EXERCISE MEDIATED REGULATION OF HEMATOPOIESIS

EXERCISE MEDIATED REGULATION OF MEDULLARY AND EXTRAMEDULLARY HEMATOPOIESIS

By

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Abstract

Bone marrow is the major site of adult hematopoiesis. Bone marrow cells exert control over the hematopoietic stem cells that reside in the niche; osteoblasts act as positive regulators while adipocytes act as negative regulators. Levels of circulating hematopoietic cytokines also regulate hematopoiesis. In this study, we demonstrate that an endurance exercise training program results in several changes that act to induce hematopoiesis. Bone marrow-derived mesenchymal stem cell differentiation is skewed away from adipogenesis and towards osteogenesis in exercise trained animals. As a result, the bone marrow cavity is remodeled during the training period and acts to facilitate hematopoiesis. Hematopoietic cytokine gene expression levels also increase in exercise trained skeletal muscle. These changes translate into increased bone marrow and blood hematopoietic stem and progenitor cell content. This study draws a link between exercise training, bone marrow niche regulation, skeletal muscle derived hematopoietic cytokines, and the regulation of hematopoiesis.

Acknowledgements

Though my M.Sc. has gone by quickly, I have learned a lot and it has been an enjoyable experience. I would like to thank my advisor Dr. Gianni Parise for his infinite patience and understanding. Another thank you must be given to my lab members for the help that they have given me with this project. Finally, I would like to thank my committee members Dr. Thomas Hawke and Dr. Stuart Phillips for their assistance and advice.

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HSC	Hematopoietic stem cell
EPO	Erythropoietin
GM-CSF	Granulocyte macrophage colony stimulating factor
M-CSF	Macrophage colony stimulating factor
IL-3	Interleukin-3
IL-6	Interleukin-3
BFU-E	Burst forming unit erythroid
CFU-GM	Colony forming unit granulocyte macrophage
CFU-GEMM	Colony forming unit granulocyte erythrocyte megakaryocyte monocyte
LSK	Lineage negative, Sca1 positive, c-kit positive hematopoietic stem cells
MSC	Mesenchymal stem cell
Mk	Megakaryocyte
SEC	Sinusoidal endothelial cell

1. Introduction

Our culture embraces the sedentary lifestyle. Sitting while travelling to work, sitting at a desk while at work, and sitting at home while watching television, reading, and using a computer describes the daily routine of many individuals [1]. We have no reason to hunt for our food or farm for our crops, any daily physical activity is a recreational choice or a workplace obligation. Physical activity and regular exercise is unquestionably healthy and can result in many positive health effects. True sedentary behavior is not just a lack of physical activity; it is a divergence in the opposite direction [2]. The negative adaptations associated with sedentary behavior mirror the positive adaptations associated with regular activity [3]. These adaptations are many and often manifest themselves in unusual places.

a. Exercise Training and Adaptations

Daily physical activity is a necessary component of a healthy lifestyle. While 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 exercise and anaerobic or resistance exercise. Each has its own particular health benefits. The body of an athlete who is highly aerobically trained is very different from the body of an athlete who is highly strength trained [4]. Resistance exercise involves applying resistance to muscular contraction in order to build strength. Weightlifting is the most common example of resistance training. Improvements in posture, cardiovascular health, and in muscle size and tone are typical adaptations to a resistance training program [5]. Though improvements in cardiovascular

health are associated with resistance training, regular aerobic exercise tends to be a better cardiovascular stimulus.

Aerobic exercise training, or endurance exercise training, is generally likened to exercises such as walking, jogging, swimming, and cycling. These exercises build endurance more so than physical strength. As one trains, aerobic exercise tends to build physical endurance by improving the efficiency of the heart, lungs, and circulatory system [6]. These changes prepare muscles for prolonged periods of exercise and further aerobic activities. Many specific bodily adaptations occur as a result of endurance exercise training. These adaptations result in changes that act to increase the ability of an individual to transport and utilize oxygen [7]. These changes can be broken down into three general categories; adaptations that improve ventilatory capacity, adaptations that improve circulatory ability, and adaptations that increase the ability of muscle to efficiently utilize oxygen (Figure I).

Oxygen first enters the body through the lungs. The ventilatory capacity of highly trained athletes is improved and this improves exercise performance; lung tidal volume, the normal amount of air displaced between inhalation and exhalation, is increased [8]. The pulmonary diffusing capacity of endurance athletes is also increased and this results in an improved capacity for oxygen diffusion from the lungs to the blood [9]. After oxygen is extracted from the environment it enters into circulation. Cardiac output increases as a result of endurance exercise training. The stroke volume of the heart, rather than heart rate, increases and this serves to increase oxygenated blood flow throughout the body [8]. Increased muscle capillary density, another adaptation associated with exercise training, results in shorter oxygen diffusion distances and a

better supply of oxygen to muscle mitochondria [10]. Increased red blood cell transit time through increased numbers of capillaries also helps to facilitate increased oxygen extraction from blood to muscle [11]. When oxygen from the blood reaches the muscle, it is used for aerobic metabolism. Increased muscle oxygen utilization efficiency results in increased aerobic energy production and increased exercise performance. Endurance training results in an upregulation of oxidative enzymes in the mitochondria along with increased mitochondrial biogenesis [12]. These metabolic changes within the muscle result in increased reliance on oxidative fat metabolism as a fuel source and decreased reliance on anaerobic glycolysis, further boosting performance [8].

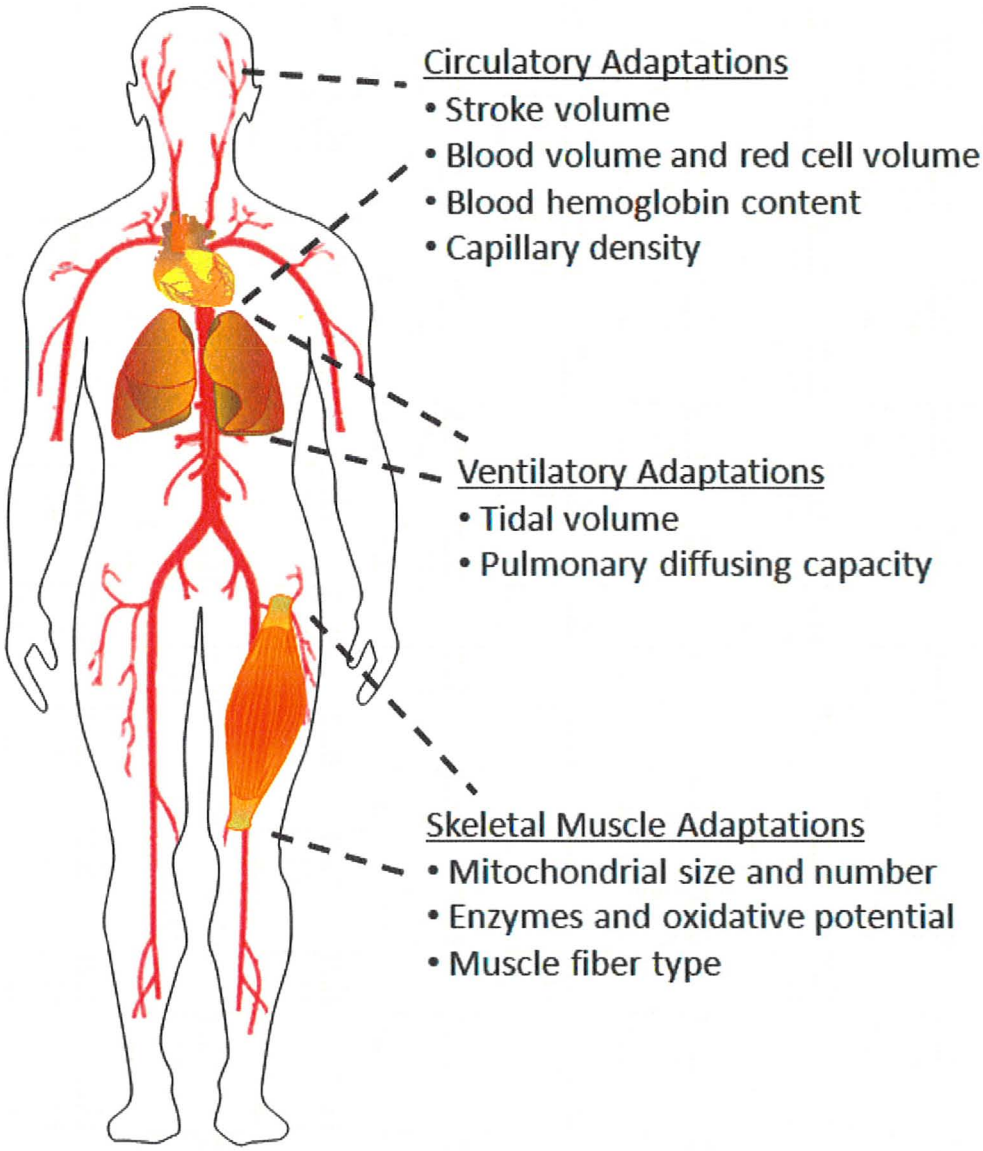

Figure I. Adaptations Associated with Endurance Exercise Training. Factors influencing oxygen uptake, transportation, and utilization in the body. Endurance exercise training results in positive adaptations to these factors, which results in increased ability of the body to use oxygen and increased exercise performance.

Of particular interest are blood based adaptations to endurance exercise training. Adaptations to exercise training tend to result in changes that allow for increased oxygen transport. Blood based adaptations account for much of the increased oxygen transportation capacity from endurance training and are some of the most interesting [13]. 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 hemoglobin of red blood cells and later, when the arterial blood is delivered to working skeletal muscle, oxygen diffuses from the hemoglobin of the red blood cells into muscle fibers. The ability of blood to carry oxygen is equally as important as the ability of the lungs to extract oxygen from the atmosphere or the ability of muscle to utilize it during aerobic metabolism.

An increased hematocrit is commonly associated with endurance exercise training. While various groups have reported differing opinions on whether white blood cell mass changes with endurance training, red blood cell mass has been shown on many occasions to increase [14]. Supplementary to this measure, many groups have reported increased blood volume as well as increased blood hemoglobin content with endurance training [15]. Increased blood red cell volume and hemoglobin content would serve to increase the capabilities of blood to transport oxygen, resulting in increased exercise performance [16]. 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 [17]. Blood adaptations in hypoxic environments are associated with increased production of kidney erythropoietin [18]; however, this has never been shown following endurance training at sea level. Indeed, exogenous erythropoietin supplementation is one means by which athletes are able to artificially boost their hematocrit and increase their exercise performance [19].

Blood changes associated with endurance training are unexplainable. No plausible mechanisms for sea level based, non-hypoxic increases in hematocrit have ever been produced. One possible means by which endurance exercise training could produce these changes is by increasing the hematopoietic capabilities of an endurance trained

organism. Increased hematopoiesis would result in increased production and turnover of healthy, oxygen carrying red blood cells. This link is not as far fetched as it may seem. Endurance exercise training has been associated with increased immune function [20] and increased immune function is often associated with increases in hematopoietic capabilities [21]. Exercise is also able to mobilize hematopoietic cells into circulation [22], possibly to support increased hematopoiesis and blood production. Given the undeniable links between exercise training and oxygen carrying capacity an investigation into the ability of exercise to modulate hematopoiesis is in order.

b. Hematopoiesis: the production of blood

Hematopoiesis is the process by which blood cells are produced. Hematopoiesis has two distinct developmental phases – embryonic and adult. Hematopoietic stem cells are one of the first cell types to develop during embryogenesis [23]. 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 [24]. Blood islands, multiple foci of primitive erythroid cells, begin to form on the yolk sac. At the onset of circulation these blood islands give rise to primitive erythrocytes that are able to enter in to circulation [23]. As development continues, the yolk sac also begins to produce cells of white blood lineages and starts to release them into circulation [25]. These circulating embryonic hematopoietic cells then begin to populate the fetal liver where they engraft and develop into adult hematopoietic stem cells [23]. Colonization of the spleen with adult hematopoietic stem cells soon follows. The cells persist in these environments temporarily and soon begin to shift to the bone marrow cavities as they form. By birth,

the majority of hematopoiesis has shifted from the fetal liver and spleen into the bone marrow cavity. During post embryonic development this recolonization continues until the majority of hematopoietic capabilities shift to the bone marrow and the liver is no longer a potent hematopoietic organ [24]. The spleen retains some hematopoietic capabilities during post embryonic development and acts as a secondary organ of hematopoiesis in adult development.

