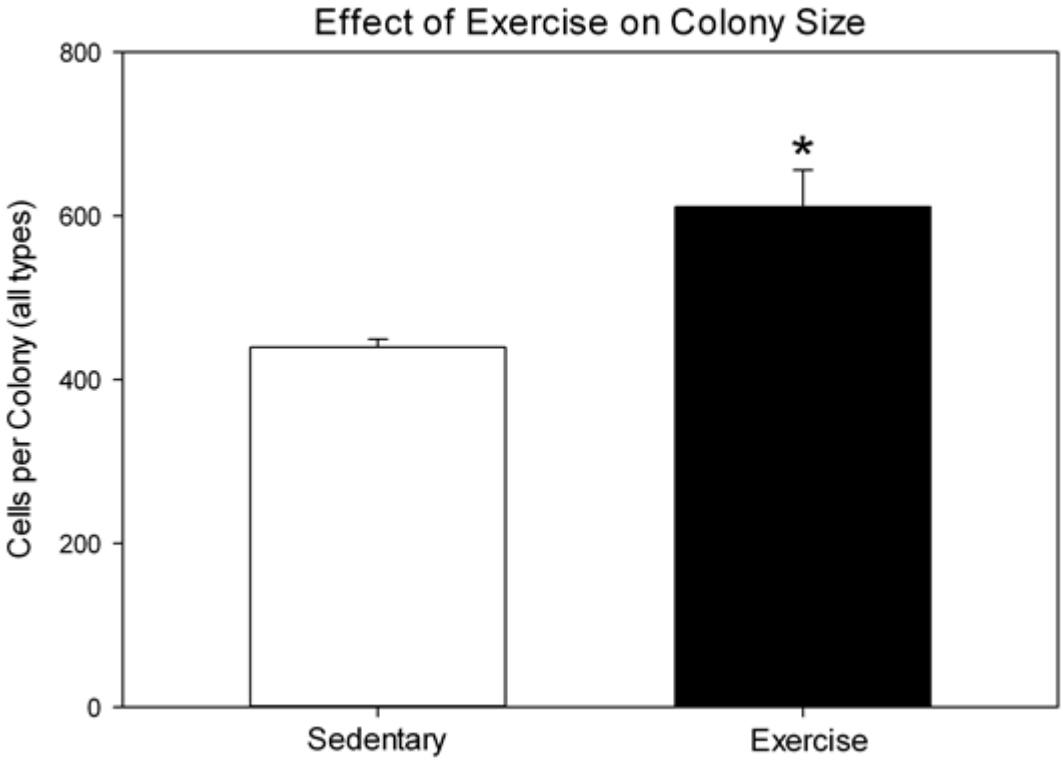

Figure 1: Cells per Colony Forming Unit. Young mice were exercise trained. Mononuclear cells were isolated from femur and tibia bone marrow. Cells were plated into Mouse Methylcellulose Complete Medium. Total colonies were scored (Sedentary 204 vs Exercise 312, $p<0.05$). Afterwards, methylcellulose media was removed, washed, and filtered. Progeny cells were pelleted and counted. Exercise colonies contained more cells per colony than sedentary colonies. * indicates $p<0.05$.

