SCALING OF METABOLIC ENZYMES: TRANSCRIPTIONAL BASIS OF INTERSPECIES VARIATION IN MITOCHONDRIAL CONTENT

by

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Abstract

Mitochondrial content, an important determinant of muscle metabolic capacity, changes in individuals during development, and in response to physiological and environmental challenges. This phenotypic plasticity is attributed to the coactivator PPARγ coactivator-1α (PGC-1α) but it remains unclear if this transcriptional regulator accounts for evolutionary variation in mitochondrial content. In an attempt to explain why some species have higher muscle mitochondrial enzyme levels than other species, I examined if the transcriptional mechanisms that control mitochondrial content of a tissue in an individual are also responsible for differences between species. If PGC-1α creates differences between the mitochondrial content of species based on variation in promoter binding motifs, then cis-factor evolution may be the guiding force in scaling trends.

In this thesis I explored the basis of size-dependent patterns by looking at layers of regulation, from catalytic activities to promoter evolution and regulation. A representative family, Rodentia, was used to collect muscle samples from a size range of approximately 20g up to 17 kg. As expected, in rodent lower limb muscles, mitochondrial and glycolytic enzyme activity exhibited reciprocal scaling patterns, though the scope differed between muscles. Very little of the variation was accounted for when the activity was related to DNA content. However, when COX activities were expressed relative to DNA, the scaling patterns were similar among the 3 muscles. To determine if interspecies differences were linked to transcriptional regulation, ~800bp of the PGC-1α promoter from 56 terrestrial mammals (5g-5000kg) was examined. The basal placental mammalian promoter possesses putative elements for Sp1, HNF3, myogenic factors and metabolic effectors, which have been retained in mammals with
little change in order or spacing. To investigate the ability of these promoters to control PGC-1α expression, rodent promoters were cloned into luciferase reporter gene constructs and transfected into a common mouse myoblast background (Sol8 cells). Unlike mitochondrial content, promoter activity did not vary with body size across the rodent family. Likewise, PGC-1α transcript levels did not vary in rodent muscles in a way that would explain differences in COX activity. This suggests that though PGC-1α may be crucial for within species variation, transcriptional regulation of PGC-1α is not responsible for interspecies variation in mitochondrial content.
Co-authorship

This study will be coauthored by Tika Kocha when in final form for her assistance in midiprep of reporter plasmids.
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<tr>
<td>AMPK</td>
<td>adenosine monophosphate kinase</td>
<td>MRF</td>
<td>myogenic regulatory factor</td>
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<td>ATF</td>
<td>activating transcription factor</td>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<td>$b$</td>
<td>scaling coefficient</td>
<td>MyoD</td>
<td>myoblast determination protein</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
<td>NFAT - nuclear factor of activated T-cells</td>
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<td>protein</td>
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<td>myocyte enhancing factor</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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Chapter 1: General Introduction and Literature Review

Thesis overview

Mitochondria are the sites of aerobic oxidation of metabolic fuels, including pyruvate and fatty acids. Here chemical potential energy, in the form of reducing equivalents, is converted by the electron transport system (ETS) to a proton motive force (PMF), which can be used to drive the F$_1$F$_0$ ATP synthase to produce ATP. The combination of oxidation by the ETS and phosphorylation by the ATP synthase is known as oxidative phosphorylation (OXPHOS), and it produces most of the ATP energy required to support cellular processes.

The metabolic rate of a tissue or organism is a reflection of flux through OXPHOS and thus differences in metabolic rate between tissues of animals can be traced back to this pathway. In the daily life of an animal, differences in metabolic rate are achieved through regulation of the existing machinery: regulation of enzymatic activity through mass action effects, allosteric modulation and covalent modification. Long-term or steady-state differences in metabolic rate between tissues and species are typically achieved by changing the amount of the machinery (i.e., enzyme levels). Thus, tissues or animals with a higher aerobic metabolic rate tend to have more mitochondria than tissues or animals with a lower aerobic metabolic rate.

There are many paradigms where differences in metabolic rate and metabolic enzyme content are obvious, but for the most part, studies that address the genetic origins of differences focus on metabolic remodelling of an individual in response to a physiological challenge or developmental cue. For example, a great deal is known about how animals modify mitochondrial content in response to physiological stressors such as activity level, and
developmental processes, such as muscle fiber type differences (reviewed in Moyes and Hood, 2003). Much of the control of mitochondrial biogenesis in these situations is attributed to proteins that act as master regulators of transcription, controlling the expression of networks of genes to coordinate the synthesis of hundreds of proteins needed to make mitochondria. In contrast to adaptive remodelling in individuals, there are few paradigms where metabolic differences between species have been studied at the level of molecular genetics. My thesis is one of the first studies to attempt to explain why some species have higher muscle mitochondrial enzyme levels than other species.

Interspecies differences in mitochondrial content are evident in many types of comparisons. For example, athletic and sedentary species differ in muscle mitochondrial content (Weibel et al., 2004). There are many challenges to employing two species comparisons to derive the mechanistic basis of underlying differences (see Garland et al., 2005). However, there is one paradigm where multiple species can be more easily compared to assess the evolutionary basis for differences in muscle mitochondria: allometric scaling. It has been known for more than 100 years that smaller animals possess higher mass-specific metabolic rates than do larger animals (reviewed in Glazier et al., 2005). Despite a rich history in the study of the allometric scaling of metabolic rate, the underlying mechanisms remains vigorously debated (see White and Seymour, 2003). Likewise, it has been known for more than 50 years that muscle mitochondrial content also shows allometric scaling (see Moyes and Genge, 2010), but the genetic and cellular mechanisms responsible for this trend remain obscure. It seems plausible that the transcriptional mechanisms that control mitochondrial content of a tissue in an individual are also responsible for differences between species.
In the following sections, I briefly review the history of studies of allometric scaling of metabolism in animals, discuss the patterns seen in muscle mitochondrial enzymes, and consider the mechanisms by which mitochondrial content is regulated in different contexts. This will lay the foundation for my studies, which address the hypothesis that the differences in the expression of PGC-1α determines differences in muscle enzymes in relation to body size in mammals.

Scaling of metabolic rates

Metabolic rate is the rate of heat production in a living system. It is the sum of the diverse metabolic processes carried out by cells and tissues, but in most cases it reflects the energy metabolism of the mitochondria. Heat production is difficult to measure without specialized calorimetry equipment, and thus metabolic rate is usually measured in terms of indirect calorimetry: oxygen consumption or carbon dioxide production.

It has long been known that the rate of oxygen consumption of animals depends on many factors. Whole animal metabolism may be influenced by the animal’s activity level, diet, state of digestion and body temperature (see McNab, 2005). Thus, there is a repertoire of terminology that distinguish between different metabolic states. Basal metabolic rate (BMR) represents the minimum energy cost of steady-state existence of a homeotherm and allows for comparison of different species by placing them in the same physiological state (post absorptive, thermoneutral, resting). Standard metabolic rate (SMR) typically refers to the minimum energy costs of existence at a particular temperature and is usually used to describe ectotherms such as fish (White and Seymour, 2005). Resting metabolic rate (RMR) is more often used with mammalian physiology. RMR is like BMR except that the physiological
conditions can be defined (e.g. at a non-neutral temperature). Because metabolic rate is influenced by activity, maximal metabolic rate (MMR), or the limits of aerobic performance (VO\textsubscript{2} max), is another important measure. With activity, over 90% of oxygen can be consumed in muscles for ATP resynthesis whereas at BMR oxygen is consumed more uniformly across cells (Weibel et al., 2004).

Species also differ in the relationship between BMR and MMR. Metabolic scope is the difference in capacity to increase metabolic rate in support of exercise, even if they have comparable BMR. Typically, larger and more athletic animals have a greater metabolic scope (Bishop et al., 1999; Weibel et al., 2004, Killen et al., 2006). Control of metabolic rate may thus be more closely linked to the ability of cells, and specifically mitochondria, to adjust the metabolic rate to a particular degree. The constitutive level of mitochondrial content may be linked to the ability to change in response to demands rather than just meeting the basic needs of cells at rest.

Metabolic rates also differ in relation to body size. More than 100 years ago, Rubner (1883) observed that larger animals had higher total metabolic rates than did smaller animals, but the increase was less than would be expected solely on the basis of size. When BMR was plotted against body mass on a log-log plot, a straight line was found. Rather than an isometric relationship (where metabolic rate and body size increase in equal proportion), the relationship is typically allometric (“allo” is derived from the Greek “allos”, meaning “other”). Rubner’s original analysis with dogs demonstrated this allometric relationship, while Klieber (1932) later demonstrated this relationship between body size and metabolic rate for a wider range of mammals and birds. This work was extended to show that the mass-specific metabolic rate of a
five-gram shrew has been measured to be approximately 100-fold higher than the value measured in the 10,000 kilogram elephant (Hawkins et al., 1960; Brody, 1945).