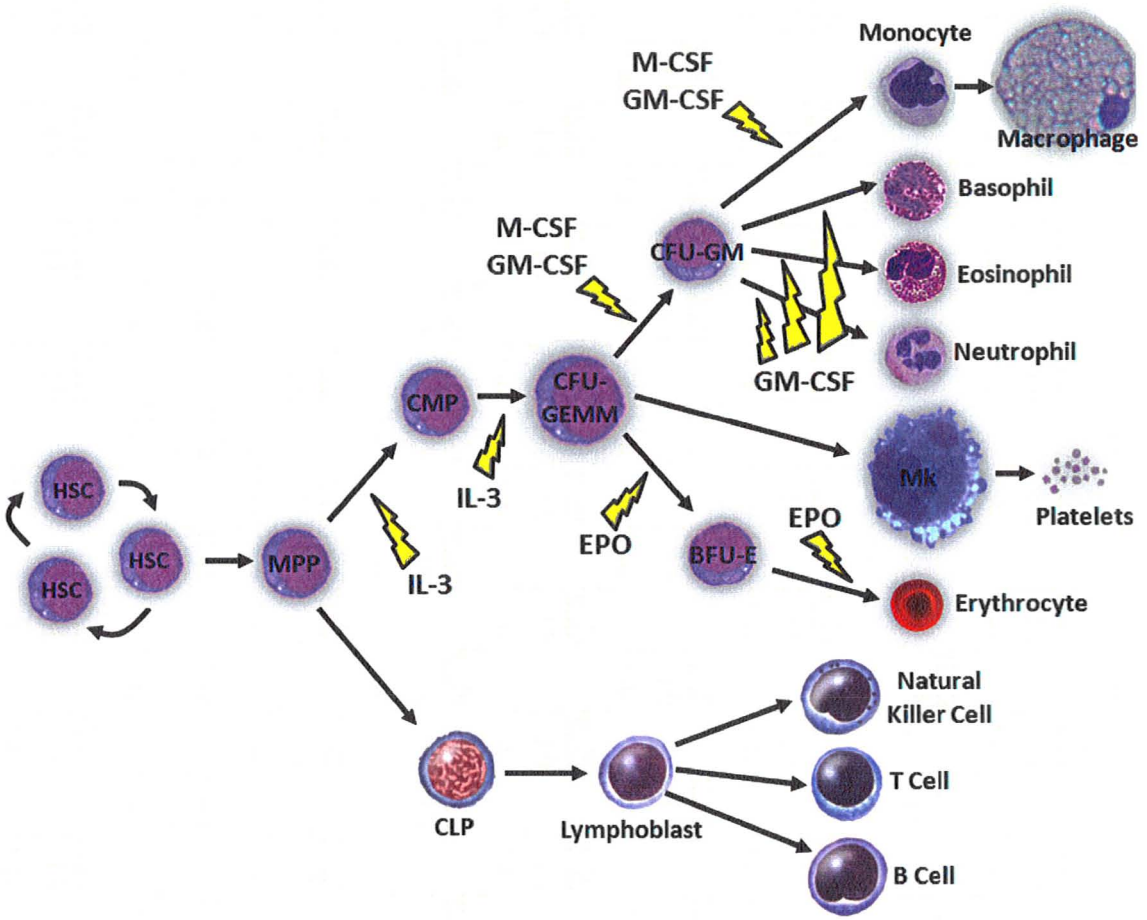

Figure II. Hematopoiesis. Hematopoietic stem cells (HSC) differentiate into multipotent progenitor cells (MPP, defined by LSK surface antigens), which further differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). IL-3 positively influences early steps in myeloid differentiation, while EPO, M-CSF, and GM-CSF positively influence later stages in myeloid differentiation. BFU-E, CFU-GM, and CFU-GEMM progenitor cell types are important to note for later discussion.

Hematopoietic stem cells have a vast capacity for self renewal, proliferation, and differentiation. During the embryonic phase of development exponential self renewal occurs with each niche shift [23]. Relatively small numbers of hematopoietic stem cells forming the blood islands on the yolk sac expand to enter circulation, expand to fill the developing fetal liver, and expand again to populate the bone marrow cavity. Enlargement of these niches during post embryonic development calls for similar expansions in the number of available hematopoietic cells [26]. The ability of hematopoietic stem cells to self renew and maintain a primitive state, still capable of massive proliferation and varied differentiation, is critical for the development of life.

While self renewal and maintenance of a primitive, multipotent hematopoietic stem cell pool is important, the ability of hematopoietic stem cells to proliferate and differentiate is equally important. Primitive hematopoietic stem cells are multipotent, meaning they can give rise to cell types of more than one lineage. In general, hematopoietic cells are divided into two broad categories; cells of the myeloid lineages and cells of the lymphoid lineages (Figure II). True hematopoietic stem cells are able to reconstitute both lineages. 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 [27]. Multipotent stem cells represent a very small pool in an adult organism. Multipotent myeloid or lymphoid progenitor stem cells represent a pool a fold higher in number. Cells committed to specific myeloid or lymphoid lineages represent a pool that is again a fold higher in number. This trend continues all the way down the

hematopoietic lineage to the final product, terminally differentiated hematopoietic cells. One primitive multipotent hematopoietic stem cell is capable of giving rise to billions of terminally differentiated hematopoietic cells [28].

Hematopoietic homeostasis requires that hematopoiesis is tightly regulated and precisely controlled. Terminally differentiated blood cells are short lived and must be continually replaced [29]. Failure of hematopoietic stem cells to adequately self renew, proliferate, and differentiate results in failures of the blood cell system. Anemias, a lack of red blood cells in the body, is one of these conditions and is characterized by a lack of energy due to reduced blood oxygen transport capabilities [30]. Immune deficiencies, either in the innate or adaptive immune systems, also derive from failure of hematopoietic stem cells and result in increased risk of infection or the inability of the immune system to clear cancers and tumors from the body [31]. Overproduction of blood cells from hematopoietic stem cells is also a problem. Leukemia is a cancer of the blood cell and is characterized by an overproduction of white blood cells [32]. Leukemia can impair immune and myeloid cell function and many dangerous and life threatening symptoms occur as a result [33]. Precise control over the hematopoietic system is important. This precise control is mediated by growth factor regulation of hematopoiesis and medullary and extramedullary niche regulation of hematopoiesis.

c. Hematopoietic Regulation: cytokines

Hematopoiesis is regulated in part by a wide variety of cytokines and growth factors. These factors can be released by cell types residing directly in the hematopoietic niche, by other hematopoietic cells, or into circulation by various tissues and organs.

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; erythropoietin (EPO), thrombopoietin (TPO), Flt3 ligand (Flt3L), and stem cell factor (SCF) are amongst the most vital. These growth factors allow hematopoietic cells to self renew, proliferate, differentiate, engraft to their niche, mobilize from their niche, assume cell quiescence, and mount an immune response [27]. Specific growth factors impact hematopoiesis in specific ways. Some growth factors controls specific steps in the hematopoietic hierarchy, such as EPO promoting the production of red blood cells from red blood progenitor cells, while other growth factors influence a wide array of cell types, such as granulocyte-macrophage colony stimulating factor (GM-CSF) promoting the production of myeloid white blood cells from myeloid white blood cell progenitors [27]. Synergistic applications of growth factors can combinatorially influence hematopoietic cells and result in stronger cell responses [34]. While many hematopoietic growth factors exert positive influences, some of the growth factors exert negative influences on hematopoietic cells. The serum profile of an individual, with all of the hematopoietic cytokines and growth factors tightly balanced and concentrations precisely controlled, gives the body exacting control over the process of hematopoiesis [27].

Of particular interest to this work are the hematopoietic cytokines interleukin 3, erythropoietin, macrophage colony stimulating factor, and granulocyte macrophage colony stimulating factor. Research into the effects of skeletal muscle derived growth factors and cytokines on non-skeletal muscle tissues is a newly emerging area. Identifying which factors in particular are released by skeletal muscle is an important part

of this effort. The following section focuses on these factors since they represent potent mediators of hematopoiesis and, taken together, are able to promote the growth of almost all hematopoietic cell types (Figure II).

i. Interleukin-3

IL-3 has a plethora of biological effects and, as such, the IL-3 receptor is expressed on almost all myeloid and lymphoid cell types [35]. One of the primary functions of IL-3 is to support the self-renewal and proliferation of almost every hematopoietic progenitor type. IL-3 also acts to stimulate differentiation of early hematopoietic progenitor cell types into more terminally differentiated, lineage committed hematopoietic cells [35]. IL-3 is an important hematopoietic growth factor in that it potentiates the effects of other hematopoietic cytokines, such as EPO, GM-CSF, and IL-6 [36]. IL-3 has this effect by increasing the expression levels of the receptors for these other cytokines on hematopoietic cells [37]. Although animals with genetically induced IL-3 deficiencies survive birth and develop as normal, their basal levels of certain hematopoietic cell types are reduced and the proliferation rates of these cells, in response to treatment with factors such as GM-CSF, are greatly reduced [38]. IL-3 has been used in a number of clinical settings. IL-3 treatment, in situations such as myelotoxicity associated with chemotherapy or in various forms of anemia, aids in inducing hematopoietic proliferation and restoration of proper blood cell homeostasis [39].

ii. Erythropoietin

EPO is a familiar hematopoietic cytokine in that it has received much attention from news outlets and the media sources. The reason for this is that many professional athletes have used EPO to artificially increase their red blood cell volume in order to increase their athletic performance [19]. The primary role of EPO is to stimulate erythropoiesis and the primary source of EPO in adult mammals is from the kidney. EPO functions by aiding in the differentiation and proliferation of hematopoietic progenitor cells that are already committed to red blood cell production [40]. As such, erythropoietin has very little influence on primitive multipotent hematopoietic stem cells or hematopoietic progenitors of other lineages [41]. EPO sees clinical usage in patients who have anemia associated with renal disease.

iii. Macrophage Colony Stimulating Factor

M-CSF was first isolated based on its ability to stimulate hematopoietic cells to form macrophage colonies *in vitro* [27]. M-CSF functions by supporting the self renewal, proliferation, and differentiation of monocytes, a hematopoietic progenitor capable of producing macrophages [27]. M-CSF also upregulates the immunogenic activity of monocytes and macrophages, allowing them to secrete various cytokines in order to mediate an immune response [42]. In conjunction with IL-3, M-CSF promotes the differentiation of multipotent hematopoietic stem cells into hematopoietic progenitors capable of giving rise to monocytes [43]. In a clinical setting M-CSF is used to accelerate the growth of white blood cells following hematopoietic ablation associated with bone marrow transplant [44].

iv. Granulocyte Macrophage Colony Stimulating Factor

In a similar fashion to M-CSF, GM-CSF was first isolated based on its ability to stimulate hematopoietic cells to form granulocyte and macrophage colonies *in vitro* [27]. The colony stimulating factors share little sequence homology with each other despite their similar biological activities [45]. As with M-CSF, GM-CSF is critical for the self renewal, proliferation, and differentiation of macrophage progenitors [27]. GM-CSF is also required for granulocyte progenitor development. GM-CSF is also able to stimulate the development of other myeloid cells, such as neutrophils and eosinophils [27]. Interestingly, treatment of hematopoietic cells with both M-CSF and GM-CSF leads to synergistic suppression of macrophage cell growth [46]. IL-3 and GM-CSF act synergistically together to promote hematopoietic cell growth [37] and interleukin-4 is also able to synergize with GM-CSF and promote hematopoietic cell growth [47]. GM-CSF deficient animals develop normally and have normal levels of hematopoiesis [48]; animals that over express GM-CSF, however, suffer from a host of conditions that result from tissue damage associated with macrophage overstimulation [49]. As with M-CSF, GM-CSF is used in clinical settings to promote hematopoietic growth in situations where hematopoiesis has been impaired, such as exposure to radiation, chemotherapy treatment, or following a bone marrow transplant [50]. GM-CSF is also given to bone marrow donors in order to mobilize their hematopoietic cells into circulation. Hematopoietic stem cells are collected from the blood of donors; GM-CSF is given to hematopoietic donors in order to increase the number of the cells that can be collected [50].

v. Interleukin-6

IL-6 is a cytokine known to be produced by muscle [51]. IL-6 is also responsive to exercise; one report demonstrates that IL-6 levels in blood increase by 8000 fold after a marathon run in adult athletes [52]. The target of IL-6 in the body is mainly lymphoid cells and it acts on these cells to modulate immune function [53]. IL-6 does have some effects on myeloid hematopoietic cells. IL-6, in combination with IL-3, promotes the self-renewal and proliferation of primitive multipotent hematopoietic stem cells [54]. IL-6 also plays a role in the development of megakaryocytes [55] and the subsequent production of platelets [56]. IL-6 is also able to mobilize hematopoietic stem and progenitor cells into circulation, though to a lesser extent than GM-CSF [57].

Alterations in hematopoietic cytokine level induced by outside sources can thus result in drastic hematopoietic changes. A common doping strategy for the competitive endurance athlete involves supplementation with exogenous EPO, which results in increased red blood cell production [58]. As discussed earlier, this serves to increase endurance exercise capabilities by increasing the oxygen carrying capacity to working muscles. However, there are risks associated with cytokine supplementation. Supplementation with EPO has been known to cause thromboembolism and has been shown to increase risk of mortality in certain patient groups [59, 60]. EPO supplementation can also increase risk of stroke [61]. As discussed earlier, GM-CSF is often used to mobilize hematopoietic stem cells and increase production of white blood cells [62]. As a result of chemotherapy, blood cell production in cancer patients is severely attenuated. GM-CSF can be used to bolster the immune systems of these patients in order to prevent infection [63]. Similarly, interferon therapy can be used to

bolster the immune system of individuals in order to decrease their risk of bacterial or viral infection [64]. However, too much interferon therapy can result in unwanted inflammation, anemia, and a host of other negative side effects [65, 66]. Regulation of hematopoietic cytokine levels is thus critical for proper hematopoietic function. Artificially induced changes in levels of circulating cytokines can have equally as many positive as negative effects.

Physiologically based changes in levels of circulating cytokines are able to mediate the hematopoietic process. The immune response is an excellent example of this. Depending on certain triggers, the cytokine profile in the body can change to direct the immune response towards either a humoral response, utilizing antibodies, or a cellular response, utilizing activated immune cells [67]. This is a dynamic process that is fine tuned and able to change depending on the threat. Release of hematopoietic cytokines and growth factors due to physiological stresses is a common occurrence. Muscle release of hematopoietic cytokines has also been associated with immune function. Post exercise concentrations of cytokines in the blood have been shown to dramatically rise; one report observes that serum IL-6 rises by 8000 fold post marathon run in adult athletes [52]. This has been suggested by some as a mechanism by which exercise can mediate the immune response, in which skeletal muscle acts as an immune potentiating organ [51].

If muscle derived cytokines can mediate lymphoid hematopoiesis and the immune response then it stands to reason that exercise may also be able to mediate the process of myeloid hematopoiesis. Unfortunately, this has not been documented to any meaningful extent. It is known that skeletal muscle is capable of releasing many different types of cytokines and growth factors into circulation as a result of an acute bout of exercise [68].

Cytokine release also changes as a result of endurance exercise training. This is demonstrated by the fact that the muscle endocrine profile of a trained animal is different than that of a sedentary animal; IL-6, TNF-α, IL-1β and IL-10 release from the skeletal muscle of chronically trained rats is decreased [69]. This is a newly emerging area of research, however, and only a few cytokines and growth factors have been demonstrated to be released by skeletal muscle. Even fewer have been shown to change with exercise. Given that muscle is collectively a very large organ and capable of increasing the serum concentration of some growth factors by a large amount [52], it may be possible that muscle derived hematopoietic cytokines are able to promote or alter hematopoiesis. If muscle were able to produce myeloid hematopoietic cytokines and growth factors and release those factors into circulation it would be one means by which exercise training could alter hematopoiesis.