6.12 – Figure 2

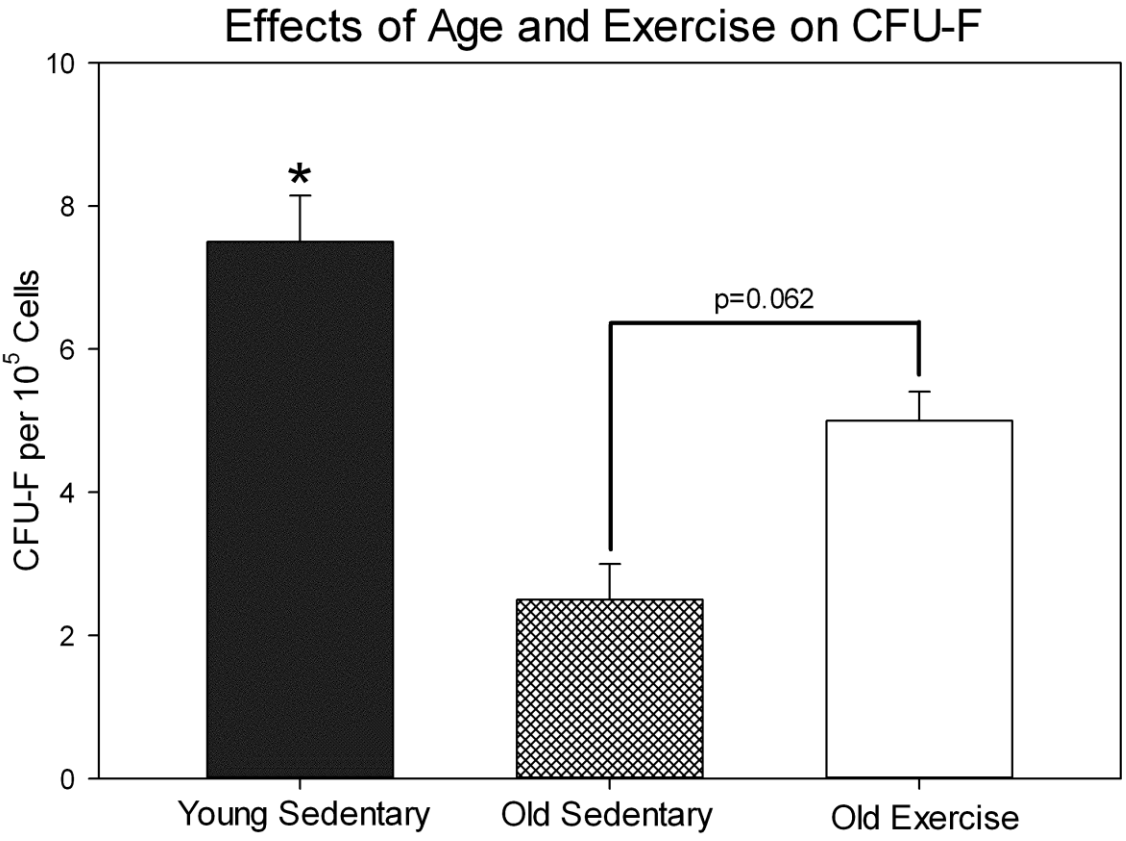

Figure 2: Colony forming unit fibroblast counts in young sedentary, old sedentary, and old exercise trained animals. Femur and tibia, cleaned of muscle and connective tissue, and flushed free of bone marrow, were crushed and digested with collagenase and dispase. Resultant cells were washed, filtered, and counted. Cells were plated, left to incubate for 1 day, and then plates were washed free of unattached cells. Cells were then left to grow for 14 days. Plates were then stained with crystal violet and counted. Only colonies containing more than 30 cells were counted. * indicates $p<0.05$.