Generally, the relationship between metabolic rate and mass is expressed as \( y = aM^b \), with the \( Y \) as metabolic rate, \( M \) as the body mass, \( a \) the normalization coefficient and the exponent \( b \) representing the scaling coefficient. When log transformed, the scaling exponent is estimated as the slope of a log-log plot. Whole body metabolic rates of terrestrial endotherms typically show scaling coefficients between +0.7 and +0.8. When data are expressed, not per animal but per gram animal, the mass-specific scaling coefficients of the allometric relations are typically between -0.2 and -0.3).

Since the original relationship was identified, the underlying explanation for these patterns in scaling of metabolic rate has been vigorously debated (reviewed in Glazier, 2005). Rubner (1883) first suggested that the allometric scaling of BMR was related to size effects on surface area-to-volume ratios. Small mammals lose heat more rapidly because of their higher surface area-to-body mass ratio. If heat loss was the driving force, the scaling relationship would show an exponent of 2/3, which is the relationship between surface area and the volume of cubes of different sizes. However, since animals are not cubes, factors, such as animal shape and insulation, also play roles in determining heat loss of animals of a given mass. A number of studies have shown that it is unlikely that the underlying basis of this relationship is simply related to heat loss. Perhaps the most compelling evidence against the heat flux explanation is that the same allometric pattern is seen in metabolic rate of poikilotherms and ectotherms (Childress and Somero, 1984), where there is no metabolic energy used to maintain a constant body temperature. Size-dependent patterns are subject to many ontogenetic, phylogenetic,
life-history and environmental factors so a single universal model of scaling is unlikely.

Metabolic rates, even within closely related or similar sized mammals, are highly variable because of many factors including body temperature, behavior, environmental conditions such as temperature, precipitation, latitude, altitude, food availability, and diet (McNab, 1995). Apart from the issues of surface-area to volume models, many researchers argue that better estimate of the scaling coefficient is 3/4 not 2/3 (Kleiber, 1932; West et al., 1997; Banavar et al., 1999). For the purpose of this thesis, the work does not depend on either of these positions being correct.

Mutifactorial control or fractal geometry in supply?

In the past 10 years, there have been two main alternative explanations for the underlying basis of allometric scaling of metabolic rate. Hochachka and coworkers posit that metabolic rate is the product of metabolic regulation at multiple levels, and that no simple explanation can account for the size-dependent patterns (Darveau et al., 2002). The alternate hypothesis, introduced by West and coworkers, is that metabolic rate is constrained by supply and supply networks are fractal in nature (West et al., 1997).

Hochachka and coworkers (Darveau et al., 2002) argue that the control of metabolic rate is multifactorial, and that ascribing the scaling pattern to one single factor is naïve. BMR is a consequence of shared control by multiple steps at multiple levels of biological organization (see Suarez and Darveau, 2005). Rather than a simple ‘single-cause’ of scaling patterns, a more likely explanation takes into account that there are multiple control sites in metabolic pathways. The ‘allometric cascade’ views scaling as being a consequence of the contributions of various processes involved in supply of materials and energy expenditure (Suarez and Darveau,
Supply and demand are inherently linked. Demand may in fact be more important at times, such as during exercise. This takes into account adaptations to lifestyle (active versus sedentary) and environment (Suarez et al., 2004).

The alternate explanation suggests that scaling of BMR is related to fractal-like networks that supply of materials to the cell (West et al. 1997). Metabolic rate can be dependent on the consumption of food and oxygen which arrive at cells by branching supply networks and thus fractal geometry of the supply network could explain 3/4 power scaling (West et al., 1997). This means that larger animals are constrained to lower metabolic rates rather than being more efficient at energy use. It would also place constraints on metabolic scope, or the capacity to increase metabolic rate in the face of stress.

Again, not all researchers have found the same scaling coefficient holds true, especially with an expanded data set. Neither 2/3 or 3/4 can be used to explain all scaling patterns. Universal trends are hard to support, since even larger sized mammals scale with steeper slopes than smaller mammals (Glazier, 2005). Therefore, though scaling trends are known to occur both on an intra- and interspecies level, the exact relationships are unclear. Regardless of the correct explanation, the metabolic phenotype of muscle clearly displays some size related trends.

**Metabolic phenotype**

Enzyme rates vary with decreasing body size, providing a way to examine the biochemical basis of these differences in metabolic rates. Kunckel and coworkers found as early as 1956 that cytochrome oxidase (COX) activity of rats scaled with body mass to the power ¾, which they concluded matched the pattern in BMR (Kunckel et al., 1956). However, more
recent studies have shown that the allometric relationships seen in BMR differ from the patterns seen in muscle metabolic enzymes; scaling coefficients for oxidative enzymes are generally lower than BMR. Emmett and Hochachka (1981) studied gastrocnemius muscle of selected mammals, showing that the oxidative enzyme, citrate synthase (CS) also scaled negatively, though with a shallower slope ($b = -0.11$) than whole animal metabolic rate ($b= -0.25$ to $-0.33$).

Skeletal muscle also has a high capacity for anaerobic metabolism. Interestingly, an inverse relationship has been shown between oxidative and glycolytic enzymes in muscles of animals of different sizes. Childress and Somero (1980) examined the muscle activities of fish in relation to size. They found the familiar inverse relationship between mass and mitochondrial enzyme specific activity, but they also noted a positive scaling relationship between mass and glycolytic enzyme activities. In other words, the muscles of larger fish showed the lower mitochondrial enzyme specific activity but higher glycolytic enzyme activities (Childress and Somero, 1984). Emmett and Hochachka (1981) showed the same inverse relationship between oxidative and glycolytic enzymes in the gastrocnemius muscle of a variety of mammals.

The study of the scaling of muscle metabolic enzymes addresses the cellular mechanisms by which muscle metabolic phenotype is determined. Muscle tissue is a large contributor to whole animal metabolic rate, both at rest and during exercise. Because of its size alone, which can be 50% of body mass in some athletic species, it plays a key role in energy balance (Weibel, 2002). During maximal exercise, 90% of metabolic demands come from skeletal muscle (Weibel et al. 2004). Most mammalian skeletal muscle consists of a mixture of 4 different fibre types: I, IIA, IIB, IIX/d, classified based on myosin heavy chain (MHC) isoform
expression. Type I fibres are slow-twitch: highly dependent on oxidative metabolism, containing high numbers of slow isoform contractile proteins, high volumes of myoglobin, high capillary densities and high oxidative enzyme capacity. Examples of muscles with a high proportion of these fibres include postural muscles, with a low maximal shortening velocity and high resistance to fatigue. Fast oxidative fibres, type IIa and IIx/d, are typically fast contracting, highly oxidative and relatively fatigue resistant. These fibres are known to have higher oxidative capacities in rats but not in humans. Type IIa and IIx/d are seen as intermediate types, differing in which myosin is expressed. The final fibre type, having comparatively low mitochondrial content, is the fast glycolytic type IIb. These fibres have low volume density of mitochondria, high glycolytic enzyme activity, high myosin ATPase activity, increased expression of fast isoforms of contractile proteins, increased rate of contraction and low resistance to fatigue (Edgerton and Roy, 1991). Fibre type is also linked to muscle fibre diameter. Small fiber size in oxidative fibers aids rapid diffusion of oxygen from the surrounding capillaries into the fiber interior core to support an increased oxidative capacity (Edgerton and Roy, 1991). Larger fibres generate more force and greater velocities.

Muscles consist of different proportions of each fibre type. For example, the soleus, a postural muscle, consists predominantly of slow-twitch oxidative fibres whereas muscle involved in more explosive activity, such as the gastrocnemius, incorporate more fast-twitch glycolytic fibres. In mammals, most skeletal muscles display a mixed phenotype, with varying proportions of each fibre type. Fibre type profile for a specific muscle differs in small and large animals, creating issues for scaling studies (Wang and Kernell, 2001). For example, the type I
fibres represent almost 100% of the soleus in the guinea pig while in the rat they represent about 84% (Ariano et al., 1973).

Muscle is relatively plastic, responding to both internal (e.g. contraction) and external (e.g. temperature) stimuli as well as through development. For a given muscle, the total number of fibres is fixed early in development, but changes can occur both in terms of fibre size and composition in response to numerous environmental and physiological challenges. Muscle structure must reflect functional needs related to the lifestyle of an individual animal, which may change throughout lifespan. Thus muscle must be able to adapt from the original phenotype laid down during development.