Cytokines only partly regulate hematopoiesis. The hematopoietic niche, the area in the body where hematopoietic stem cells reside, also imparts a level of control over hematopoiesis. The niche serves as an environment for hematopoietic growth and development. Perturbations in the niche can have large impacts on hematopoietic regulation.

d. Hematopoietic Regulation: the niche

Just as adult muscle stem cells have a specific niche, between the sarcolemma and basal lamina of a muscle fiber, hematopoietic stem cells also have specific areas in which they must reside (Figure III). The primary organ of hematopoiesis in adult mammalian organisms is the bone marrow cavity. Within the marrow cavity are multiple cell types

that form the hematopoietic niche. These cells allow hematopoietic cells to attach to a physical matrix and provide the cues needed for the cells to self-renew, proliferate, differentiate, mobilize from the niche, home back to the niche, and assume a quiescent state [23]. The cell types within the niche can act as positive regulators, promoting hematopoiesis, or negative regulators, suppressing hematopoietic stem cell function. Changes in the balance of these niche cells result in changes to the hematopoietic stem cells that reside in the marrow cavity with them.

The general consensus in the literature describing the medullary hematopoietic niche is that it is made up of two distinct regions. The first region is the endosteal niche. This is the niche adjacent to the surface of the bone. Hematopoietic stem cells that reside here physically attach to osteoblasts that line the endosteum [70]. They receive physical signals from these cells, in the form of cell to cell membrane receptor contacts, and also receive paracrine signals from these cells, in the form of cytokines and growth factors [23]. Hematopoietic stem cells associated with the endosteal niche tend to be quiescent, long term, multipotent progenitor stem cells [70]. Ablation of osteoblasts from the bone marrow cavity reduces numbers of hematopoietic cells. Conversely, increasing the number of osteoblasts in the bone marrow cavity has been shown to increase numbers of hematopoietic cells [71]. Mesenchymal cells within the marrow cavity can also impact hematopoietic cell number if their levels are perturbed in any way [70]. A possible mechanism by which endurance exercise training can mediate hematopoiesis is through altering the osteoblast content of the bone marrow cavity.

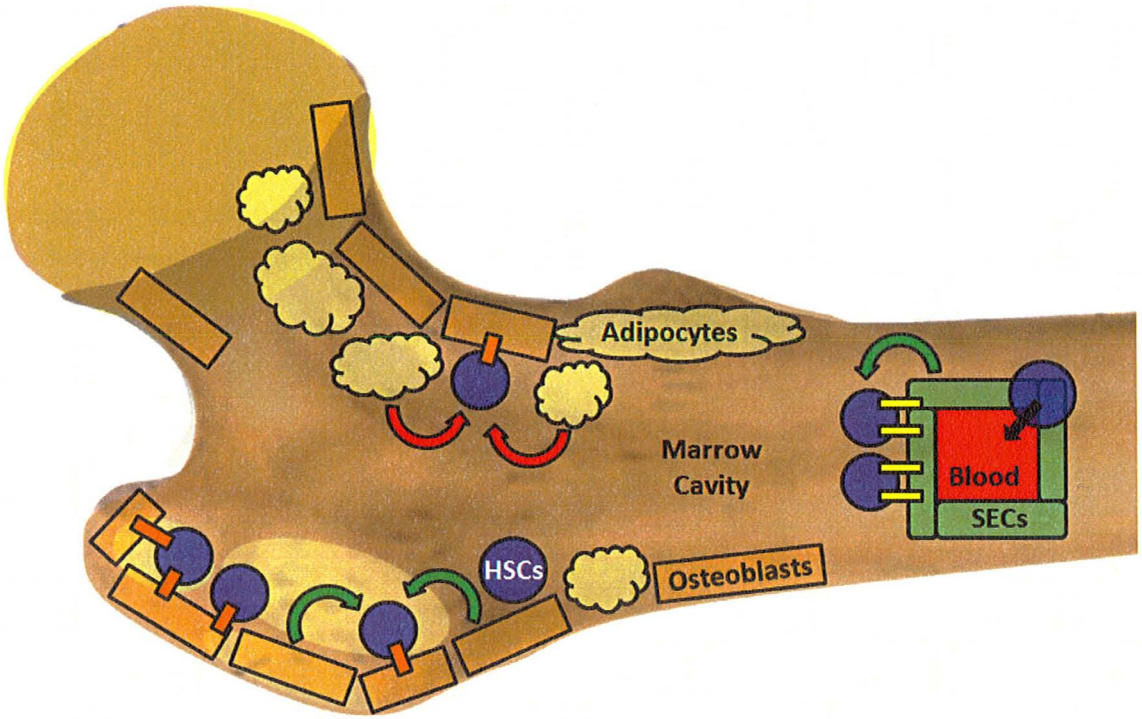

Figure III. The Bone Marrow Hematopoietic Niche. The hematopoietic niche in bone marrow is made up of the endosteal niche, osteoblasts that are adjacent to the endosteum, and the vascular niche, sinusoidal endothelial cells (SECs) adjacent to capillaries. Hematopoietic stem cells physically attach to both niche cell types. Adipocytes can exert negative paracrine influences on HSCs (red arrows) while osteoblasts and SECs can exert positive paracrine influences on HSCs (green arrows). HSCs are able to enter into circulation through the vascular niche.

The second niche region within the bone marrow cavity is the vascular niche. Hematopoietic stem cells form close associations with endothelial cells that make up the sinusoidal blood vessels within the marrow cavity [23]. 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 [70]. Cells in association with the vascular niche tend to be more activated than cells in association with the endosteal niche. These cells are more capable of immediate proliferation and differentiation. These cells are also able to mobilize into circulation and return back to the marrow cavity, which makes sense given their localization next to sinusoidal blood vessels [72]. Just as with the endosteal niche, endothelial cells regulate hematopoietic cells through physical attachment and paracrine signaling. Endothelial cell specific

deletions of surface proteins that serve to attach hematopoietic and endothelial cells are able to massively ablate bone marrow cellularity [70].

The shift of hematopoietic stem cells from fetal liver to bone marrow that occurs after birth makes the bone marrow cavity the primary site of hematopoiesis in adult animals. However, extramedullary hematopoiesis still occurs in adults. Extramedullary hematopoiesis, meaning hematopoiesis outside of bone, occurs in the spleen and to a lesser extent the liver [73]. Though the capacities of these organs to support hematopoiesis declines with age, they always retain it to some degree [74]. The hematopoietic niche in the liver and spleen strongly resembles the vascular niche in the bone marrow cavity, though very little is known about the differences in the makeup of the niche between the bone marrow and these tissues [70]. If hematopoiesis in the bone marrow is altered by a medical condition, such as age or cancer, extramedullary hematopoiesis in the spleen, and to a lesser extent the liver, increases [75]. This is a mechanism to help compensate for reduced blood cell production. Given the increased demand for oxygen transport that results from endurance exercise training, increased extramedullary hematopoiesis is a probable result. In the spleen myeloid hematopoietic stem cells reside within the red pulp. Lymphoid hematopoietic cells that make up the immune cells reside within the white pulp. The hematopoietic niche in adult liver is even less well characterized and very little is known about its composition.

The hematopoietic stem cell niche is essential for regulation of the hematopoietic stem cell. Changes in the niche result in direct changes to the hematopoietic stem cell pool. Though hematopoietic stem cells can survive in circulation for some time they will end up dying or terminally differentiating without a niche to support them [76].

e. Rationale for Research

Though they have been demonstrated on many occasions, the links between endurance exercise training and hematopoiesis are less than clear. The relationship is tenuous, no mechanisms or means of influence have been described in the literature. That endurance exercise is associated with changes in immune system function, hematopoietic cell mobilization, and red blood cell volume cannot simply be due to chance. A more focused look at the interactions between hematopoietic cells and endurance training is needed. General mechanisms controlling the link between endurance exercise and hematopoiesis must be uncovered before any further research between the two can continue.

It is well know that physical activity levels can influence bone morphology. Sedentary levels of activity negatively influence bone health while higher levels of physical activity seem to promote bone health [77]. It has also been suggested that physical activity can influence bone mesenchymal stem cell regulation [78]. Given that change in the hematopoietic niche result in changes to hematopoietic capabilities, this is one plausible mechanism by which endurance training could impact hematopoiesis. Studying the effects of training on the hematopoietic niche is justifiable and niche changes, positive or negative, could help uncover one link between endurance training and hematopoiesis.

The idea that skeletal muscle is able to release copious amounts of cytokines and growth factors into circulation is receiving more attention in the literature and is, as an area of study, slowly becoming more developed. Some of these muscle derived

cytokines, or myokines, include cytokines and growth factors associated with hematopoietic stem cell regulation, such as IL-6 [51]. There is a possibility that true hematopoietic cytokine family members, such as IL-3 and GM-CSF, can also be released by skeletal muscle. It is also possible that exercise training can increase muscle derived hematopoietic cytokine production, as it is able to do with IL-6 and various other growth factors. The skeletal muscle endocrine profile in an endurance trained individual, compared to a sedentary individual, could promote hematopoietic activity. This is another possible mechanism by which training and hematopoiesis can be linked.

Aside from demonstrating plausible mechanisms, definite and unquestionable links between endurance exercise training and hematopoiesis must be demonstrated. The previously mentioned associations between the two only hint at a link. Specific evidence must be gathered. Demonstrating that endurance exercise could increase hematopoiesis could be potentially beneficial to many patient populations, such as anemic and autologous bone marrow transplant patients. It is also very interesting from a basic exercise physiology perspective and could serve to explain the blood oxygen carrying capacity adaptations associated with endurance training.

f. Statement of Research Questions and Hypothesis

Exercise and hematopoiesis are linked but the mechanisms that link them are not known. The goal of this research was to explore possible links between endurance exercise training and hematopoiesis. That endurance training could induce positive changes in the medullary hematopoietic niche was one possible link. This was investigated by analyzing bone mesenchymal stem cell differentiation capacity and

exercise-trained bone morphology. Increased hematopoietic growth factor and cytokine release from skeletal muscle as a result of endurance training was another possible link. This was investigated with the use of real time PCR to demonstrate increased hematopoietic cytokine and growth factor expression in endurance trained muscle. Finally, direct proof that endurance exercise training can promote hematopoiesis was collected. Hematopoietic stem and progenitor cell content in both medullary and extramedullary hematopoietic niches was analyzed in trained and untrained animals. This was analyzed through use of flow cytometry and *ex vivo* hematopoietic stem cell culture. Absolute quantification of the stem cell pool demonstrates undeniably that hematopoietic activity is increased as a result of exercise training.

We propose the following hypotheses:

1. Measures of hematopoiesis will increase in animals that have been endurance exercise trained, as compared to sedentary controls
2. These changes will be demonstrated in multiple hematopoietic stem cell types and in both medullary and extramedullary locations
3. The capabilities of endurance trained skeletal muscle to produce hematopoietic cytokines and growth factors will be increased
4. The physical composition of the medullary hematopoietic niche will be altered as a result of the endurance training program into an environment better capable of supporting hematopoiesis
5. Medullary niche remodeling will occur as a result of endurance exercise induced changes in bone mesenchymal stem cell differentiation capacity

g. References for Section 1

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2. Manuscript Section

The following manuscript has been submitted to Blood for review

Exercise Mediated Regulation of Bone Marrow Hematopoiesis

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Abstract

Bone marrow is the major site of adult hematopoiesis. Bone marrow cells exert control over the hematopoietic stem cells that reside in the niche; osteoblasts act as positive regulators while adipocytes act as negative regulators. Levels of circulating hematopoietic cytokines also regulate hematopoiesis. In this study, we demonstrate that an endurance exercise training program results in several changes that act to induce hematopoiesis. Bone marrow-derived mesenchymal stem cell differentiation is skewed away from adipogenesis and towards osteogenesis in exercise trained animals. As a result, the bone marrow cavity is remodeled during the training period and acts to facilitate hematopoiesis. Hematopoietic cytokine gene expression levels also increase in exercise trained skeletal muscle. These changes translate into increased bone marrow and blood hematopoietic stem and progenitor cell content. This study draws a link between exercise training, bone marrow niche regulation, skeletal muscle derived hematopoietic cytokines, and the regulation of hematopoiesis.

Introduction

Exercise is an unquestionably healthy activity. The physiological and psychological benefits of a well defined exercise regime are numerous. Sedentary behaviour, on the other hand, has been linked to a number of health problems, such as heart disease [1] and type 2 diabetes [2]. Highly aerobic endurance training programs, such as treadmill running or cross country skiing, provide many health benefits and have been implicated in improving sedentary associated health outcomes [3]. The multitude of

health benefits associated with exercise likely extend into many bodily processes, bone marrow based hematopoiesis being one possible candidate.

Exercise has been tenuously linked with hematopoietic regulation in a few different of ways. Adaptations to exercise training include increases in blood volume, red cell volume, and blood hemoglobin content [4]. These adaptations serve to increase oxygen transport to working skeletal muscle and thereby increase exercise performance in trained athletes [4, 5]. Exercise has also been associated with modulation of immune function. Moderate exercise can boost immune function [6] while intense exercise can decrease immune function, causing decreased lymphocyte concentrations, natural killer cell activity, and lymphocyte proliferation [7]. Exercise is also a potent means of mobilizing hematopoietic cells into circulation; an acute bout of maximal exercise in adult athletes can double circulating CD34+ cells, increase white blood cell counts, and increase circulating burst forming unit erythrocyte (BFU-E) and colony forming unit granulocyte erythrocyte monocyte megakaryocyte (CFU-GEMM) progenitors [8].

The mechanisms by which exercise can mediate hematopoiesis and hematopoietic cell mobilization are unknown. Altitude training has been shown to increase hematocrit through hypoxia mediated release of erythropoietin from the kidney [9]; however, sea level increases in hematocrit have never been adequately explained. Skeletal muscle is known to release an assortment of growth factors and cytokines into circulation; IL-6, IL-8, and IL-15 being just a few [10]. Some of these muscle derived cytokines are hematopoietic in nature, providing a potential mechanism through which exercise may play a role in regulating hematopoiesis. Bone health and structure are also mediated by physical activity; exercise promotes bone health, sedentary behaviour does the opposite

[11]. This is another possible means by which exercise can mediate hematopoiesis in the medullary niche.