6.13 – Figure 3

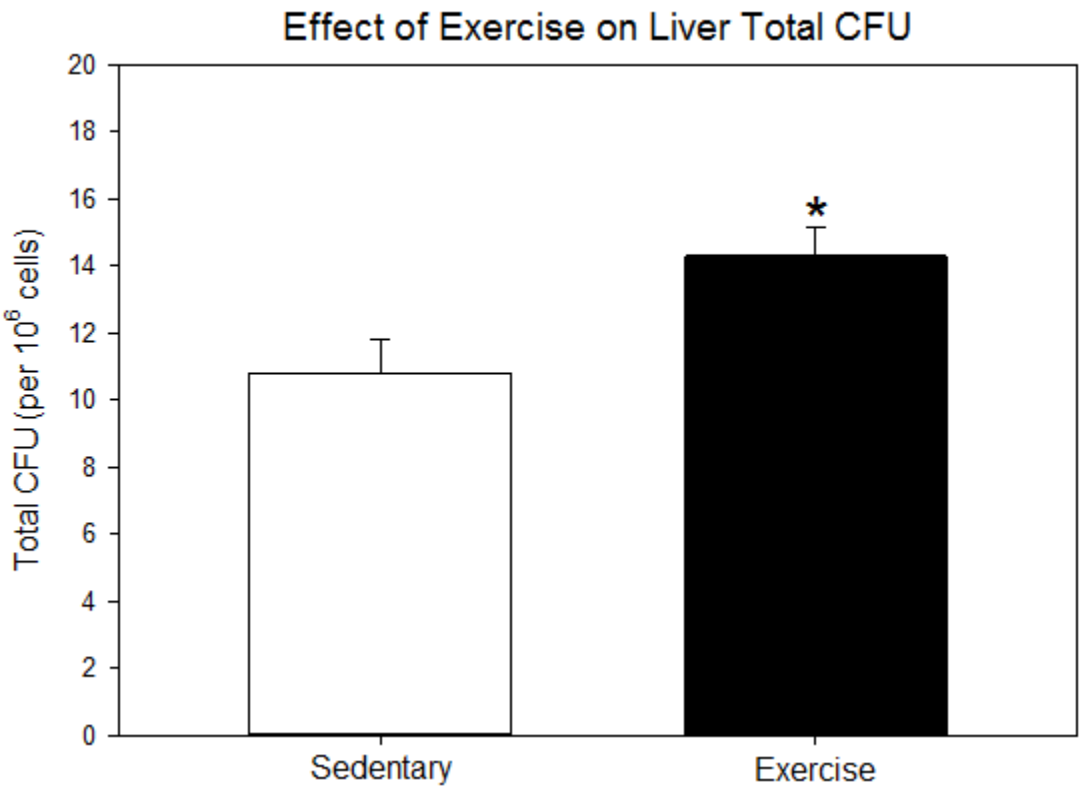

Figure 3: Total CFU in liver of young sedentary and exercise trained animals. Mononuclear cells were isolated from the right lobe of the liver in mice. Cells were plated into Mouse Methylcellulose Complete Medium. Total colonies were scored. * indicates $p<0.05$.

6.14 – Figure 4

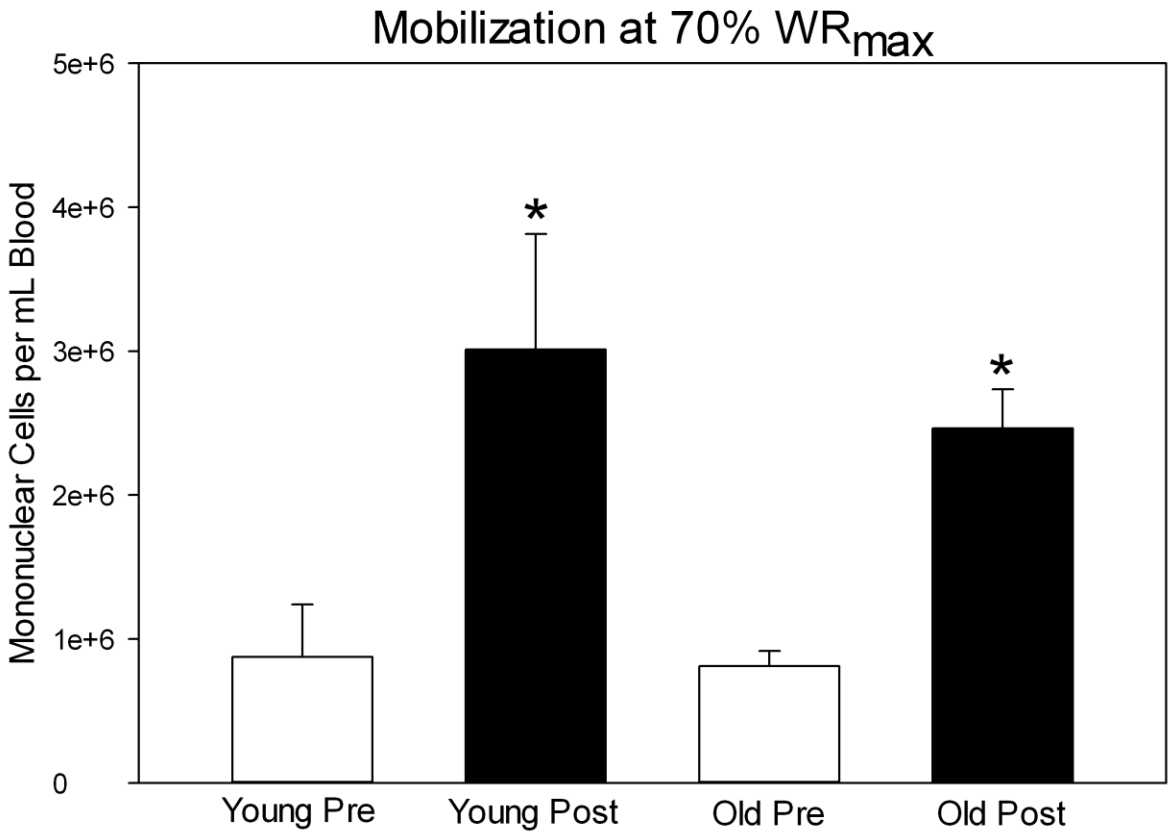

Figure 4: Comparison of mobilization, pre exercise to immediately post exercise, at 70% WR_{max} to failure for young (average 21 years old) and old (average 70 years old) subjects. Blood was collected at the indicated time points. Mononuclear cells were isolated from blood and counted. * indicates $p<0.05$ between pre and post exercise.