Increases in fibre size may occur through transcriptional mechanisms and increases in rates of protein synthesis. The myogenic factors are a group of DNA-binding proteins that regulate the expression of various structural proteins necessary for muscle development. MyoD is required for the differentiation potential of skeletal myoblasts whereas Myf5 regulates their proliferation rate and homeostasis. This occurs during development and also in response to activity. Hypertrophy of skeletal muscles involves an increase in the size of the fibres without an increase in their number, regardless of any increase in the number of nuclei per fibre (Paul and Rosenthal, 2002). This means that sometimes the ratio of cytoplasm-to-nucleus will increase as well. The number of myonuclei and thus the size of the myonuclear domains appear critical in establishing the adaptive potential of muscle. The importance of myonuclear domain in relation to mitochondrial content will be discussed further later on.

Inherently linked to fibre type profiling is the organelle responsible for OXPHOS, the mitochondrion. Major responses to challenges may involve changes in fibre type itself (e.g.
shifting to more type I) or simply changing in mitochondrial density in the existing fibres. The differences in fibre type proportion between tissues and thus mitochondrial content is indicative of the bioenergetic demands of various tissues (Fluck and Hoppeler, 2003). The mitochondrial content indicates the relative importance of mitochondria to the supply of energy (Leary et al., 2003). Higher aerobic capacity is related to mitochondrial content of locomotor muscles, as shown in both humans (Hoppeler et al., 1973) and athletic mammals (Hoppeler et al., 1987). Interspecies differences in mitochondrial content can thus be related to differing demands. Constitutive mitochondrial levels are likely maintained at levels needed to support the demands at the highest steady state metabolic rates (see Moyes and Le Moine, 2005). Animals differ in muscle mass according to activity level, with larger relative muscle mass in active animals than sedentary. Fibre type profile also varies in relation to body size, leading to the expectation that mitochondrial content also varies in animals differing in size.

**Mitochondrial content and myonuclear domain**

In the earliest studies (George and Talasera, 1961), muscle fibre diameter was the single best predictor of muscle mitochondrial enzyme. This suggests that muscles of large animals have fewer mitochondria, not because they are large animals, but rather because they make muscles with large fibers. The link between muscle fiber diameter and mitochondrial content ties in with the more modern concept of myonuclear domain. Myonuclear domain, the cytoplasm-to-myonucleus ratio, is the inverse of nuclear content. It determines the capacity of a muscle to support protein turnover rates. Myonuclear number usually increases proportionally with fibre size to maintain a constant myonuclear domain (Allen et al., 1995). However, myonuclear domain also varies with fibre type. In slow oxidative fibres myonuclear
domain size is smaller than in fast glycolytic (Burleigh, 1977) possibly due to higher rates of protein turnover (Edgerton and Roy, 1991). Mitochondrial content per myonucleus is much more conserved than mitochondrial content per fiber (see Moyes and Le Moine, 2005). Theoretically, if myonuclei produce the same mitochondrial precursors regardless of fiber type, then small fibers would end up with more mitochondria than large fibers (expressed per g tissue).

On a transcriptional level, with higher levels of both nuclei and mitochondria per g tissue, higher levels of nuclear gene transcripts (per g tissue) can arise even if there are no differences in gene expression *per se*. In fact, expressing mitochondrial enzyme content per nucleus largely removes differences in mitochondrial enzymes arising in relation to body size (of a given species) (Davies and Moyes, 2007) and between fiber types (Dalziel et al., 2005) but not between species (Dalziel et al., 2005). The myonuclear content may be the mechanism by which differences in mitochondrial content arise between fiber types of an individual, between muscle of a species in relation to size, or between homologous muscles of different species (Moyes and Genge, 2010).

**Transcriptional regulation of mitochondrial biogenesis**

Apart from the question of why the metabolic enzymes should show the observed patterns, the more proximal question is how do muscle cells determine their desired enzyme patterns? When animals experience long-term changes in energy demands, it is likely that changes in enzyme levels are increasingly important. One mechanism by which enzyme content can be changed is through control of gene expression. Thus, understanding the transcriptional
regulation of mitochondrial content may be crucial to understanding differences in metabolic capacity.

**DNA-binding transcriptional regulators**

The first step in establishing the molecular origins of a muscle metabolic phenotype in relation to body size is to assess the relative importance of transcriptional versus non-transcriptional regulation. Changes mediated by transcriptional regulation would be seen as increases in mRNA to a new steady state followed, with sufficient time, by an increase in the level of enzyme protein to a new steady state. The lag in the protein response is due to the different half-lives of mRNA and protein. In the absence of a sequence-specific factor, a typical cytoplasmic mRNA has a half-life of about 4 h. Thus, with an instantaneous doubling of gene expression, it would take about 24 h (about 7 half-lives) for the steady state level of mRNA to double. The half live of a given mRNA would also be influenced by elements in the 3’ and 5’ untranslated region that can bind proteins that accelerate or impede RNAase activity. The rate of change in protein would also depend on its half-life, but for a protein with a half life of 2 days, it would take about 2 weeks for the new steady state level to be achieved. In this simple scenario, by the end of 2 weeks, the mRNA and protein levels would both have doubled in this response generated entirely through transcriptional regulation.

If enzyme levels change without a change in mRNA levels, then the case could be made for a greater role for post-transcriptional origin of the new phenotype. When exploring the origins of enzyme differences between animals of different sizes, assessing the role of transcriptional regulation is complex because of the potential influence of differences in protein turnover and RNA stability. Nonetheless, measuring the relationship between mRNA and
protein is an important first step in assessing if the metabolic phenotype is a result of differences in genetics (e.g., promoter sequence) or gene expression (e.g., promoter activity). Either way, transcriptional regulation must be guided by specific factors.

Changes in mitochondrial content require the generation of new organelles. Mitochondria possess a circular double-stranded genome encoding 13 OXPHOS genes as well as 22 tRNA and 2 ribosomal RNAs. However, the remaining structural and functional mitochondrial proteins are transcribed from nuclear genes, including transcription factors essential in controlling biogenesis. Proteins, from nuclear-encoded genes, and phospholipids, must be synthesized in the cytosol, imported into mitochondria and incorporated appropriately for successful mitochondrial biogenesis. The separation of mitochondrial genes into two genomes requires complex coordination to ensure proper assembly and function of the organelle.

Mitochondrial DNA transcription is regulated by nuclear-encoded transcription factors such as mitochondrial transcription factor A (TFAM) and transcription specific factors 1 and 2 (TFB1M and TFB2M). An additional level of control is necessary to ensure coordination with the nuclear genome as well as regulation of these factors. Biochemical remodeling of an entire pathway, such as OXPHOS or glycolysis is coordinated through shared sensitivity to transcription factors (Moyes and Le Moine, 2005). Transcription factors can regulate gene expression in response to stimuli. There are two main axes of transcription factors that regulate bioenergetic genes; in general, nuclear respiratory factors (NRFs) regulate genes involved in OXPHOS and peroxisome proliferator-activated receptors (PPARs) regulate genes associated with fatty acid metabolism.

NRF-1 is a homodimeric DNA-binding protein that was first identified as an activator of the rat somatic cytochrome c gene (Evans and Scarpulla 1989). It has since been shown to
regulate multiple genes involved in OXPHOS and mitochondrial proliferation (see Scarpulla 2008). NRF-2, though unrelated in structure, is a transcription factor that plays a similar role to NRF-1 in the coordination of genes encoding mitochondrial proteins. NRF-2 can activate the expression of all nuclear-encoded COX subunits (Ongwijitwat et al. 2006, Ongwijitwat and Wong-Riley 2005), as well as several NRF-1 responsive genes involved in the regulation of mitochondrial transcription and protein translocation (Blesa et al. 2007, Virbasius and Scarpulla 1994). Both NRF-1 and NRF-2 regulate TFAM, which is responsible for replication, maintenance and transcription of mitochondrial DNA, thus extending their impact to both the mitochondrial and nuclear genomes (Virbasius and Scapulla, 1994).

Mitochondrial genes are also regulated by PPARs, members of the large family of nuclear hormone receptors (NHR). PPARs are highly conserved in vertebrates and have a wide variety of functions. They regulate transcription primarily through interactions with ligands, and exert long term regulation of expression of key enzymes (Desvergne et al., 2006). The PPARs act through forming heterodimers with other NHRs, such as retinoid-X-receptor. Heterodimers then bind to a specific sequence of DNA, known as the peroxisome proliferator response element (PPRE), which have been found in numerous genes involved with lipid metabolism (van Bilson et al., 2002).