In this study, we directly investigate the influence of endurance exercise training on the regulation of hematopoietic stem cells and the bone marrow hematopoietic niche. We demonstrate that endurance exercise training significantly alters the structure of the bone marrow hematopoietic niche, resulting in increases in the hematopoietic progenitor cell content of the bone marrow as well as increases in basal levels of circulating hematopoietic progenitors. We also demonstrate that endurance trained skeletal muscle adopts a phenotype associated with increased release of hematopoietic growth factors into circulation.

Methods

Animals

Adult male C57Bl/6 mice (Jackson Laboratories), aged 4 weeks, were used for the exercise training experiments. No more than 5 mice were housed per cage (27 x 12 x 15.5 cm) and were provided food and water *ad libitum*. Mice were maintained on a 12:12 hour light: dark cycle at 22 ± 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 Training

Mice were exercise trained (n=15) on a Exer 6M Treadmill (Columbus Instruments Inc) 3 days per week (Monday, Wednesday, Friday) for ten weeks. The mice

were allowed to acclimatize to the treadmill a week prior to training. For the 10 week training period, mice were subjected to a progressive exercise protocol with the training portion of the protocol beginning at 14 m/min for 45 min (week 1) and increasing to 24 m/min for 45 min (week 10). The training portion of the protocol 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 hind limb stimulation with the bristles of a paint brush. Sedentary control mice (n=15) were exposed to the treadmill on the same days as exercised mice, but were not exercised.

Animal Sacrifice

Mice were sacrificed two days after their last training period. Mice were briefly anaesthetized with isoflurane (Abraxis BioScience) and blood was collected into heparinised tubes (Sigma-Aldrich) via cardiac puncture. Mice were then euthanized via cervical dislocation. Both femurs and tibias were excised, dissected of muscle and fat, and flushed with 1 ml of Iscove's modified Dulbecco medium (Sigma) with 2% fetal bovine serum (FBS) (Gibco) to collect bone marrow. Kidneys and lower leg muscle groups were isolated and flash frozen in liquid nitrogen.

Mesenchymal Stem Cell Collection

Femurs and tibias, previously flushed of marrow and cleared of muscle and fat, were cracked open with a mortar and pestle. Bone fragments were washed twice for 5 minutes each using phosphate buffered saline (PBS) (Sigma) containing 1 mM EDTA (Sigma) and 2% FBS at 37 °C with 225 RPM agitation. Bones were then submerged in 5 mL PBS containing 20% FBS and 0.25% Collagenase I (Sigma), chopped into smaller

pieces with scissors, and agitated at 225 RPM at 37 °C for 45 minutes. α-minimum essential medium (α-MEM) (Sigma), with 15% FBS and penicillin streptomycin (Gibco), was added to the resultant cell suspension, which was then filtered twice with 70 μm and 30 μm cell strainers (Partec), centrifuged, and plated in 35mm tissue culture plates at $1x10^6$ cells per plate.

Mesenchymal Stem Cell Differentiation

Half of the mesenchymal stem cell (MSC) plates were used to assay osteogenesis. Cells were maintained in α-MEM, with 15% FBS and penicillin streptomycin, supplemented with 50 μg/mL ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma), for 28 days. Cells were stained with Alizarin Red S (Sigma). To quantify osteogenesis the bound Alizarin Red S was eluted, as described elsewhere [12], and measured at 405 nm. The other MSC plates were used to assay adipogenesis. Cells were maintained in α-MEM, with 15% FBS and penicillin streptomycin, for 21 days. Cells were stained with Oil Red O (Sigma). To quantify adipogenesis the bound Oil Red O was eluted with isopropanol and measured at 500 nm. Representative images were captured using a Nikon Eclipse 90i (Nikon) with Nikon Elements AR 3 (Nikon) software. Dye measurements were taken using an Ultraspec 3000 Pro (GE Healthcare). Additional wild type or GFP positive male C57Bl/6 animals, bred in house and exercised trained as above, were used to achieve statistical significance in these experiments (n=5).

Histology

Both humeri from each animal were also harvested, fixed (4% formaldehyde in PBS, 48 h), and decalcified (10% EDTA, pH 7.5, 14 d). The bones were then paraffin-

embedded, sectioned transversely, stained with haematoxylin and eosin, and visualized using a Nikon Eclipse 90i (Nikon). Marrow cavity fat was determined by comparing adipocyte surface area to total marrow cavity surface area using the Nikon Elements AR 3 software package (Nikon).

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR Reaction

Total RNA was isolated from trained and sedentary gastrocnemius muscle using a combination TRIzol (Invitrogen) and Total RNA Kit (Omega Bio-Tek) method [13]. RNA was reverse transcribed using a commercially available kit (Applied Biosystems High Capacity cDNA Reverse Transcription Kit) with a Mastercycler epgradient Thermal Cycler (Eppendorf). Quantitative RT-PCR reactions were conducted using a Stratagene Mx3000P real-time PCR System (Stratagene) or a Mastercycler ep Realplex 2S QPCR (Eppendorf). All samples were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fold changes in gene expression were calculated using the delta-delta Ct method [14]. Previously published primer sequences were used for interleukin-3 (IL-3) [15], erythropoietin (EPO) [16], macophage colony stimulating factor (M-CSF) [17], and granulocyte macrophage colony stimulating factor (GM-CSF) [18].

Hematopoietic Colony Forming Assays

Mononuclear cells were isolated from blood and bone marrow cell suspensions using Ficoll-Paque PLUS (GE Healthcare) and $2x10^4$ cells were plated in triplicate into Mouse Methylcellulose Complete Media (R&D Systems). BFU-E, colony forming unit granulocyte monocyte (CFU-GM), and CFU-GEMM colonies were scored 10 days later

on a Zeiss Axiovert 200 microscope (Carl Zeiss). Bone marrow mononuclear cells were also used for the cobblestone area forming cell (CAFC) assay [19, 20] using the FBMD-1 cell line as a feeder layer [21]. CAFC were scored at day 35 post plating. FBMD-1 were a generous gift from Dr. Ronald van Os and were subcultured as described elsewhere [20].

Flow Cytometry

The number of lineage negative (lineage panel, BD Pharmingen, neat), c-kit positive (anti-mouse c-Kit 2B8, eBiosciences, 1:10), and Sca-1 positive (anti-mouse Sca-1 E13-161.7, eBiosciences, 1:10) (LSK) cells in each mononuclear bone marrow sample was measured with an Epics XL flow cytometer (Beckman Coulter). Gaiting and compensation were established based on single stained controls. Cells were quantified as a percentage of total input.

Statistics

Data are expressed as mean ± standard error with $p \leq 0.05$ considered significant. Statistical differences between sedentary and exercise groups were determined using 2-tailed t-tests with SigmaStat 3.1 (Systat Software Inc). CAFC frequency was determined with use of L-Calc software (Stemcell Technologies).

Results

Exercise alters mesenchymal cell differentiation potential

To understand the effects that endurance exercise training had on the bone marrow hematopoietic niche, MSCs were isolated from the femurs and tibias of sedentary

and exercise trained animals. The marrow cavity had been flushed from these bones prior to MSC extraction. These bones were used for MSC isolation as they represent load bearing bones that would be impacted by the treadmill training program. The isolated cells were allowed to differentiate with minimal stimulation for lineage commitment. Adipocyte differentiation was allowed to spontaneously occur while osteoblast differentiation proceeded without stimulation from strong osteogenic factors, such as BMP-4. This was done in order to assess the natural propensity of the cells to differentiate into these lineages.

Oil Red O was used to measure lipid droplets as a proxy measure of adipocyte differentiation. There were noticeable differences in adipocyte lipid formation between the sedentary (Figure 1A) and endurance trained (Figure 1B) samples. Solubilization of the dye from the cells allowed for quantification of staining (Figure 1C) and revealed a 42% decrease ($p < 0.05$) in the amount of lipids in exercise trained samples as compared to the sedentary controls. Alizarin Red S was used to measure calcification as a proxy measure of osteoblast differentiation. Differences in calcification between the sedentary (Figure 1D) and exercise trained (Figure 1 E) samples were noticeable. Solubilization of the dye from the cells in each group allowed for quantification of staining (Figure 1F). There was a 52% increase ($p < 0.05$) in calcification in the exercise trained samples as compared to the sedentary controls. A within mouse adipocyte to osteoblast differentiation potential ratio verifies these findings; exercise trained animals had a 64% decrease ($p < 0.05$) in the ratio of adipocyte to osteoblast differentiation compared to sedentary controls (Supplementary Figure 1).

Exercise remodels the bone marrow hematopoietic cavity

MSCs residing within compact bone and inside the bone marrow cavity can differentiate into osteoblasts and adipocytes, the cells that comprise the bone marrow niche. Given the differences in mesenchymal differentiation potential observed between sedentary and exercise trained animals, the niche itself may have been remodelled during the course of the exercise training program. Humeri from each animal, another load bearing bone that would be impacted by the treadmill training program, were used to examine the appearance of the marrow cavity. Adipocyte content in the marrow cavity and overall marrow cellularity were assessed with a haematoxylin and eosin stain.

Large deposits of fat were seen adjacent to the endosteum in sedentary animals (Figure 2A). Fat also marbled the center of the bone marrow cavity in sedentary animals (Figure 2C). Much less fat was seen adjacent to the endosteum in exercise trained animals (Figure 2B). Less fat was also seen in the center of the bone marrow cavity in exercise trained animals (Figure 2D). A quantification of total fat in the bone marrow cavity revealed that exercise trained animals had 78% less ($p < 0.05$) fat in their marrow cavities than sedentary control animals (Figure 2E).

Exercise increases hematopoietic cytokine expression in skeletal muscle

Contracting skeletal muscle is a rich source of growth factors that can enter circulation. Skeletal muscle also undergoes extensive metabolic and structural remodelling during exercise training [22]. Taking these factors into consideration, it is feasible to suggest that exercise training is able to alter the endocrine footprint of skeletal muscle. Real time PCR was used to analyze changes in skeletal muscle hematopoietic

growth factor expression in order to isolate their potential contribution to circulating hematopoietic growth factor levels.

Increases in the expression of several hematopoietic growth factors were observed in exercise trained muscle as compared to sedentary muscle. Interleukin-3 expression was increased in exercise trained muscle by 116% ($p < 0.05$) (Figure 3A). Erythropoietin expression was increased in exercise trained muscle by 93% ($p < 0.05$) (Figure 3B). The expression of both macrophage colony stimulating factor (Figure 3C) and granulocyte macrophage colony stimulating factor (Figure 3D) was increased by 66% ($p < 0.05$) and 67% ($p < 0.05$) in exercise trained muscle, respectively.

The kidney is a primary source of erythropoietin for the body and acts to regulate erythropoiesis through erythropoietin production. The ability of exercise to alter kidney erythropoietin production at sea level conditions is unknown. Interestingly, kidney expression of erythropoietin decreased by 87% ($p < 0.05$) in exercise trained animals as compared to sedentary controls (Supplementary Figure 2).

Exercise increases bone marrow hematopoietic stem cell capacity

Positive changes in the bone marrow niche and increases in the potential contribution of skeletal muscle to circulating hematopoietic cytokine levels hint that exercise training may facilitate increased hematopoiesis. To be sure of this the hematopoietic cell content in the bone marrow cavity was assessed. Several methods were used in order to capture the spectrum of hematopoietic stem and progenitor cell development. Flow cytometry and the CAFC assay measured hematopoietic stem cells

while the methylcellulose colony forming assay was used to measure more differentiated hematopoietic progenitor cells.

An increase of 208% ($p < 0.05$) was observed in the number of BFU-E colonies forming from the marrow of exercise trained animals as compared to sedentary controls (Figure 4A). In the same groups, an increase of 24% ($p < 0.05$) was seen in the numbers of CFU-GM progenitors (Figure 4B) and an increase of 234% ($p < 0.05$) was seen in the number of CFU-GEMM progenitors (Figure 4C). Overall, there were 49% more ($p < 0.05$) total colony forming cells in exercise trained marrow as compared to sedentary control marrow (Figure 4D). The frequency of CAFCs at day 35 increased by 76% ($p < 0.05$) in exercise trained samples (Figure 4E). There was a trend suggesting a 20% increase in the number of LSK cells in the bone marrow of exercise trained animals (Figure 4F) but the difference did not achieve statistical significance ($p = 0.176$). Greater increases were seen in the numbers of BFU-E and CFU-GEMM progenitors than were seen in the number of CFU-GM progenitors ($p < 0.05$ for all differences) (Supplementary Figure 3).

Exercise supports higher basal levels of circulating hematopoietic progenitors

A bout of exercise can temporarily elevate levels of circulating hematopoietic stem and progenitor cells. An elevation in the basal levels of circulating hematopoietic progenitors is not seen as a result of an acute bout of exercise. This is instead indicative of increased levels of hematopoiesis. Observing increases in basal levels of circulating progenitors would verify the increases in the hematopoietic content observed in the bone marrow cavity. Blood was taken from exercise trained and sedentary animals and

assayed for hematopoietic progenitor cells with the methylcellulose colony forming assay.

The number of BFU-E was increased by 180% ($p < 0.05$) in the blood of exercise trained animals as compared to sedentary controls (Figure 5A). An increase of 218% ($p < 0.05$) was seen in the number of circulating CFU-GM progenitors (Figure 5B). The number of CFU-GEMM progenitors in the blood of exercise trained animals increased by approximately 869% ($p < 0.05$) (Figure 5C). Overall, there were approximately 229% more ($p < 0.05$) total colony forming cells in the blood of exercise trained animals as compared to sedentary controls (Figure 5D).

Discussion

Our findings demonstrate that endurance exercise training increases hematopoiesis (Figures 4 and 5). This may be due in part to increased endocrine signalling from skeletal muscle (Figure 3), as well as a remodelling of the medullary hematopoietic niche (Figures 1 and 2). Discussed below are current ideas in the hematopoietic literature that support these claims.