6.15 – Figure 5

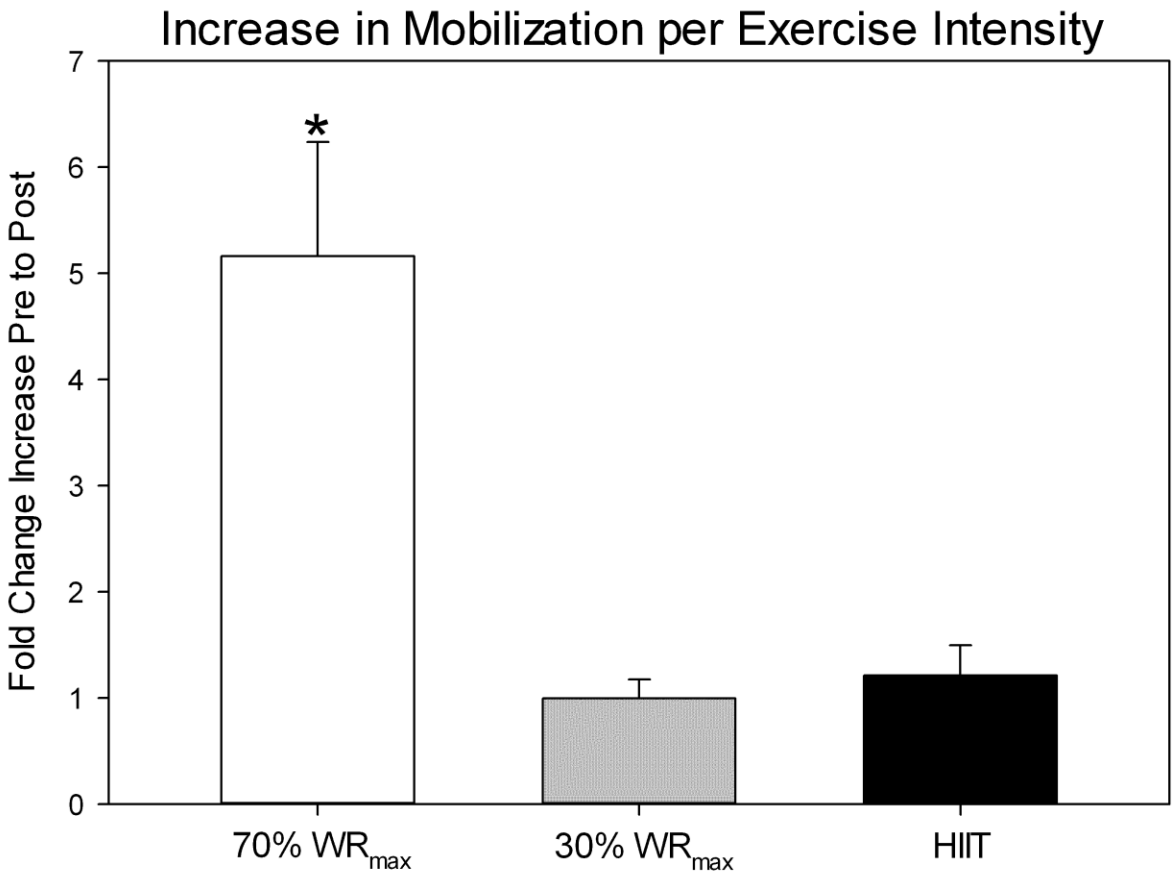

Figure 5: Fold change increase in mononuclear cell number pre exercise to immediately post exercise in young subjects. 70% WR_{max} was to failure, 30% WR_{max} was work matched to the 70% WR_{max} condition, and a typical HIIT protocol was used. Blood was collected at the indicated time points. Mononuclear cells were isolated from blood and counted. * indicates $p<0.05$.