Although many genes related to oxidative metabolism and mitochondrial metabolism share elements that bind NRFs and NHRs, no single transcription factor binds all of the required genes. However, a coactivator has emerged as the ‘master regulator’ of mitochondrial biogenesis through its’ interactions with multiple transcription factors.
**PGC-1**

Coactivators are able to modulate gene expression through specific protein-protein interactions without binding directly to DNA. Members of the PGC-1 family of coactivators can regulate genes involved with OXPHOS through association with DNA-binding transcription factors such as NRFs and PPARs. PGC-1α was initially described as an activator of the thermogenic response in brown adipose tissue, but its role in mammalian tissues is now known to extend to a variety of metabolic programs (Finck and Kelly 2002, Puigserver et al. 1998, Rodgers et al. 2008). Its role has been examined in phenotypic adaptations including development (Buroker et al. 2008, Lin et al. 2002), exercise (Baar et al. 2002, Watson et al. 2007, Wright et al. 2007) and dietary manipulations (Nakazato and Song 2008, Sparks et al. 2005).

PGC-1α expression is tissue specific, and enriched in highly oxidative tissues such as muscles, liver and BAT (Puigserver et al. 1998). Levels also correlate with muscle mitochondrial levels in fibre types in mammals, showing a greater abundance in type I fibers than type II fibres (Lin et al., 2002). Cells transfected with PGC-1α increase mitochondrial content, indicating a direct role in regulating mitochondrial content (Lehman et al., 2000). In fact, PGC-1α has been shown to be involved in coordinated increases in oxidative enzymes, mitochondrial biogenesis and the production of type I muscle fibre proteins, all indicators of muscle fibre type switching (Handschin et al., 2003; Akimoto et al., 2005). Coactivators rely on interactions with multiple transcription factors. PGC-1α has specific binding domains for NRF-1, through which it can increase expression of components of OXPHOS through the induction of a transcriptional cascade where the coactivation of NRF-1 and NRF-2 allow for increases in levels of TFAM and
subsequently components of mitochondrial OXPHOS (Finck and Kelly, 2003). The transcription of myofibillar genes typical of oxidative muscle fibres is driven through coactivation of myocyte enhancer factor MEF2c (Lin et al., 2002).

Binding of PGC-1α to transcription factors bound to elements of target genes causes a conformational change in PGC-1α itself that recruits other proteins, increasing transcriptional activity (Wu et al., 1999). For efficient transcription, large protein complexes are recruited to the transcription factor and facilitate opening of the chromatin structure and initiation of transcription. PGC-1α mediates interactions with histone remodelling proteins and general transcription machinery to facilitate the initiation of transcription. This activity is resulted through phosphorylation (AMPK, p38 MAPK), deacetylation (Sirt1) or methylation (Finck and Kelly, 2006).

Post-translational modifications play an important role in regulating the activity of PGC-1α dependent pathways, but expression of the PGC-1α gene itself is responsive to many stimuli (Cao et al. 2004, Daitoku et al. 2003, Handschin et al. 2003). Understanding this regulation in various species may be important to understanding interspecies differences of both PGC-1α and the expression of genes it controls.

**Transcriptional regulation of PGC-1α**

Understanding the interspecies differences in mitochondrial content may begin with the transcriptional regulation of PGC-1α. The regulatory sequences found in the promoter region of a gene are an important part of understanding the control of gene activity. Thus, understanding the promoter activity of PGC-1α can help to understand the control of downstream pathways. The induction of PGC-1α gene responds to numerous stimuli such as catecholamines (Cao et al.,
glucagon (Yoon et al., 2001) and exercise (Handschin et al., 2003). There are multiple binding domains on the promoter region of the PGC-1α gene (located immediately upstream of the transcription start site) that are sensitive to transcription factors involved in energy balance related to these stimuli. Each of these factors may be responsible for creating differences in the expression of PGC-1α that guide changes in metabolic capacity.

![Figure 1: Transcription factor binding sites identified in the human PGC-1α promoter.](image)

Some key sites with a specific number correspond to base pairs from the transcription start site (TSS) as identified in the studies listed below. E1 binds to bHLH family members such as MyoD, myogenin and MRF4 (Chang et al., 2006). Sp1 is capable of binding to either specificity factor 1 or 3, performing either activating or repressing role (Li et al., 2004). Hepatic nuclear factor (HNF3) is a forkhead transcription factor regulated by insulin and is primarily involved in hepatic gluconeogenesis. NFκB binds to TNF as part of a pro-inflammatory response in cardiac tissue (Palomer et al., 2003). The CRE site can bind ATF which is phosphorylated by p38 MAPK (Cao et al., 2004) or CREB which is phosphorylated by PKA (Herzig et al., 2001). Both are involved in CAMK signaling (Handschin et al., 2003). NFAT is also activated in response to CAMK (Schulz and Yutzey, 2004). MEF2 is responsive to CAMKIV and can-mediated changes in phosphorylation status (Czubryt et al., 2003). PPAR is part of a positive autoregulatory loop controlling the PGC-1α gene through coactivation of PPAR responsive to TZDs by PGC-1α itself (Hondares et al., 2006). TRE is responsive T3 (Wulf et al., 2008).

One factor controlling PGC-1α expression is cAMP levels. cAMP activates protein kinase A (PKA) which phosphorylates a cAMP-responsive element binding protein (CREB), which binds CRE on the promoter to activate genes. CREB is also coactivated by TORC2 (transducer of regulated CREB activity 2) another important regulator of PGC-1α expression (Wu et al, 2006).

In C2C12 myotubes mitochondrial biogenesis was shown to be induced in a PGC-1α dependent
manner with TORC2 overexpression (Wu et al., 2006). Conditions inducing mitochondrial 
biogenesis, such as the elevated calcium levels from exercise, increase promoter activity.
Exercise stimulates calmodulin kinase (CAMK) which activates CREB to stimulate the PGC-1α 
promoter through the binding of CREB. This is thought to be an autofeedback loop, where 
calcium-signalling pathway results in stable induction of PGC-1α (Handschin et al., 2003). Since 
CAMK mediates type I fibre conversion in response to contractile activity (Wu et al., 2002), this 
regulation plays an important role in the response of muscle to exercise. However, factors 
other than CREB may be important in the induction of PGC-1α, as shown in greater effects of 
CAMK when in combination with calcineurin A (CnA) (Handschin et al., 2003).

Other known targets of CAMK and CnA in muscle fibre type determination, specifically 
nuclear factor of activated T-cells (NFAT) and myocyte enhancer factor 2 (MEF2) also possess 
binding sites in the promoter of PGC-1α. Through insulin growth factor 1 (IGF-1) growth factor 
Cn-mediated signalling is increased resulting in enhanced NFAT and GATA2 activity involved in 
muscle hypertrophy, while elevated NFAT and MEF2 activities lead to myofibre type switching 
(Schulz and Yutzey, 2004). Thus these transcription factors play a role in guiding the PGC-1α 
fibre-switching response. NFAT is important in immune response as well as the development of 
skeletal muscle. It is activated via CAM and important in transcriptional activation of slow fibre 
specific responses. Expression of NFAT itself has little effect on the PGC-1α promoter, but its 
activity increases when coexpressed with CnA and MEF2, indicating that interaction between 
these factors has a positive effect on the promoter (Handschin et al., 2003).

MEF2 is a transcription factor regulating muscle development and calcium dependent 
gene expression. Its activity is replaced by class II histone deacetylases (HDACS) which
dissociate upon phosphorylation in response to calcium signalling (Czubryt et al., 2003). Overexpression of PGC-1α in skeletal muscle results in slow twitch fibre increases which are MEF2 dependent (Lin et al., 2002) suggesting a link between PGC-1α and increases in mitochondrial number in response to CAMK signalling. The PGC-1α promoter contains two MEF2-binding sites mediating transcriptional activation by MEF2 and repression by HDAC5. This interaction provides a method of modulating PGC-1α levels in response to changing energy demands during myocyte hypertrophy. Both MEF2 and NFAT are responsive to CAMKIV and CnA-mediated changes in phosphorylation status. The major effect of CAMKIV appears to be through CREB acting on the PGC-1α promoter, but CnA is able to further increase the activity of MEF2 to stimulate transcription (Handschin et al., 2003). Interestingly, MEF2 and the PGC-1α protein also control PGC-1α gene transcription in an autoregulatory loop since MEF2 is responsive to PGC-1α itself (Czubryt et al., 2003; Handschin et al., 2002). Activation of MEF2 results in binding to the PGC-1α promoter, which in turn would lead to coactivation of bound MEF2, leading to a stable feed-forward regulatory loop. This may allow for high levels of PGC-1α to be sustained in muscle cells, promoting mitochondrial biogenesis and expression of muscle fibre type 1 genes (Handschin et al., 2003).