The cells that make up the medullary hematopoietic niche play important roles in the regulation of hematopoiesis. Osteoblasts, osteogenic cells that line the endosteum, have been shown to act as positive mediators of hematopoiesis. Hematopoietic stem cells (HSCs) are found in close proximity to osteoblasts and it has been suggested that they directly attach to these cells through N-cadherin-mediated adhesion [23, 24]. Increases in osteoblast number correlate with increases in HSC number [23, 24]. Conversely, ablation of osteoblasts results in an immediate loss of some hematopoietic progenitor types and is

later followed by a decline in the LSK population [25, 26]. Osteoblasts are known to produce growth factors that can support hematopoiesis. Angiopoietin and thrombopoietin, both released by osteoblasts, have been shown to promote HSC quiescence [27, 28]. CXCL12 is also released by osteoblasts and plays a role in HSC homing and localization to the niche [29]. Osteoblasts also express the Notch ligand, Jagged1, which can activate Notch signalling in HSCs and promote HSC maintenance [24].

Conversely, other cell types in the marrow cavity, such as adipocytes, play a role in negatively regulating hematopoiesis. Adipocytes are the most abundant stromal cell phenotype in adult human marrow [30]. Early in life hematopoietically active red marrow predominates in the bone marrow cavity but is slowly replaced by fatty, hematopoietically inactive, yellow marrow [31]. Evacuation of marrow cavity fat results in a regeneration of hematopoietically active red marrow though hematopoiesis soon declines as adipocytes begin to repopulate the evacuated area [32]. While it was once thought that fatty marrow conversion negatively impacted hematopoiesis by simply occupying marrow space, it is now known that adipocytes are able to negatively regulate the hematopoietic microenvironment in an active manner. Mice with genetically or pharmacologically impaired adipogenesis show accelerated hematopoietic recovery after bone marrow ablation [33]. While adipocytes are able to support the differentiation of hematopoietic progenitor cells in culture, they are unable to maintain hematopoietic stem cell survival and self renewal [34]. Compared to their undifferentiated precursors and other marrow cell types, bone marrow adipocytes show reduced production of the hematopoietic growth factors GM-CSF and G-CSF [34, 35]. Furthermore, adipose tissue

can secrete factors, such as neuropillin-1 [36], lipocalin 2 [37, 38], adiponectin [39], and TNF-α [40, 41], which act to impair hematopoietic proliferation.

We demonstrate that MSCs from endurance trained animals have decreases in adipocyte differentiation potential (Figure 1C) and increases in osteoblast differentiation potential (Figure 1F). The changes in mesenchymal differentiation potential act to decrease marrow cavity fat over the training period (Figure 2E). These results may be due to the mechanical forces applied to the bone during the treadmill training program. Mechanical strain *in vitro* inhibits adipogenesis [42] and is able to enhance mineralization in differentiating MSCs [43]. Mechanical forces act to down regulate PPARγ, a receptor responsible for adipocyte-specific gene expression and adipose cell formation, resulting in increased osteogenesis and decreased adipogenesis in bone marrow stromal cells [44]. A study investigating the effects of climbing exercise on MSC differentiation potential and bone remodelling demonstrates similar results [45].

Given the important regulatory role played by osteoblasts and adipocytes on HSCs, the data presented herein suggest that exercise training alters the HSC niche in a matter that supports hematopoiesis. Exercise training reduced adipocyte content in the marrow cavity (Figures 1 and 2). Adipocytes act as negative hematopoietic regulators and reducing their number would serve to increase hematopoiesis. Exercise training increased the osteoblast differentiation potential of MSCs (Figure 1). Osteoblasts act as positive hematopoietic regulators and increasing their number would serve to increase hematopoiesis. If these findings were chemically or genetically induced, as in some of the studies discussed previously [23, 24, 33], the result one would expect is an increase in bone marrow and blood hematopoietic cell content. Indeed, these are the results of the

exercise training program – an increase in bone marrow hematopoietic stem cell content (Figure 4) and an increase in the number of basal circulating hematopoietic progenitors (Figure 5).

While the bone marrow niche and the cells that reside within it are important, endocrine signalling from extramedullary tissues is critical for regulation of hematopoiesis. The result of interrupting such signalling, such as a marked reduction in erythropoiesis and hemoglobin synthesis when erythropoietin signalling is ablated by nephrectomy [46], is severely attenuated hematopoiesis. Positive effects can be seen through interventions that enhance or mimic increased extramedullary endocrine signalling; subcutaneous injections of the hematopoietic cytokines IL-3 and IL-6 increase the number of hematopoietic colony forming cells found in bone marrow [47] and administration of GM-CSF or GM-CSF in conjunction with IL-3 can facilitate hematopoietic recovery following bone marrow ablation [48, 49] . Blood doping with erythropoietin is a common way in which athletes can increase their oxygen carrying capacity by greatly increasing their hematocrit. Interestingly, a simple physiological intervention like exercise can boost levels of circulating hematopoietic cytokines in a considerable manner. Increases of 8000 fold in circulating levels of IL-6 have been observed in athletes after finishing a marathon race [50]. Elevations in circulating angiopoietin, c-kit ligand, Flt3 ligand, TGF-β1, and many other hematopoietic growth factors are also commonly seen post exercise [8, 51]. Recent reports suggest that skeletal muscle is able to produce some growth factors associated with hematopoiesis, such as IL-6, IL-8, IL-15 [10], BDNF [52], and LIF [53].

Here we demonstrate that the result of an endurance exercise training program is increased basal expression of IL-3, erythropoietin, M-CSF, and GM-CSF in trained skeletal muscle (Figure 3). IL-3 is a cytokine that supports the development of many different types of hematopoietic progenitors. Erythropoietin supports red blood cell progenitor growth. M-CSF and GM-CSF, while potent hematopoietic mobilization factors, also support macrophage and granulocyte progenitor cell growth and development. As with the examples mentioned previously, increasing the levels of these hematopoietic cytokines in blood should serve to increase hematopoietic capacity. Indeed, increases in bone marrow hematopoietic stem cell content (Figure 4) and basal circulating hematopoietic progenitors (Figure 5) were observed following exercise training. These results suggest that skeletal muscle may be an important regulator of hematopoiesis.

The kidney regulates levels of red blood cells through release of erythropoietin. Erythropoietin stimulates red blood cell progenitors to proliferate and differentiate, resulting in new red blood cells. When oxygen levels are low, as in situations involving hypoxia, the kidney releases erythropoietin in order to compensate by increasing red blood cell synthesis [54]. Interestingly, there was an 87% decrease in the gene expression level of erythropoietin in the kidneys of exercise trained animals (Supplementary Figure 2). This is counterintuitive given the increases seen in the number of BFU-E in the bone marrow and blood (Figures 4A and 5A). Indeed, hematopoietic progenitors capable of giving rise to red blood cells increased in number more so than progenitors only capable of giving rise to white blood cells (Supplementary Figure 3). One would expect the kidney to have produced more erythropoietin in order to

facilitate these increases. Skeletal muscle expression of erythropoietin, however, was increased 93% in exercise trained animals (Figure 3B). This suggests that the 87% decrease in kidney erythropoietin expression may be compensated for and even surpassed by the 93% increase in skeletal muscle erythropoietin expression. Our results indicate that skeletal muscle, as a cytokine and growth factor releasing organ, may play a meaningful role in the regulation of hematopoiesis. Though changes in erythropoietin gene expression do not necessarily mean changes in erythropoietin secretion, expression levels and secretion levels of some cytokines produced by skeletal muscle do correlate with one another; changes in muscle IL-6 expression are associated with similar changes in muscle IL-6 secretion [55]. Profiling erythropoietin expression in this way also allows for identification of the contribution of specific organs to blood erythropoietin levels.

The potential clinical implications for exercise training mediated improvements in hematopoiesis are extensive. Anemias are characterized by an inability of the bone marrow to replenish blood cells and maintain blood cell homeostasis. Treatments for anemia involve anything from hematopoietic growth factor supplementation to bone marrow transplant [56]. The marrow cavity in anemic individuals is often filled with adipocytes [57], similar to the phenotype observed in our sedentary animals. Indeed, bedrest in otherwise healthy adults results in marrow fat accumulation and symptoms of anemia [58]. An endurance exercise training program that is capable of remodelling the marrow cavity sufficiently enough to improve hematopoiesis, along with the extra hematopoietic growth factor supplementation from exercise trained skeletal muscle, could serve as a first step therapy for some forms of anemia.

Endurance exercise could also serve as a physiological adjuvant for autologous bone marrow transplantation. The transplantation process replaces hematopoietic stem cells but does not replace a damaged or failing bone marrow niche. Using endurance exercise training to remodel the bone marrow niche, such that it is better able to support newly transplanted and engrafting hematopoietic stem cells, could potentially improve transplant success. Another concern with autologous transplantation is acquiring sufficient numbers of hematopoietic stem cells from blood or bone marrow [59]. Given the increases in the number of hematopoietic stem cells we see in the bone marrow and blood of endurance trained animals, our data suggests that an endurance training conditioning program could make finding sufficient numbers of hematopoietic cells much easier.

In summary, we demonstrate changes occurring in exercise trained animals that act synergistically to facilitate increased hematopoiesis. Changes in the bone marrow cavity begin to reduce adipocyte content and expand bone marrow hematopoietic stem cell carrying capacity (Figures 1 and 2). Potential increases in levels of circulating hematopoietic growth factors, mediated by increased expression of these factors in skeletal muscle, then allow the hematopoietic stem and progenitor cells in the bone marrow to fill their expanding niche (Figure 3). The result is increased numbers of hematopoietic stem and progenitor cells in the bone marrow cavity (Figure 4). These changes are reflected in the blood by higher basal levels of circulating hematopoietic progenitor cells (Figure 5). The observed increases in the number of hematopoietic progenitors in the marrow cavity and blood were not small; large increases, upwards of

800%, were seen in some experiments. These findings suggest that both physical activity and skeletal muscle itself may act as an important regulatory organ for hematopoiesis.

Authorship

Contribution: J.B. designed and performed experiments, analyzed data, and wrote the manuscript; M.D. performed dissections and edited the manuscript; G.P. designed experiments, supervised the project, and edited the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests. Correspondence: Correspondence should be directed to:

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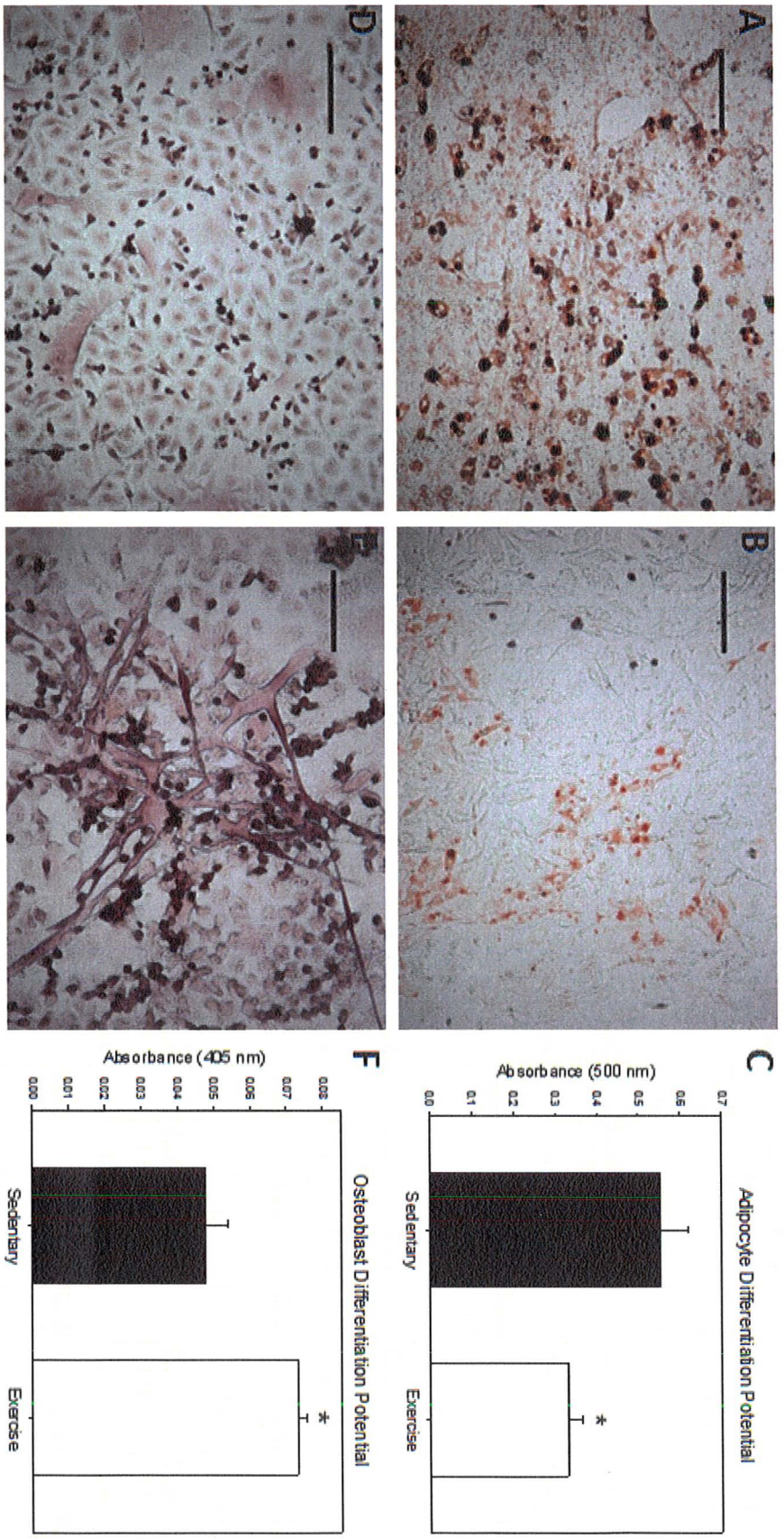


Figure 1. Mesenchymal stem cell differentiation potential. Scale bars represent 500 μM. Representative images of 21 day, Oil Red O stained, differentiated MSCs taken from the bones of **(A)** sedentary or **(B)** exercise trained animals. **(C)** Quantification of Oil Red O staining between sedentary and exercise trained plates. Representative images of 28 day, Alizarin Red S stained, differentiated MSCs taken from the bones of **(D)** sedentary or **(E)** exercise trained animals. **(F)** Quantification of Alizarin Red S staining between sedentary and exercise trained samples. * $p < 0.05$ or is as otherwise indicated.