6.16 – References

1. Joyner M. VO2MAX, blood doping, and erythropoietin. Br J Sports Med. 2003 Jun;37(3):190–1.

2. Philpott NJ, Prue RL, Marsh JC, Gordon-Smith EC, Gibson FM. G-CSF-mobilized CD34 peripheral blood stem cells are significantly less apoptotic than unstimulated peripheral blood CD34 cells: role of G-CSF as survival factor. Br J Haematol. 1997 Apr;97(1):146–52.

3. Ohmori K, Luo Y, Jia Y, Nishida J, Wang Z, Bunting KD, et al. IL-3 induces basophil expansion in vivo by directing granulocyte-monocyte progenitors to differentiate into basophil lineage-restricted progenitors in the bone marrow and by increasing the number of basophil/mast cell progenitors in the spleen. J Immunol Baltim Md 1950. 2009 Mar 1;182(5):2835–41.

4. Naets J-P. The Role of the Kidney in the Production of the Erythropoietic Factor. Blood. 1960 Dec 1;16(6):1770–6.

5. Naets J-P. THE ROLE OF THE KIDNEY IN ERYTHROPOIESIS*. J Clin Invest. 1960 Jan;39(1):102–10.

6. Baker JM, De Lisio M, Parise G. Endurance exercise training promotes medullary hematopoiesis. FASEB J Off Publ Fed Am Soc Exp Biol. 2011 Dec;25(12):4348–57.

7. Emmons R, Niemiro GM, Owolabi O, De Lisio M. Acute exercise mobilizes hematopoietic stem and progenitor cells and alters the mesenchymal stromal cell secretome. J Appl Physiol Bethesda Md 1985. 2016 Mar 15;120(6):624–32.

8. Margeli A, Skenderi K, Tsironi M, Hantzi E, Matalas A-L, Vrettou C, et al. Dramatic Elevations of Interleukin-6 and Acute-Phase Reactants in Athletes Participating in the Ultradistance Foot Race Spartathlon: Severe Systemic Inflammation and Lipid and Lipoprotein Changes in Protracted Exercise. J Clin Endocrinol Metab. 2005 Jul 1;90(7):3914–8.

9. Henningsen J, Rigbolt KTG, Blagoev B, Pedersen BK, Kratchmarova I. Dynamics of the Skeletal Muscle Secretome during Myoblast Differentiation. Mol Cell Proteomics. 2010 Nov 1;9(11):2482–96.

10. Metcalf D. Hematopoietic cytokines. Blood. 2008 Jan 15;111(2):485–91.

11. Kuznetsov SA, Mankani MH, Bianco P, Robey PG. Enumeration of the colony-forming units–fibroblast from mouse and human bone marrow in normal and pathological conditions. Stem Cell Res. 2009 Jan;2(1):83–94.

12. Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE, et al. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. J Orthop Res Off Publ Orthop Res Soc. 2004 Nov;22(6):1283–9.

13. Riddle RC, Taylor AF, Genetos DC, Donahue HJ. MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation. Am J Physiol Cell Physiol. 2006 Mar;290(3):C776-784.

14. Ghazanfari S, Tafazzoli-Shadpour M, Shokrgozar MA. Effects of cyclic stretch on proliferation of mesenchymal stem cells and their differentiation to smooth muscle cells. Biochem Biophys Res Commun. 2009 Oct 23;388(3):601–5.

15. Menuki K, Mori T, Sakai A, Sakuma M, Okimoto N, Shimizu Y, et al. Climbing exercise enhances osteoblast differentiation and inhibits adipogenic differentiation with high expression of PTH/PTHrP receptor in bone marrow cells. Bone. 2008 Sep;43(3):613–20.

16. Mori T, Okimoto N, Sakai A, Okazaki Y, Nakura N, Notomi T, et al. Climbing exercise increases bone mass and trabecular bone turnover through transient regulation of marrow osteogenic and osteoclastogenic potentials in mice. J Bone Miner Res Off J Am Soc Bone Miner Res. 2003 Nov;18(11):2002–9.

17. Ruan X-Y, Jin F-Y, Liu Y-L, Peng Z-L, Sun Y-G. Effects of vibration therapy on bone mineral density in postmenopausal women with osteoporosis. Chin Med J (Engl). 2008 Jul 5;121(13):1155–8.