Also important for modifying PGC-1α levels in muscle development are myogenic factors, which are members of the basic helix loop helix (bHLH) family. This includes factors such as MyoD, Mrf5, myogenin and Mrf4. Myogenic determining factor (MyoD) plays a key role in muscle differentiation (Amat et al., 2009). This occurs through its’ ability to work with p21 to halt cell proliferation. It has also been shown to be involved in muscle repair. MyoD is found at higher levels in fast twitch muscles, and must be deactivated to promote a shift to a more
oxidative muscle phenotype (Hu et al., 1997). MyoD can interact with PGC-1α through E-box regions located proximal to the transcription start site in the promoter (Chang et al., 2006). These E-box motifs are found in the promoters of many different muscle specific genes. MyoD is also involved with a positive auto-regulatory PGC-1α expression loop with SIRT1 (Amat et al., 2009). SIRT1, a member of the sirtuin family of protein deacetylases, is involved with post-translational modifications of PGC-1α associated with up-regulation of fatty acid oxidation genes in skeletal muscle. As well, when SIRT1 is co-expressed with MyoD, increased PGC-1α transcriptional expression occurs (Amat et al., 2009).

Another element located on the promoter is responsive to insulin. This peptide hormone increases blood glucose uptake in muscle thus serving as a primary regulator of blood glucose levels. Insulin suppresses PGC-1α expression but the binding of FKHR to the insulin responsive sequence (IRS) allows for activation of transcription mediated through protein kinase B (Daitoku et al., 2003). Forkhead transcription factor (FKHR) controls the expression of glucose metabolic enzymes such as glucose-6-phosphatase and phosphoenoilpyruvate carboxykinase (Nakae et al., 2001). However, FKHR induces gene expression by an indirect transactivation mechanism through mediation of coactivators rather than acting on the promoters of these genes themselves (Daitoku et al., 2003). This reflects the important role PGC-1α plays in gluconeogenesis. These IRS elements in the promoter of PGC-1α are required for both FKHR-induced and basal promoter activity.

Because the PGC-1α promoter lacks a TATA box, specificity protein 1 (Sp1) may regulate constitutive promoter activity. The Sp1 family of transcription factors bind and act through GC boxes to regulate gene expression of multiple targets. All members are identified by three
Cys2His2 zinc fingers, binding sequence-specific fashion to GC-rich promoter elements (Li et al., 2004). Sp1 and Sp3 share more than 90% sequence homology in the DNA-binding domain and they bind to the same cognate DNA element (Li et al., 2004) but they have very different functions. The Sp1 family shows an example of positionally constrained motifs. Sp3 binds the promoter as a monomer, whereas Sp1 can form multimers that allow Sp1 sites to act synergistically. When Sp3 binds to the promoter, it prevents Sp1 multimers from binding multiple sites, disrupting their synergistic effect. Therefore, the distance between binding sites can have important functional consequences for the target gene (Rodenburg et al., 1997). Sp1 is involved in the expression of many genes including structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors and signalling receptors (Li et al., 2004).

These factors along with others collectively control expression of PGC-1α through their interaction with specific motifs. Binding sites may not necessarily be functional. Both a transcription factor must bind and the level of transcription of the target gene must be affected for a functional binding event to occur (Tabach et al., 2007). This must be shown experimentally. The sites discussed here have been shown to affect PGC-1α expression. However, most work has examined either the human or mouse promoter. Studies have shown that many positionally constrained motifs of promoters of various genes are not conserved between even human and mouse (Vardhanabhuti et al., 2007). Thus, these motifs identified in PGC-1α are not necessarily conserved across all mammals. Many computational analyses comparing the regulatory regions in different species to identify functionally conserved motifs can help to determine relative importance (Tabach et al., 2007). The nature of lineage specific
gain and loss of transcription factor binding sites could generate new functions or expression patterns that would in turn provide the basis for interspecies differences in metabolism.

**Evolution of transcriptional regulation**

Interspecies differences in mitochondrial content may not simply be the result of different genes, but other factors such as alternative splicing and post-translational modifications as well as differential expression of certain genes. To determine the basis of expressional differences requires the examination of transcriptional regulatory networks. Changes in transcriptional regulatory networks are achieved through evolution of transcription factors (trans-acting regulatory factors) and their DNA-binding sites (cis-acting elements).

Regulation through cis-elements has been studied in greater detail and is believed to be the dominant source for the gradual evolution of transcriptional regulation.

The evolution of these binding domains, the cis elements, could be crucial in interspecies differences (Carroll 2004, Wray 2007). These regions act as switches to control either the on/off state of genes in particular cell types or regulate gene expression levels to adapt to changing physiological conditions and environmental influences. Changes in trans-factors will have a much broader impact on a network because all targets will be affected, whereas more gradual changes can occur through individual cis elements. Since these regulatory sequences are not coding functional proteins they are thought to generally evolve faster than coding regions of DNA (Ludwig et al. 2000, Wray et al. 2003). Mutations of coding regions of genes may have multiple deleterious effects, while changes in the regulatory region would only affect some aspects of its expression (Carroll 2004). The motifs recognized by various transcription factors are typically small (4-10 nucleotides) and prone to degeneracy,
thus random mutation can easily affect appearance or multiplication of these regulatory sites (Ludwig, 2000; Scemama et al., 2002). Across mammalian genomes a small proportion of regulatory regions is relatively conserved but the majority exhibit a high degree of plasticity (Dermitzakis and Clark, 2002).

Sequences regulating transcription of genes do not occupy a fixed position relative to coding regions and are often diffuse and widely dispersed. To a certain extent the functionality of a transcription factor depends on the location of a binding site. Some transcription factors are location-specific and will only affect transcription if bound within a narrow range of distances from transcription start site (Tabach et al., 2007). This occurs when interactions are necessary between the transcription factor and polymerase (Vardhanabhuti et al., 2007). Others may operate completely independently of position (Tabach et al., 2007), a category that includes many enhancers. Transcriptional regulation also often depends on interactions among transcription factors, which can also impose constraints on the binding elements. For example, in human promoters, significant positional constraint is shown in a large fraction of known motifs, with motif-pairs having a tendency to be co-expressed (Vardhanabhuti et al., 2007).

Regardless of position, not all mutations have functional consequences in non-coding regions. A single nucleotide substitution may not actually affect the ability of transcription factors to bind to that region, leaving it still a functional motif. Because of the small size of the binding sites, DNA regions identical to functional binding sites occur by chance with high frequency, which makes recognition of true binding sites difficult. As well, multiple motifs can allow for the same transcription factor to bind, allowing for a certain amount of variation. Therefore to identify functional mutations in regulatory regions putative regulatory elements
must be tested experimentally from different species. While compensatory changes may maintain the overall functionality of the cis-regulatory region, lineage-specific gains and losses could be responsible for new functions or expression patterns (Dermitzakis and Clark, 2002).

**Hypotheses**

Since PGC-1α plays such an important role in determining individual animal muscle fibre type differences it may also be guiding interspecies differences. Thus trends seen in metabolic rates may be guided transcriptionally by this coactivator. If transcriptional regulation of PGC-1α displays differences between species based on variation in promoter binding motifs, then cis-factor evolution may be the guiding force in scaling trends.

My thesis will test the following specific hypotheses:

1. Muscle mitochondrial enzyme content/activity will show negative scaling when comparing the muscle enzymes of various rodent species.

2. The differences in muscle cytochrome oxidase activities between species will be a reflection of transcriptional regulation.

3. The mRNA levels for the master regulator of mitochondrial biogenesis, PGC-1α, will reflect mitochondrial content in muscles of different rodents.