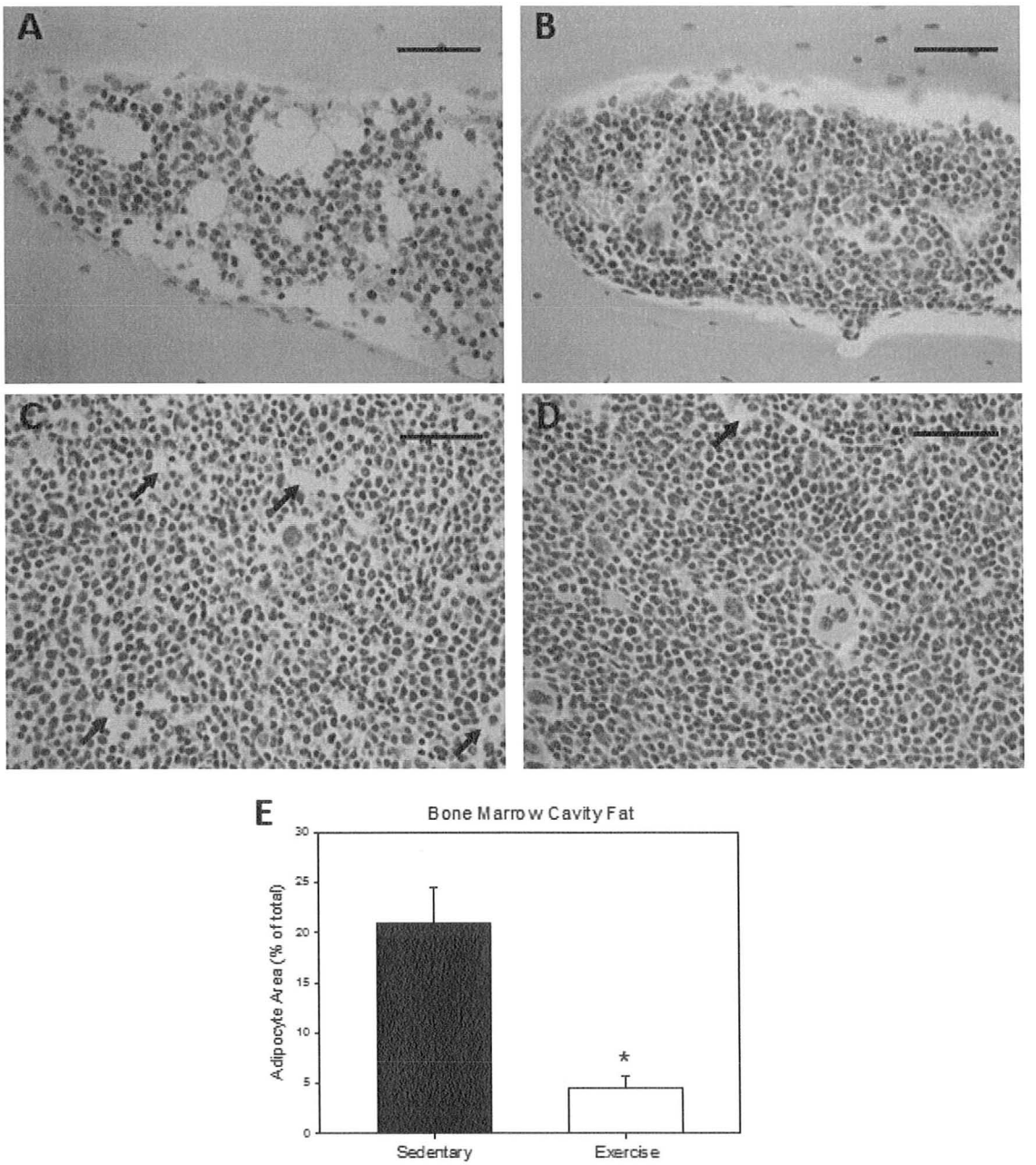


Figure 2. Sedentary and exercise trained bone marrow cavity morphology. Scale bars represent 50 μM. Representative images, taken adjacent to the endosteum, of hematoxylin and eosin stained bones from **(A)** sedentary animals or **(B)** exercise trained animals. Representative images, taken center marrow cavity, of hematoxylin and eosin stained bones from **(C)** sedentary animals or **(D)** exercise trained animals. **(E)** Quantification of bone marrow cavity, on a surface area basis, for the entire bone marrow cavity. Arrows indicate adipocytes in panels **C** and **D**. * $p < 0.05$.

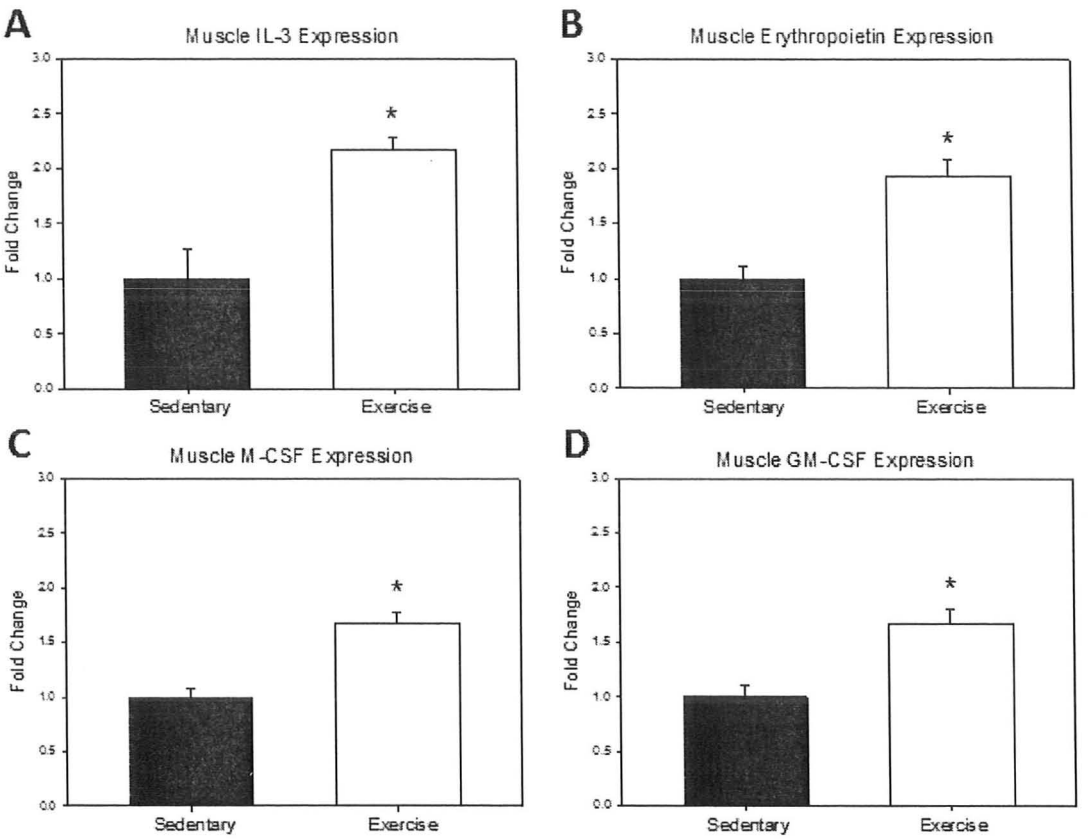


Figure 3. Hematopoietic growth factor gene expression profiling in skeletal muscle. Differences in **(A)** IL-3 expression, **(B)** EPO expression, **(C)** M-CSF expression, and **(D)** GM-CSF between sedentary and exercise trained skeletal muscle. * $p < 0.05$.

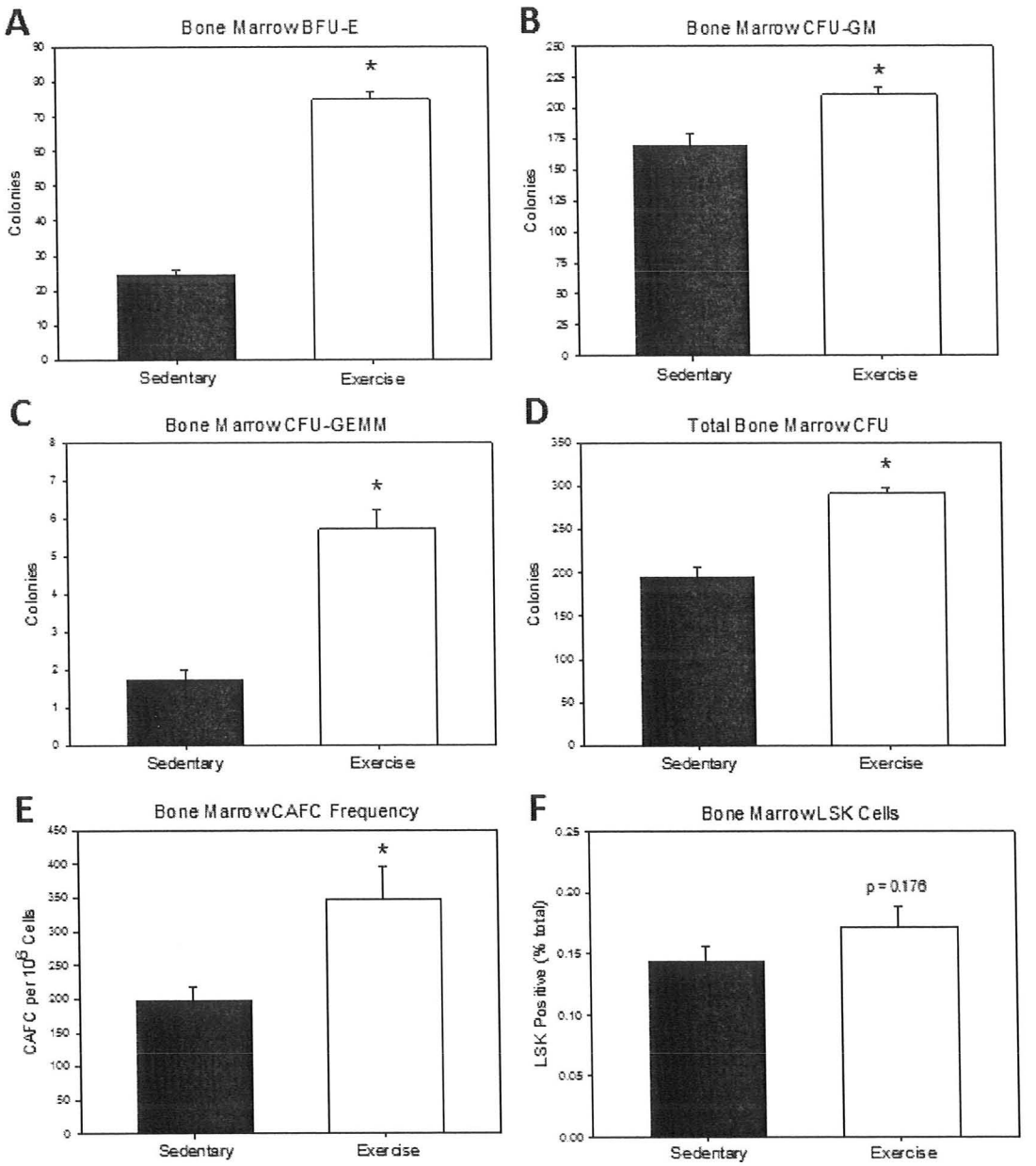


Figure 4. Bone marrow hematopoietic stem and progenitor cell content. Quantification of bone marrow **(A)** BFU-E, **(B)** CFU-GM, and **(C)** CFU-GEMM colonies between sedentary and exercise trained animals. **(D)** Total bone marrow colony forming units in each group. **(E)** Bone marrow CAFC frequency between sedentary and exercise trained animals. **(F)** Percentage of LSK bone marrow monocuclear cells detected in sedentary and exercise trained animals. * $p < 0.05$ or is as otherwise indicated.