18. Prè D, Ceccarelli G, Visai L, Benedetti L, Imbriani M, Cusella De Angelis MG, et al. High-Frequency Vibration Treatment of Human Bone Marrow Stromal Cells Increases Differentiation toward Bone Tissue. Bone Marrow Res. 2013;2013:803450.

19. Brodal P, Ingjer F, Hermansen L. Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. Am J Physiol. 1977 Jun;232(6):H705-712.

20. Hudlicka O, Brown M, Egginton S. Angiogenesis in skeletal and cardiac muscle. Physiol Rev. 1992 Apr;72(2):369–417.

21. Olfert IM, Howlett RA, Tang K, Dalton ND, Gu Y, Peterson KL, et al. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. J Physiol. 2009 Apr 15;587(Pt 8):1755–67.

22. Baum O, Vieregge M, Koch P, Gül S, Hahn S, Huber-Abel FAM, et al. Phenotype of capillaries in skeletal muscle of nNOS-knockout mice. Am J Physiol Regul Integr Comp Physiol. 2013 Jun 15;304(12):R1175-1182.

23. Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JFP, Hidalgo J, et al. PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am J Physiol Endocrinol Metab. 2009 Jul;297(1):E92-103.

24. Richardson RS, Noyszewski EA, Kendrick KF, Leigh JS, Wagner PD. Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. J Clin Invest. 1995 Oct;96(4):1916–26.

25. Lindholm ME, Rundqvist H. Skeletal muscle hypoxia-inducible factor-1 and exercise. Exp Physiol. 2016 Jan 1;101(1):28–32.

26. Rowell LB, Taylor HL, Wang Y, Carlson WS. Saturation of arterial blood with oxygen during maximal exercise. J Appl Physiol. 1964 Mar 1;19(2):284–6.

27. Pietras EM, Warr MR, Passegué E. Cell cycle regulation in hematopoietic stem cells. J Cell Biol. 2011 Nov 28;195(5):709–20.

28. Brecher G, Ansell JD, Micklem HS, Tjio JH, Cronkite EP. Special proliferative sites are not needed for seeding and proliferation of transfused bone marrow cells in normal syngeneic mice. Proc Natl Acad Sci U S A. 1982 Aug;79(16):5085–7.

29. Saxe DF, Boggs SS, Boggs DR. Transplantation of chromosomally marked syngeneic marrow cells into mice not subjected to hematopoietic stem cell depletion. Exp Hematol. 1984 May;12(4):277–83.

30. Bhattacharya D, Czechowicz A, Ooi AGL, Rossi DJ, Bryder D, Weissman IL. Niche recycling through division-independent egress of hematopoietic stem cells. J Exp Med. 2009 Nov 23;206(12):2837–50.

31. Bowers M, Zhang B, Ho Y, Agarwal P, Chen C-C, Bhatia R. Osteoblast ablation reduces normal long-term hematopoietic stem cell self-renewal but accelerates leukemia development. Blood. 2015 Apr 23;125(17):2678–88.

32. Butler JM, Nolan DJ, Vertes EL, Varnum-Finney B, Kobayashi H, Hooper AT, et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell. 2010 Mar 5;6(3):251–64.

33. Cawthorn WP, Scheller EL. Editorial: Bone Marrow Adipose Tissue: Formation, Function, and Impact on Health and Disease. Front Endocrinol [Internet]. 2017 [cited 2017 Jul 12];8. Available from: http://journal.frontiersin.org/article/10.3389/fendo.2017.00112/full

34. Schwartz AV. Marrow Fat and Bone: Review of Clinical Findings. Front Endocrinol [Internet]. 2015 Mar 30;6. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378315/

35. Geiger H, de Haan G, Florian MC. The ageing haematopoietic stem cell compartment. Nat Rev Immunol. 2013 May;13(5):376–89.

36. Burkhardt R, Kettner G, Böhm W, Schmidmeier M, Schlag R, Frisch B, et al. Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: A comparative histomorphometric study. Bone. 1987 Jan 1;8(3):157–64.

37. Pang WW, Price EA, Sahoo D, Beerman I, Maloney WJ, Rossi DJ, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci. 2011 Dec 13;108(50):20012–7.

38. Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. J Exp Med. 2000 Nov 6;192(9):1273–80.

39. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G. Aging-induced proinflammatory shift in cytokine expression profile in coronary arteries. FASEB J Off Publ Fed Am Soc Exp Biol. 2003 Jun;17(9):1183–5.

40. Bellantuono I, Aldahmash A, Kassem M. Aging of marrow stromal (skeletal) stem cells and their contribution to age-related bone loss. Biochim Biophys Acta. 2009 Apr;1792(4):364–70.

41. Wagner W, Horn P, Bork S, Ho AD. Aging of hematopoietic stem cells is regulated by the stem cell niche. Exp Gerontol. 2008 Nov;43(11):974–80.

42. Xing Z, Ryan MA, Daria D, Nattamai KJ, Van Zant G, Wang L, et al. Increased hematopoietic stem cell mobilization in aged mice. Blood. 2006 Oct 1;108(7):2190–7.

43. Coglianese EE, Qureshi MM, Vasan RS, Wang TJ, Moore LL. Usefulness of the blood hematocrit level to predict development of heart failure in a community. Am J Cardiol. 2012 Jan 15;109(2):241–5.

44. Tohgi H, Yamanouchi H, Murakami M, Kameyama M. Importance of the hematocrit as a risk factor in cerebral infarction. Stroke. 1978 Aug;9(4):369–74.

45. Brækkan SK, Mathiesen EB, Njølstad I, Wilsgaard T, Hansen J-B. Hematocrit and risk of venous thromboembolism in a general population. The Tromsø study. Haematologica. 2010 Feb;95(2):270–5.

46. Decker AM, Jung Y, Cackowski F, Taichman RS. The role of hematopoietic stem cell niche in prostate cancer bone metastasis. J Bone Oncol. 2016 Feb 19;5(3):117–20.

47. Vessella RL, Corey E. Targeting factors involved in bone remodeling as treatment strategies in prostate cancer bone metastasis. Clin Cancer Res Off J Am Assoc Cancer Res. 2006 Oct 15;12(20 Pt 2):6285s–6290s.

48. Cheshier SH, Prohaska SS, Weissman IL. The effect of bleeding on hematopoietic stem cell cycling and self-renewal. Stem Cells Dev. 2007 Oct;16(5):707–17.

49. Jelkmann W. Regulation of erythropoietin production. J Physiol. 2011 Mar 15;589(Pt 6):1251–8.

50. De Lisio M, Baker JM, Parise G. Exercise promotes bone marrow cell survival and recipient reconstitution post-bone marrow transplantation, which is associated with increased survival. Exp Hematol. 2013 Feb;41(2):143–54.

51. De Lisio M, Phan N, Boreham DR, Parise G. Exercise-induced protection of bone marrow cells following exposure to radiation. Appl Physiol Nutr Metab Physiol Appl Nutr Metab. 2011 Feb;36(1):80–7.

52. Epigenetic Regulation of Hematopoietic Stem Cells [Internet]. [cited 2017 Jul 14]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4961102/

53. Sousa-Victor P, Muñoz-Cánoves P, Perdiguero E. Regulation of skeletal muscle stem cells through epigenetic mechanisms. Toxicol Mech Methods. 2011 May;21(4):334–42.

54. McGee SL, Fairlie E, Garnham AP, Hargreaves M. Exercise-induced histone modifications in human skeletal muscle. J Physiol. 2009 Dec 15;587(Pt 24):5951–8.

55. Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, et al. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. Cell Metab. 2012 Mar 7;15(3):405–11.

56. Steinmann K, Richter AM, Dammann RH. Epigenetic silencing of erythropoietin in human cancers. Genes Cancer. 2011 Jan;2(1):65–73.

57. Tavassoli M, Maniatis A, Crosby WH. Induction of sustained hemopoiesis in fatty marrow. Blood. 1974 Jan;43(1):33–8.

58. Sahebekhtiari HA, Tavassoli M. Studies on bone marrow histogenesis: morphometric and autoradiographic studies of regeneration marrow stroma in extramedullary autoimplants and after evacuation of marrow cavity. Cell Tissue Res. 1978 Sep 26;192(3):437–50.

59. Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF. Roles of spleen and liver in development of the murine hematopoietic system. Exp Hematol. 2002 Sep;30(9):1010–9.

60. Freedman MH, Saunders EF. Hematopoiesis in the human spleen. Am J Hematol. 1981 Nov;11(3):271–5.