4. The promoter strength of the proximal PGC-1α promoter will reflect PGC-1α mRNA levels in rodents.

5. Across mammals, the PGC-1α promoter will vary in ways that reflect a general trend due to the effects of body size.
Chapter 2: Methods

Tissue Samples

Samples for DNA analysis

Material used for PGC-1α promoter analysis was obtained opportunistically from numerous sources (Table 1). Samples from many local species were obtained from buccal cell swabs (Homo sapiens, Canis familiaris, Felis catus, Equus caballus), the carcass material purchased from a commercial meat supplier (Bos taurus, Sus scrofa, Oryctolagus cuniculus, Ovis aries), collected as roadkill (Scuirius carolinensis, Tamiasciurus hudsonicus, Erethizon dorsatum, Marmota monax), culls from colonies (Mus musculus, Rattus norvegicus, Phodopus campbelli, Cavia porcellus, Meriones unguiculatus, Mesocricetus auratus) or obtained from hunters and trappers (Alces alces, Odocoileus virginianus, Ursus americanus, Castor canadensis, Mustela nivalis, Neovison vison, Martes pennanti, Procyon lotor, Canis lupus, Canis latrans, Peromyscus maniculatus, Microtus pennsylvanicus, Tamias striatus). Many samples of exotics were obtained from colleagues in Biology (Ovis aries orientalis, Rupicapra rupicapra, Ovibos moschatus, Giraffa camelopardalis, Diceros bicornis, Tapirus bairdii, Lynx canadensis, Hyaena brunnea, Ursus maritimus, Sorex cinereus, Sorex palustris, Condylura cristata, Scapanus orarius, Bradypus tridactylus, Macaca mulatta, Pongo pygmaeus, Gorilla gorilla, Chrysochloris asiatica, Procavia capensis, Elephantulus intufi, Orycteropus afer, Loxodonta africana) or donated from zoos (Hippopotamus amphibius, Hydrochoerus hydrochaeris, Panthera leo).
### Table 1: Species used for promoter analysis.

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<tr>
<td></td>
<td>Hippopotamus amphibious</td>
<td>hippopotamus</td>
<td>1700</td>
</tr>
<tr>
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<td>Sorex cinereus</td>
<td>masked shrew</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>Sorex palustris</td>
<td>water shrew</td>
<td>.013</td>
</tr>
<tr>
<td></td>
<td>Condylura cristata</td>
<td>star-nosed mole</td>
<td>.055</td>
</tr>
<tr>
<td></td>
<td>Scapanus orarius</td>
<td>coast mole</td>
<td>.062</td>
</tr>
<tr>
<td></td>
<td>Tapirus bairdii</td>
<td>tapir</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Equus caballus</td>
<td>horse</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Diceros bicornis</td>
<td>rhino</td>
<td>2500</td>
</tr>
</tbody>
</table>
Samples for Enzyme and RNA analysis

Lab mice (*Mus musculus* CD-1 males), gerbils (*Meriones unguiculatus*) and hamsters (*Mesocricetus auratus*) were ordered from Charles River Canada while rats (*Rattus norvegicus* Sprague-Dawley) were sentinel males from the animal care centre at Queen’s University. These samples were euthanized using pentobarbital 150mg/kg. Female guinea pigs (*Cavia porcellus*) were control animals used in the Reynold’s and Brien lab at Queen’s University and were anesthetized and decapitated. Dwarf hamsters (*Phodopus campbelli*) were retired from the Wynne-Edwards colony at Queen’s University and were sacrificed by cervical dislocation. Groundhogs (*Marmota monax*) were first generation provided by the Cornell University colony. Wild rodents were trapped using Sherman box traps for deer mice and chipmunks or Havahart squirrel traps for grey squirrels and red squirrels. Animals were euthanized using 150 mg/kg pentobarbital immediately after being removed from the trap. Beaver (*Castor canadensis*) samples were contributed by a local trapper as part of a pest removal, killed by gunshot.

All animals were weighed (Table 2), then gastrocnemius, soleus and tibialis anterior tissue were excised from the animal and flash frozen in liquid nitrogen. The only exception was groundhog, where the tibialis cranialis and gastrocnemius were the tissues collected. This species apparently lacks a separate soleus and the tibialis cranialis was treated as the tibialis anterior.
Table 2: Rodents used for analysis of lower limb muscles.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample mass (g)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Beaver</td>
<td>7000</td>
</tr>
<tr>
<td>Eastern grey squirrel</td>
<td>650</td>
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<tr>
<td>Chipmunk</td>
<td>115</td>
</tr>
<tr>
<td>Deer mouse</td>
<td>24.2</td>
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<tr>
<td>Dwarf hamster</td>
<td>28.5</td>
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<tr>
<td>Gerbil</td>
<td>95.1</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1069</td>
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<tr>
<td>Hamster</td>
<td>187.4</td>
</tr>
<tr>
<td>Mouse</td>
<td>34.1</td>
</tr>
<tr>
<td>Rat</td>
<td>354</td>
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<td>Red squirrel</td>
<td>180</td>
</tr>
<tr>
<td>Woodchuck</td>
<td>4650</td>
</tr>
</tbody>
</table>

Enzyme Analysis

Between 50 and 75 mg of powdered tissue were homogenized in 20 vol of extraction buffer (20mM Tris (pH 8.0), 0.6 mM lauryl maltoside) using a ground glass homogenizer on ice.

COX was assayed with 20mM Tris (pH 8.0), 0.6mM lauryl maltoside, and 100µM cytochrome C. Cytochrome c was reduced with an excess of ascorbate, dialyzed exhaustively against 20mM Tris (pH 8.0), then frozen in aliquots. Each sample was assayed in a 96-well spec plate using a Molecular Devices SpectroMax Plus spectrophotometer held at a temperature of 25°C. This assay was done immediately after homogenization in quadruplicate with samples diluted 40-fold in extraction buffer and read for 90 sec at a wavelength of 550nm.

Lactate dehydrogenase was assayed in 20 mM Hepes (pH 7.0), with 1 mM pyruvate and 0.2mM NADH. Homeogenates were diluted 40-fold in assay buffer (20mM Hepes, pH 7.0) immediately prior to the assay in order to obtain acceptable rates.
RNA Analysis

RNA was extracted from approximately 30 to 50 mg of powdered rodent muscle tissue sample using the Qiagen RNeasy kit (Mississauga, ON) following manufacturer’s instructions. RNA was quantified using the 260nm absorbance on the Molecular Devices SpectroMax Plus spectrophotometer. From this total RNA, cDNA was made from 2 µg RNA using Qiagen RT kit (Mississauga, ON) as per the manufacturer’s instructions.

Sections of rodent genes for PGC-1α and β-actin were sequenced prior to specific real-time primer design. Sequences for these genes in multiple species were lined up using Clustal-W software, then primers were designed using Primer 3 software. Portions of these genes were amplified using PCR with consensus primers (see Table 3). This reaction contained 1x PCR buffer (Qiagen), 1.5 mM MgCl₂ (Qiagen), 200 µM dNTP (Promega), 2.5 units Taq polymerase (Qiagen), 40 ng template, and water to make a final volume of 25 µl. The PCR conditions were 2 min initial denaturation at 94°C, three step cycle of 1 min at 94°C denaturation followed by a 30 s gradient annealing temperature in the gradient of 57-61°C and a 1 min extension at 72 °C for 35 cycles, with a final extension at 72°C for 10 min. After extraction of the specific bands from a 1% agarose gel following electrophoresis, the bands were extracted from gels (Qiagen PCR Purification kit). Following ligation into Qiagen pDrive cloning vector following manufacturer’s instructions, the product was transformed in DH5α cells (Invitrogen). Inserts were detected by an EcoRI digestion after purification of the plasmid (Qiagen MiniPrep). Plasmids were sequenced at Robarts Sequencing (London ON) in both directions using sequencing primers M13F and M13R (Table 3).
Table 3: Primers used for rodent mRNA analysis.

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Primer Set</th>
<th>Amplicon size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing</td>
<td>PGC-1α</td>
<td>F: AAGCAGGTCTCTCCTTGAGAGCA</td>
<td>800</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACGGTCATTCCAATTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pDrive</td>
<td>M13F: CGCCAGGGTTTTCCAGTCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>universal</td>
<td>M13R: TCACACAGGAAACAGCTATGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real time</td>
<td>PGC-1α</td>
<td>F: AAGGTCCCAGGCAGTAGAT</td>
<td>192</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTGCGGATTACATCCCTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
<td>F: AGGCTGTGCTGTCCTGTAT</td>
<td>202</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCTGTGGTGGTGAGCTGTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Real-time analysis to quantify RNA in each of the three tissues for all 12 rodents was performed on an ABI 7500 RT-PCR system (Foster City, CA, USA) using the following conditions: an initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s denaturation at 95°C, 30 s annealing at optimal primer temperature (Table 3) and 36 s extension at 72°C. Each sample was assayed in duplicate in a 25 μl reaction volume containing 100ng cDNA, 12.5 μl SYBR green master mix (Roche) and 0.3 μM of each primer. Negative controls (no template) were run as well to ensure the absence of contamination. A dissociation curve was run on dilution series to check efficiency and ensure single fluorescence peak for each set of primers. Analysis was performed according to the ΔCt method using β-actin as the housekeeping gene.
DNA analyses

DNA content

DNA content of rodent muscle (mg DNA/g tissue) was measured to determine nuclear content. Because the most commonly used technique - determination of absorbance at 260 nm for measuring DNA concentration, is not selective for double-stranded DNA and thus prone to measuring contaminants, a sensitive detecting dye, picogreen, was used. DNA content per gram of tissue was assessed using picogreen as per manufacturer’s instructions after digesting 100 µl of enzyme homogenate with 5 µg/ml proteinase K solution (consists of buffer (200 mM NaCl, 20 mM Tris, 50 mM EDTA, 0.10% SDS, pH 8.0) with proteinase K (0.2 mg/ml)). The digest was treated with RNase A (10 µg/ml) prior to quantification. Each sample was assayed in duplicate using a Molecular Devices fluorometer at excitation of 480 and 520.