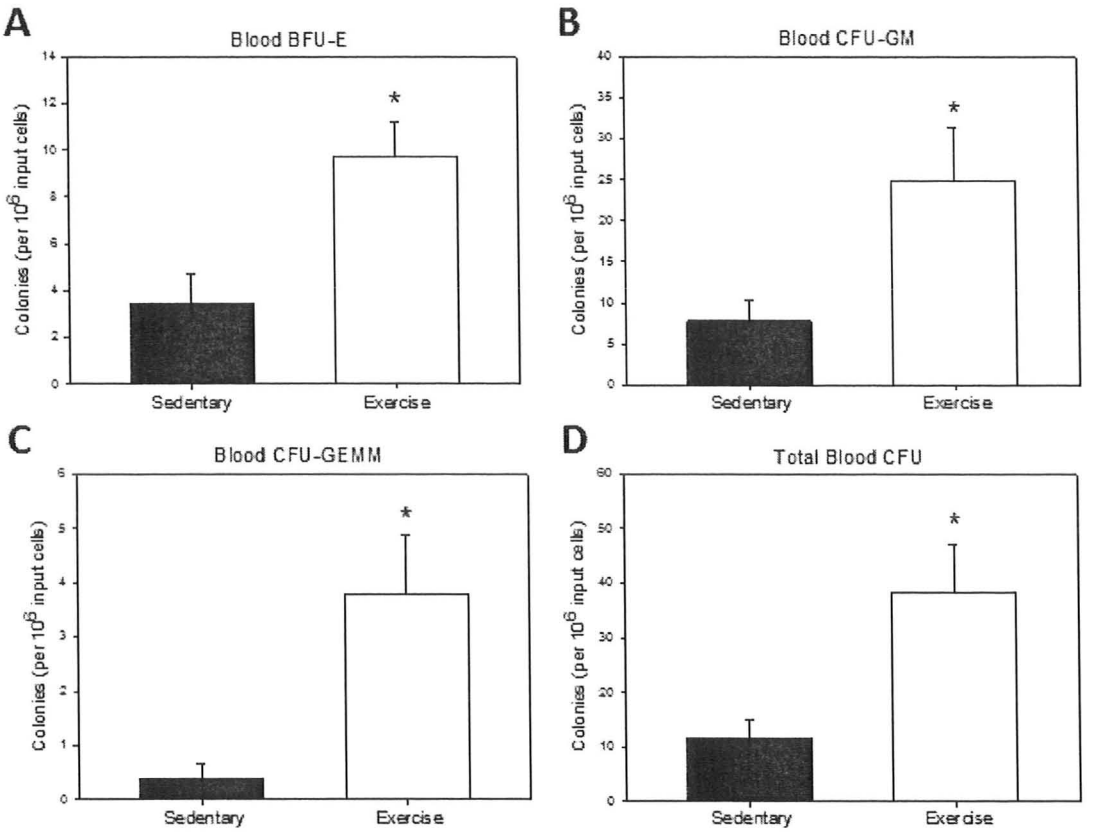
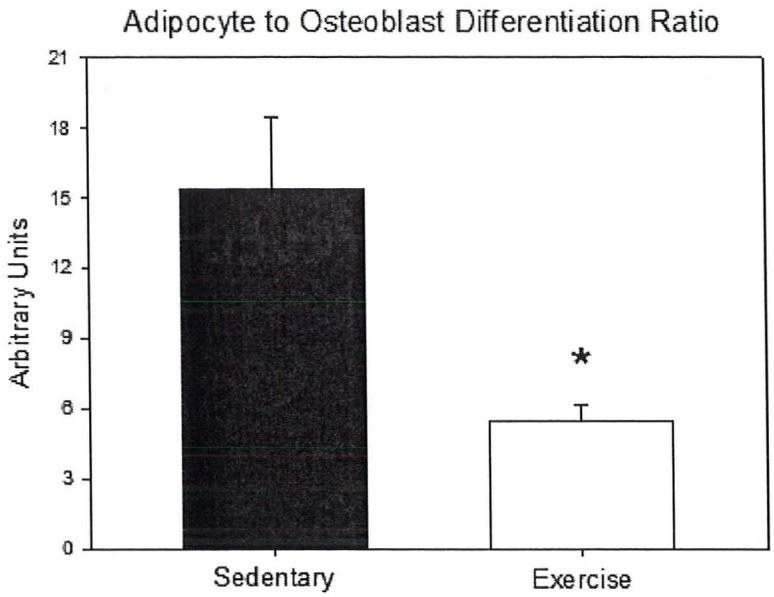
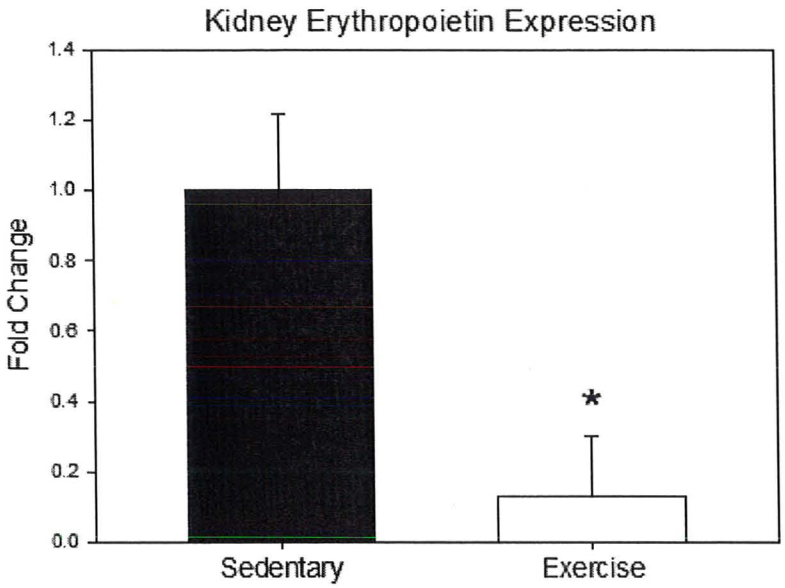


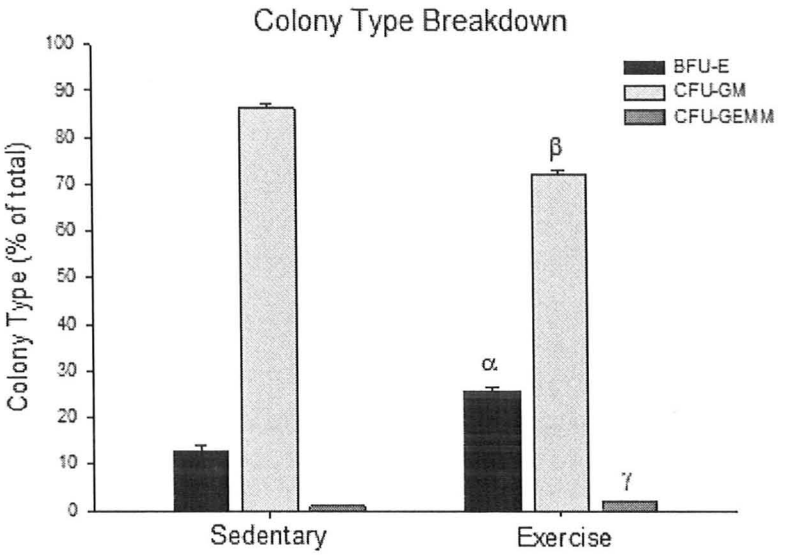
Figure 5. Circulating hematopoietic progenitor cell content. Quantification of blood **(A)** BFU-E, **(B)** CFU-GM, and **(C)** CFU-GEMM colonies between sedentary and exercise trained animals. **(D)** Total blood colony forming units in each group. Blood was harvested two days post final exercise bout. * $p < 0.05$.



Supplementary Figure 1. Within mouse ratio of adipocyte to osteoblast differentiation potential. The propensity of sedentary or exercise trained MSCs to differentiate towards adipocytes. Expressed as a ratio calculated within mouse. Sedentary MSC potential for differentiation into adipocytes is nearly double that of exercise trained MSC. * $p < 0.05$.



Supplementary Figure 2. Erythropoietin gene expression profiling in kidney. Differences in erythropoietin gene expression between sedentary and exercise trained kidneys. Exercise trained animal expression is decreased by eightfold compared to sedentary animals. * $p < 0.05$.



Supplementary Figure 3. Bone marrow hematopoietic progenitor colony type breakdown. The percent total of each colony type in the bone marrow of sedentary and exercise trained animals. While there were greater total numbers of each colony type, there were greater increases in BFU-E and CFU-GEMM and smaller increases in CFU-GM in the bone marrow of exercise trained animals. α - $p < 0.05$ for differences in BFU-e, β - $p < 0.05$ for differences in CFU-GM, and γ – $p < 0.05$ for differences in CFU-GEMM.

3. Expanded Discussion

a. Major Findings

The current work describes two proposed mechanisms by which endurance exercise training is able to positively influence hematopoiesis. The first potential mechanism involves the effect of mechanical forces on the bone marrow cavity. Treadmill trained animals have increased levels of mechanical forces applied to their bones, as a result of footfall impacts while running on the treadmill [1]. The effect of this is to alter mesenchymal differentiation potential in the bone marrow cavity away from adipogenesis and towards osteogenesis. Similar stresses, such as mechanical strain applied in vitro to mesenchymal stem cells, show similar results [2]. The marrow cavity is remodeled over the exercise training period as a result of this. The end effect is additional niche space for hematopoietic cells to occupy and less adipocytes in the marrow cavity to act as negative regulators of hematopoiesis.

The second potential mechanism involves hematopoietic growth factor and cytokine release from skeletal muscle. Endurance trained skeletal muscle expressed higher levels of GM-CSF, G-CSF, IL-3, and EPO than sedentary muscle. Presumably, the effect of this is increased levels of hematopoietic cytokines in the blood of trained animals. These increased levels of cytokines could then help hematopoietic stem cells in the bone marrow niche grow and develop. Artificial manipulations of hematopoietic cytokine levels in vivo show similar results [3]. The cells would then be able to proliferate and fill their expanding niche.

The net result of both of these mechanisms (summarized in Figure IV) is increased levels of hematopoiesis in the bone marrow cavity. Hematopoietic progenitor cell content in the bone marrow cavity, as measured by the CFU-GEMM assay, was increased. Hematopoietic stem cell content in the bone marrow cavity, as measured by the CAFC assay as well as through flow cytometry for LSK cells, was also increased. These results provide definite proof that hematopoiesis is positively regulated and increased as a result of endurance exercise training. Increased levels of hematopoiesis in the bone marrow cavity also result in increased levels of hematopoietic progenitors circulating in blood. This may be due to a spillover effect from the marrow cavity or may be due to increased skeletal muscle production of the hematopoietic mobilization factors GM-CSF and G-CSF.

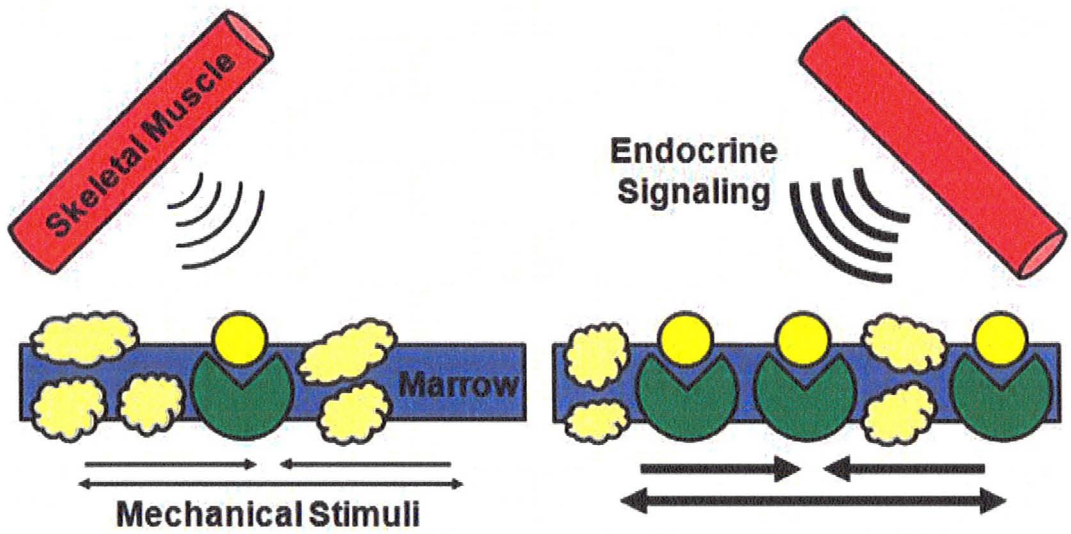


Figure IV. Potential mechanisms. Increased endocrine signaling from skeletal muscle, combined with additional mechanical stimulation of the marrow cavity, results in remodeling of the bone marrow niche. As a result of the increased mechanical forces present on the bone, the adipocyte content (white) of the marrow cavity is reduced and the osteoblast content (green) of the marrow cavity is increased. The increased growth factor signaling from skeletal muscle then allows for HSCs (yellow) to proliferate and fill their expanding niche.

b. Extramedullary Hematopoiesis

Endurance exercise trained animals had greater levels of extramedullary hematopoiesis than sedentary controls. Red blood cell progenitors, measured as BFU-E in the CFU-GEMM assay, were increased by over 300% in exercise trained animals (Figure A1). Interestingly, white blood cell progenitors, measured as CFU-GM in the CFU-GEMM assay, were decreased by 30% in the spleens of exercise trained animals (Figure A2). Multipotent hematopoietic progenitors, measured as CFU-GEMM in the same assay, increased by over 300% in the spleens of exercise trained animals (Figure A3). Though white blood progenitor cells and white red multipotent progenitors cells were not detected in the liver CFU-GEMM assay, trained animals had 40% more liver red blood cell progenitors as compared to sedentary controls (Figure A4).

This data is particularly interesting in the context of adaptation in response to endurance training. Typically, extramedullary hematopoiesis in adult mammalian organisms occurs when the hematopoietic cell system is trying to compensate for a stress [4]. For example, if hematopoietic cells in the bone marrow cavity are ablated as a result of cancer chemotherapy, extramedullary hematopoiesis is upregulated in order to help maintain blood cell pool homeostasis [5]. Endurance training causes red blood cells to be in high demand – their turnover rate is increased and steady state levels of red blood cells are also increased [6]. This helps increase the oxygen carrying capacity of blood in order to increase oxygen transport to the muscles and improve exercise performance [7].

It is possible that endurance exercise may represent a stress similar to that of chemotherapy. Hematopoiesis in the bone marrow cavity may be increased due to the

demand of endurance training, but not enough to maintain adequate levels of red blood cells. Extramedullary hematopoiesis could then act to supplement bone marrow blood cell production. This idea is supported by the fact that both red blood cell progenitors (Figure A1) and multipotent hematopoietic cells (Figure A3) increased in number in the extramedullary niches. These cell types are capable of producing red blood cells. White blood cell progenitors did not increase in number in the extramedullary niches (Figure A2). This could be due to the fact that there was no stimulus for white blood cell production. In fact, the white blood cell progenitor content in the spleen was decreased as compared to sedentary controls. Decreased production of white blood cells from the spleen could potentially be a balancing response to the increased white blood cell hematopoiesis that was seen in the medullary hematopoietic niche (see manuscript section).

c. Study Limitations

The potential mechanisms described above, through which endurance training can promote hematopoiesis, are plausible and are supported by the literature (see manuscript section). However, these mechanisms were never directly demonstrated and were not challenged in any way. A more thorough investigation would identify these mechanisms and then proceed to try and disrupt them. Muscle specific knockouts of hematopoietic growth factors would be one means by which this could be accomplished. For example, the mechanism of increased muscle growth factor production could be verified if, after training, muscle specific IL-3 knockout animals did not show increased levels of hematopoiesis. The mechanism concerning marrow cavity remodeling could be tested by treating animals with a drug that promotes development of fat in the marrow cavity. If,

post training, hematopoiesis still increased in these animals then it would suggest that marrow cavity remodeling is not the key mechanism for promoting hematopoiesis. The logical complexities of the experiments performed in this study were severely lacking.