DNA Purification

DNA from tissues was used to provide material for promoter characterization. Tissue samples were suspended in buffer (200 mM NaCl, 20 mM Tris, 50 mM EDTA, 0.10% SDS, pH 8.0) with proteinase K (0.2 mg/ml) and digested overnight. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, the sample was mixed thoroughly and centrifuged for 10 min at 1700 g. The aqueous phase was retained and DNA was precipitated by adding of 0.1 vol ammonium acetate (7.5 M) and 2 vol of 100% ethanol. The solution was centrifuged for 3 min at 1700 g and washed with 70% ethanol. The pellet was air-dried and resuspended in 250 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA purity was assessed using absorbance at 260 nm and 280 nm, then quantified based on the 260 nm reading.
Promoter analyses

**Sequencing novel promoters**

Promoter sequences for available placental mammals were downloaded from Genbank or ENSEMBL databases for the 800 bp immediately proximal to transcription start site of the PGC-1α gene. Sequences for the promoter in multiple species were lined up using Clustal-W software, then primers were designed using Primer 3 software. Consensus primers were developed for an 800 base pair section that area and the promoter was amplified in terrestrial mammals with tissue available to us (Table 4). This reaction contained 1xPCR buffer (Qiagen), 1.5 mM MgCl₂ (Qiagen), 200 µM dNTP (Promega), 2.5 units Taq polymerase (Qiagen), between 20-50 ng template, and water to make a final volume of 25 µl. The PCR conditions were 2 min initial denaturation at 94°C, three step cycle of 1 min at 94°C denaturation followed by a 30 s gradient annealing temperature in the gradient of 57-61°C and a 1 min extension at 72 °C for 35 cycles, with a final extension at 72°C for 10 min. After extraction of the specific bands from a 1% agarose gel following electrophoresis, the bands were extracted from the gel (Qiagen PCR Purification kit). Following ligation into Qiagen pDrive cloning vector following manufacturer’s instructions, the product was transformed in DH5α cells (Invitrogen). Inserts were detected by an EcoRI digestion after purification of the plasmid (Qiagen MiniPrep). Plasmids were sequenced at Robarts DNA Sequencing facility (London, ON).
Table 4: Primers used for amplification of PGC-1α proximal promoter for sequencing

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Species amplified</th>
<th>Amplicon size</th>
<th>Tm</th>
</tr>
</thead>
</table>
| F: CGCTTTCAAACACTCCCTCA  
R: TCACCTCGATGTCACTCCAT | Primate, Eulioptera, Xenartha, Afrotheria | 693 | 57 |
| F: TTGCCTTCAAACACTCTCTA  
R: AGCTCACCTCTATGTCACTCCAT | Rodentia | 688 | 57 |
| F: TTTCAAACACTCTCCCCAAAT  
R: TCACCTCGATGTCACTCCAT | Carnivora | 690 | 59 |
| F: CATCCTCTGCTTTCAAACACTC  
R: TCACCTCGATGTCACTCCAT | Cetartiodactyla | 672 | 57 |
| F: CATTCCTTGCTTTCAAACACT  
R: TCACCTCGATGTCACTCCAT | Perissiodactyla | 686 | 57 |

Transcription factor binding site identification

Inspection of this sequence for the presence of consensus transcription factor binding sites was performed by high stringency searches using PATCH (Pattern search for transcription factor binding sites) and TRANSFAC 6.0. The odds of identifying false positives were minimized by excluding non-canonical sequences, or sequences that contained nucleotide mismatches. Sites of importance were identified through their presence in this search as well as published data primarily from mouse and human.

Reporter gene constructs

An 800 bp fragment of the PGC-1α promoter was generated by PCR using the insert from the original plasmid from each species as a template. These PCR primers were designed from the species-specific PGC-1α promoter sequence and included the restriction sites XhoI and Mlu (Table 5). The cloned fragment was first ligated into Qiagen pDrive cloning vector following
manufacturer’s instructions, the product was transformed in DH5α cells (Invitrogen). This product was then cut out using MluI and XhoI, and then ligated into the MluI- and XhoI-digested sites of the calf intestinal phosphatise (CIP) treated pGL3-basic vector containing the luciferase reporter gene. The insert was confirmed using both restriction digest as well as sequencing of the plasmid. Plasmid concentration was determined using a picogreen assay with excitation at 490nm and emission at 520nm.

### Table 5: Primers used for generation of reporter gene constructs.
Restriction sites are highlighted.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Species amplified</th>
<th>Amplicon size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAAACCGCGTATTTCTCGAGACAGACTCCTGGCT</td>
<td>Muridae</td>
<td>582</td>
<td>59</td>
</tr>
<tr>
<td>AAAAACCGCGTATTTCTCGAGACAGACTCCTGGCT</td>
<td>Capybara</td>
<td>602</td>
<td>59</td>
</tr>
<tr>
<td>AAAAACCGCGTATTTCTCGAGACAGACTCCTGGCT</td>
<td>Squirrel Beaver</td>
<td>599</td>
<td>59</td>
</tr>
<tr>
<td>AAAAACCGCGTATTTCTCGAGACAGACTCCTGGCT</td>
<td>Human</td>
<td>614</td>
<td>57</td>
</tr>
</tbody>
</table>

**Cell Culture**

Different cell lines were examined to see which would be an optimal common background for transfections. To mitigate potential effects of differences in culture media, all cells were grown in Dulbecco's Modified Eagle Medium (DMEM: high glucose with glutamine, Gibco, Burlington, ON). C2C12 murine myoblasts and L6 rat myoblasts were supplemented with 10% fetal bovine serum (FBS, Gibco, Burlington, ON) while Sol8 murine myoblast cells were
supplemented with 20% FBS. All were also grown with penicillin–streptomycin-neomycin (Gibco, Burlington, ON), with 5% CO₂ and 95% humidity at 37 °C.

Once the cells had reached 90% confluence, differentiation was induced by changing the medium to DMEM supplemented with 2% horse serum with penicillin–streptomycin-neomycin. Within 2–3 days of serum starvation, myotubes begin to form.

Cells used for RNA analysis were homogenized in buffer RLT using a 21-gauge needle and RNA was extracted using the RNeasy extraction kit (Qiagen, Mississauga, ON). Complimentary DNA was synthesized using AMV RT (Promega, Madison, WI), oligo (dT) primers, and random hexamers as per manufacturer’s instructions. Real-time analysis was performed on an ABI 7500 RT-PCR system (Foster City, CA, USA) using the following conditions: an initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s denaturation at 95°C, 30 s annealing at optimal primer temperature (Table 5) and 36 s extension at 72°C. Each sample was assayed in duplicate in a 25 μl reaction volume containing 100 ng cDNA, 12.5 μl SYBR green master mix (Qiagen) and 0.58 μM of each primer. Negative controls (no template) were run as well to ensure the absence of contamination. Analysis was performed according to the ΔCt method using TATA-binding protein (TBP) as the housekeeping gene.

Transient transfections

Plasmids were prepared for transfection using Plasmid Midiprep kit according to manufacturer’s directions (Qiagen, Mississauga, ON). Cells were transfected using FuGENE 6 (Roche, Mississauga, ON). Briefly, cells grown to ~80% confluence in 12-well cell culture plates were transfected with 1.5ml fresh growth medium with 1 μg of firefly luciferase plasmid construct and 50 ng of pRL-TK, a TK promoter-driven Renilla luciferase expression vector used
as an internal reference. After 24 h medium was changed to differentiation media. After 3 additional days, luciferase measurements were performed on the differentiated myocytes.