Another limitation in this study was the use of very basic science techniques. Gross physiological measures were observed, and as a result, gross physiological data was collected. The result of this is that the interpretation of the data is limited. A summary of the findings would involve the words 'more' and 'less' used over and over again; more hematopoiesis, less adiposity in the marrow cavity, more cytokine production by muscle. No signaling work was performed in this study. We can say that there are more osteoblasts and less adipocytes but the signaling pathways responsible for transforming the mechanical stimuli of running into marrow cavity remodeling were not identified. More specific observations and less general observations would make the data from this study much more compelling. However, this work was only a pilot study and will act as starting point for years of future research. Upcoming work will focus on specific signaling pathways that are impacted as a result of endurance training.

Figure IV illustrates how mechanical stimuli on the femur and tibia could result in a remodeling of the marrow cavity and how this could subsequently promote hematopoiesis. It is reasonable to assume that treadmill running submits the long, load bearing bones to increased mechanical forces. This assumption is supported by various studies in the literature [8, 9]. However, this does not change the fact that this is only an assumption. Mechanical forces as a result of training were never measured. We only assume that the difference between a sedentary animal and a trained animal lies in these

mechanical forces. The marrow cavity remodeling changes could solely be based on differences in metabolism between a sedentary and endurance trained animal.

More does not always mean better. As mentioned earlier, increased concentrations of hematopoietic cytokines in the body can be positive but only to a certain point. Negative consequences can result from overstimulation of the hematopoietic system. The muscle data from the current study examined four of potentially a hundred or more hematopoietic cytokines and growth factors. It could be that negative regulators of hematopoiesis were also produced by muscle, or that training decreased the production of negative hematopoietic regulators. The data is limited in that it simply suggests that more cytokines were being produced, albeit known hematopoietic cytokines. The absolute levels of increase cannot be described with the techniques that were used. Future studies should measure circulating levels of these cytokines.

As the ideas of hematopoiesis and endurance exercise training have never been tied directly, conducting this study was somewhat difficult. Educated guesses at what to look for decided the direction of the research. Two potential mechanisms were uncovered; however, other mechanisms could be at work. Metabolic changes in the endurance trained animals could play a role in increasing hematopoietic activity. The tissues harvested in this pilot study could only go so far.

d. Future Directions

This work but opened the door. The basic concepts and ideas described in this study must be verified and expanded upon. Many interesting research ideas can be taken from the above work. A long road of future studies lie ahead.

One future direction will be to expand on the extramedullary hematopoiesis data collected from this study. Increased production of GM-CSF and G-CSF from skeletal muscle explains mobilization of hematopoietic cells into circulation but does not explain engraftment of the cells into the extramedullary niches. It is possible that the hematopoietic niche in spleen and liver may change as a result of endurance training such that it is better able to support engraftment. It is unlikely that mechanical forces play a role in this adaptation as both the spleen and liver are well cushioned within the abdominal cavity. Muscle growth factor signaling could potentially induce changes in these extramedullary niches. Alteration of the hematopoietic stem cells themselves is another possibility. Perhaps when hematopoietic cells are supported by a rich milieu of growth factors and cytokines they are better able to survive and develop outside of the medullary hematopoietic niche. These are all questions that must be answered.

Another direction for future studies will be to further investigate the capacity of skeletal muscle to produce hematopoietic cytokines and growth factors. The expression profile of only four growth factors, in a family that contains more than a hundred, was investigated. A more complete profiling will need to be undertaken. Skeletal muscle may act as a hematopoietic regulating organ through release of hematopoietic growth factors and cytokines. Endurance training may only potentiate this effect. Investigating hematopoietic activity in animals with muscle specific knockouts of certain hematopoietic growth factors could be one way to answer this question. Understanding how endurance training can increase the expression of these hematopoietic growth factors will also be important. Exercise induced hypoxia may play a role in this. Another area of potential interest involves muscle fiber type. Muscle fiber types differentially express

growth factors in response to certain stimuli [10]. A possibility is that endurance trained slow twitch fibers are better able to produce hematopoietic cytokines than fast muscle with its abundance of fast twitch fibers. Again, these are all questions that must be answered.

Another path will be to further investigate the niche changes that occur as a result of endurance training. Changes in the vascular niche were not assessed at all in this study. The hematopoietic niche in extramedullary tissues closely resembles the vascular niche in the bone marrow cavity [11]. The increases in extramedullary hematopoiesis may result from exercise training being able to positively influence the vascular niche in extramedullary tissues. Whether or not the vascular niche within the marrow cavity is also positively influenced is unknown. Muscle is able to produce angiogenic factors, such as VEGF [12]. These angiogenic factors are partly responsible for one of the adaptations commonly seen with endurance training, increased muscle capillary density. If these factors are able to enter into circulation it may be possible that they promote sinusoidal vessel formation within the medullary and extramedullary hematopoietic organs and promote the vascular niche. It will be important to perform research into this side of the story as well.

Deriving potential clinical applications of new research is always important. This research points to several possible clinical studies that could be performed. Endurance training to treat anemia is one possibility. Endurance training would be able to promote hematopoiesis, resulting in increased blood cell production, and in doing so help anemic patients. Deficiencies of the immune system, resulting from the hematopoietic organs being unable to produce sufficient white blood cells to maintain immune defenses, could

also be improved by endurance exercise training. Endurance training bone marrow recipients, remodeling their hematopoietic niches to allow them to better accept engrafting cells, is another possible clinical application. Exercise as a means to mobilize hematopoietic stem cells in preparation for cell collection from a bone marrow donor is another.

Overall, much work is left to be done. Fortunately, the work is as interesting as it is novel. Many future studies will be published as a result of this study.

e. References for Section 3

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4. Appendix A

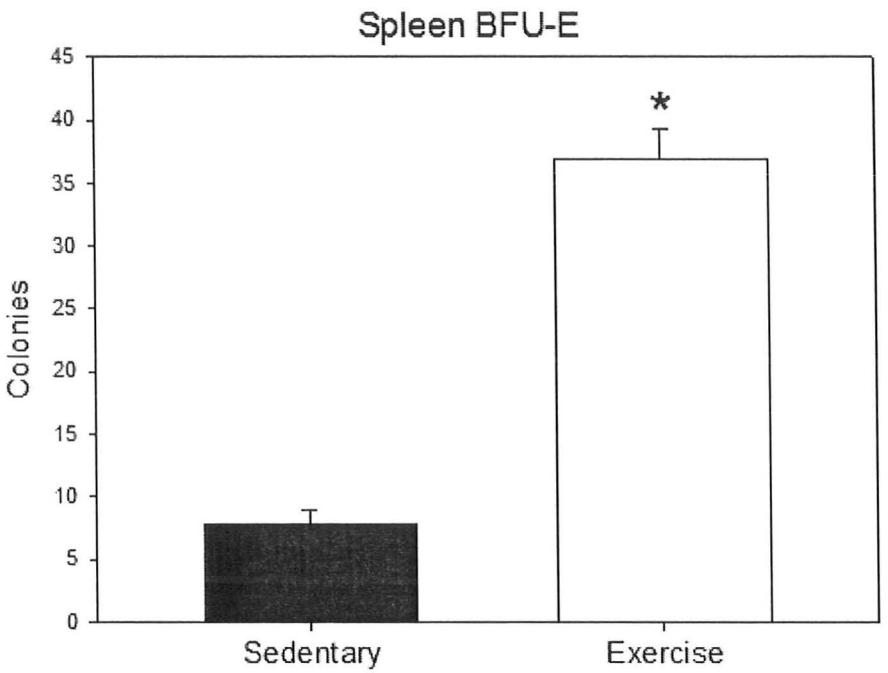

Figure A1. Spleen red blood cell progenitors. Spleen contains higher levels of red blood cell progenitors in trained animals than in sedentary animals. * $p < 0.05$

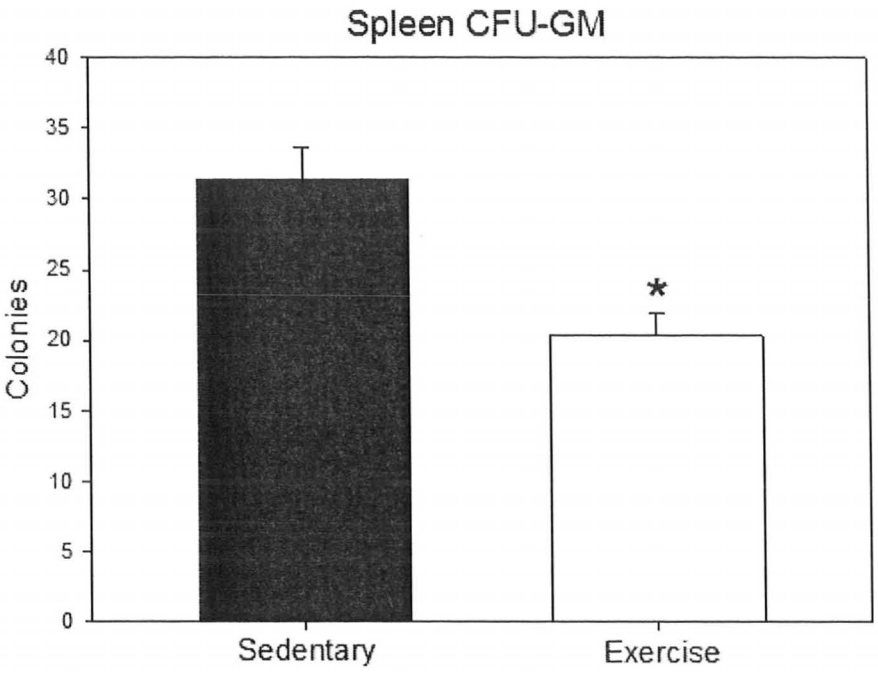

Figure A2. Spleen white blood cell progenitors. Spleen contains lower levels of white blood cell progenitors in trained animals than in sedentary animals. * $p < 0.05$

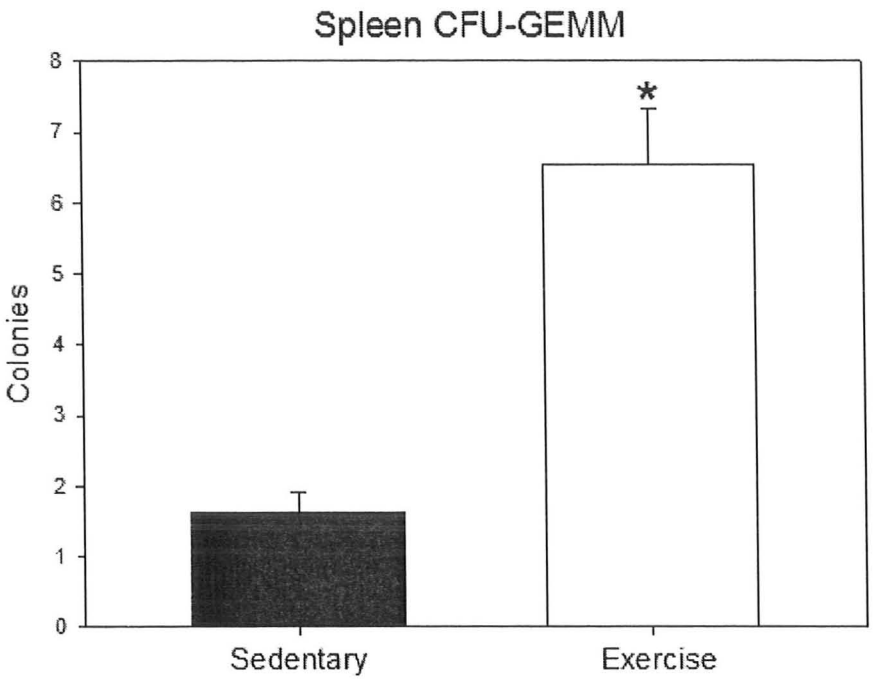

Figure A3. Spleen white red multipotent progenitors. Spleen contains higher levels of white red progenitors in trained animals than in sedentary animals. * $p < 0.05$

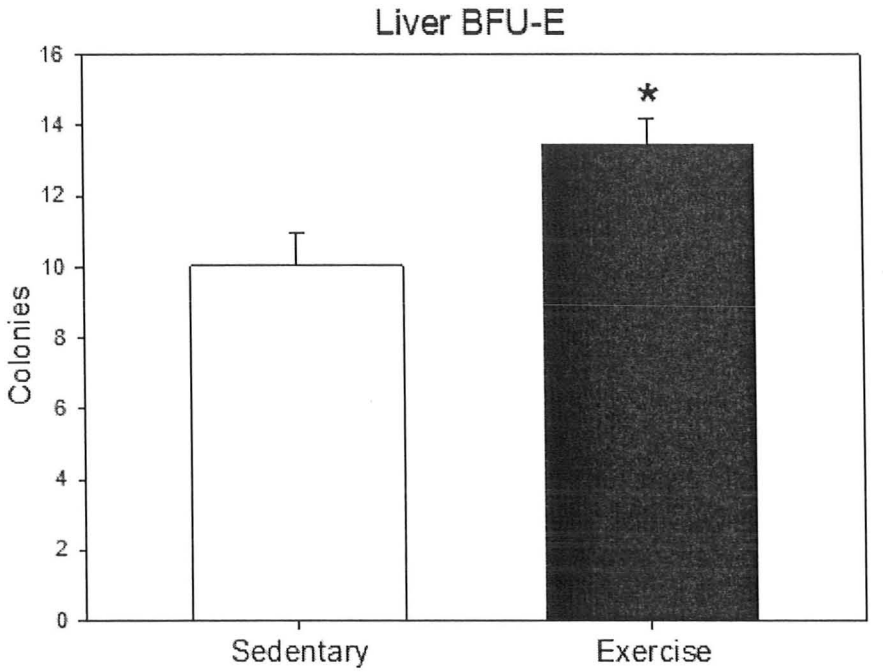

Figure A4. Liver red blood cell progenitors. Liver contains higher levels of red blood cell progenitors in trained animals than in sedentary animals. * $p < 0.05$