Luciferase expression was determined using the Dual Luciferase Assay System (Promega, Madison WI) according to the manufacturer’s instructions on a luminometer (Lmax; Molecular Devices, Sunnydale, CA) with automatic injector. Briefly, cells grown in 12-well plates were harvested in 100 µl of 1x passive lysis buffer and frozen at −80°C. To measure firefly luciferase, 100 µl of luciferase assay reagent (LARII) were added to 10 µl of room-temperature cell lysate in a white 96-well plate. Relative light units were measured for 10 s, with a 2-s premeasurement delay. To measure Renilla luciferase, 100 µl of Stop & Glow solution (Promega) were then added to the same well, and relative light units were again measured for 20 s, with a 2-s delay.
Chapter 3: Results

Enzyme analysis

Enzyme measurements for the muscles of twelve rodents are summarized in Table 6. The activity of COX differed between the three tissues collected from rodent lower limb: gastrocnemius, soleus and tibialis anterior (TA) from rodents ranging in size from the 20 g deer mouse to a 17 kg beaver. Each species contributed to a single point corresponding to the mean for that tissue. Enzyme activity for COX scaled negatively with body mass in the TA and gastrocnemius, with a slope of -0.07 and -0.18 respectively, while no significant scaling trend was detected in the soleus (Figure 2).

DNA/g follows this trend of negative scaling with body size in the TA and the gastrocnemius but displays positive scaling in the soleus (Figure 4). When expressed per g of DNA (to account for differences in myonuclear content), mitochondrial enzyme activity still scaled negatively with body mass but the differences between tissues decreased (slope in gastrocnemius: -0.05, soleus: -0.13, TA: -0.14).

The enzyme activity for LDH (Figure 3) scaled positively with body mass in all three tissues (slope in gastrocnemius: 0.08; soleus: 0.16; TA: 0.12). When expressed per g of DNA, activity still scaled positively with body mass but greater difference in slopes between tissues (gastrocnemius: 0.1; soleus: 0.06; TA: 0.2).

When activity for COX is expressed relative to LDH the slopes of all three become more negative (Figure 4).
<table>
<thead>
<tr>
<th>Species</th>
<th>DNA content</th>
<th>COX activity (U/g)</th>
<th>LDH activity (U/g)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
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</tr>
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<td>55.1</td>
</tr>
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<td>0.1</td>
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<td>0.2</td>
<td>48.8</td>
</tr>
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<td>0.6</td>
<td>106.3</td>
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<td>0.1</td>
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<td>0.4</td>
<td>47.5</td>
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<tr>
<td><strong>Soleus</strong></td>
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<tr>
<td><em>Peromyscus maniculatus</em></td>
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<td>0.1</td>
<td>86.6</td>
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<td>72.1</td>
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<td>0.5</td>
<td>59.3</td>
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<td><em>Castor canadenis</em></td>
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<td><strong>Tibialis Anterior</strong></td>
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<td><em>Peromyscus maniculatus</em></td>
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<td>0.6</td>
<td>82.1</td>
</tr>
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</tr>
<tr>
<td><em>Tamiasciurus hudsonicus</em></td>
<td>2.4</td>
<td>0.6</td>
<td>108.2</td>
</tr>
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<td>50.0</td>
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<td><em>Scurius carolinensis</em></td>
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<td>0.1</td>
<td>67.9</td>
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<td><em>Cavia porcellus</em></td>
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<td>46.6</td>
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<tr>
<td><em>Marmota monax</em></td>
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<td>0.1</td>
<td>31.4</td>
</tr>
<tr>
<td><em>Castor canadenis</em></td>
<td>1.5</td>
<td></td>
<td>33.2</td>
</tr>
</tbody>
</table>
Figure 2: Cytochrome oxidase activity in rodent lower limb muscle.

For each species, a single point (mean) was used because of differences in sample size.
Figure 3: Lactate dehydrogenase activity in rodent lower limb muscle.
For each species, a single point (mean) was used because of differences in sample size.
Gene expression

If the observed specific enzyme activities were due to transcriptional regulation of their respective genes, then the master regulator PGC-1α may also correlate with body size. The relative gene expression of PGC-1α normalized to a housekeeping gene β-actin was examined in the three tissues of the rodents collected. No significant scaling trend was observed across species for any of the three muscle tissues (Figure 6).
Figure 6: PGC-1α mRNA expression in rodent lower limb muscles normalized to B-actin expressed relative to body size.

PGC-1 promoter analysis

Placental mammals

The first 800 bp immediately proximal to the transcriptional start site were determined for this analysis to be the proximal promoter. This region contains several regions that have been identified as crucial for constitutive activity of the PGC-1α gene. The promoter does not contain a TATA box, but a putative GC box approximately -75 bp from TSS is known to bind Sp1 in humans and mice (Figure 1). A CRE site is found around position -170 from TSS and two IRS around -330 and -480. Multiple E-boxes responsive to MyoD and myf5 were found at positions such as -15 and -150 as well as a serum response element (SRE). NFAT appears in mouse but not human.
Table 7: Trends in putative binding sites identified in mammals across suborders.
Representative refers to number of species in which promoter was examined.

<table>
<thead>
<tr>
<th>Suborder</th>
<th>Rep</th>
<th>E1</th>
<th>IRS</th>
<th>NFAT</th>
<th>CRE</th>
<th>Sp1</th>
<th>Exceptions</th>
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</thead>
<tbody>
<tr>
<td>Afrotheria</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lagomorpha</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Muridae (see figure)</td>
</tr>
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<td>Primate</td>
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<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Rodentia</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
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<tr>
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<tr>
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<td>13</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Ursidae: 2 Sp1</td>
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<tr>
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<td>1</td>
<td>1</td>
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<td>Suridae 1 Sp1</td>
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<td>2</td>
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</tr>
</tbody>
</table>

Across mammalia the promoter appears to be well conserved. However, the promoter does not appear to reflect the accepted mammalian phylogeny (Figure 7). Putative binding sites in promoters of mammalian species of sequenced rodents were identified and compared to the relatively well characterized human and mouse promoters. Many of these binding sites appear to be well conserved across species, both in terms of presence and location. One site that is generally well conserved across Mammalia appears to be Sp1 (Table 7). However, tandem motifs may be seen in certain species while others only have one. This tandem Sp1 condition appears to be basal to the supraorder laurasiatheria, but is lost in the majority of the carnivores. However, 2 sites are present in the largest members of the carnivores: the ursids, but the reverse is seen in perissodactyla where the largest member, the rhino, possesses one site.
Figure 7: Neighbour-joining phylogeny of PGC-1α promoter on the right in contrast with accepted mammalian phylogeny on the left (adapted from Springer and Murphy, 2007).

Certain sites are seen only within specific orders (Table 7). For example, the NFAT site is only found within Lagomorpha and Rodentia, including the mouse where the promoter is well characterized. Even within other Euarchontoglires, such as the primates, a NFAT site is not
seen within the proximal promoter. However, the CRE site is found in animals lacking this NFAT motif whereas many rodents are lacking the CRE motif. Lagomorpha appears to be one order possessing both these sites. Even within Rodentia there are a few species possessing both CRE and NFAT motifs, such as rat and groundhog.

Rodentia provides a within-family basis to examine these differences based on both size and relatedness (Figure 8). However, some selective trends both size-specific and family-specific are observed. The binding site for CRE at approximately -133 from transcription start site is present only in larger rodents with everything smaller than a red squirrel lacking this site. This includes all members of the subfamily Myodonta except rat and beaver, both the largest members of their family. A putative GC box shown to bind Sp1 in humans can be seen across the rodents. A single Sp1 site is present in the majority of rodents while the two largest species (beaver and capybara) possess two located approximately 15 bp apart. The IRS regions also vary between species in terms of number, though this pattern is more variable. In humans, two sites occur seen in the 800 base pairs immediately before the transcription start site, whereas only the smallest rodents display both.

Since trends do not always hold within all members of the family, and it is difficult to assess through sequence examination whether changes are functional or not, an experimental approach too is necessary.
Figure 8: Neighbour joining phylogeny of family Rodentia and gain/loss of putative binding sites in the proximal promoter of PGC-1α.

Promoter activity

To determine the appropriate background in which to measure promoter activity to be measured in, three different muscle cell lines were examined, two murine (Sol8, C2C12) and one rat (L6). The expression of PGC-1α transcripts was assessed to determine the best point at which transfections should be harvested. The gene expression of PGC-1α normalized to TBP was highest in rat L6 cells with the lowest in C2C12. Transcript levels increased in all cell lines as differentiation occurred but levelled off or decreased by day 7. Based on these results, we used Sol8 largely to ensure that measurable levels of PGC-1α promoter activity would be found, based upon more measurable levels of mRNA. Transfection of both an empty vector and a reporter construct into the common background of Sol8 cells were conducted to determine
which day yielded optimal results. The highest activity of firefly luciferase relative to *Renilla* was found in cells harvested at day 3, 4 days after transfection.

To determine whether the differences between species in promoter sequence result in functional changes, reporter constructs were made for representatives from the family Rodentia. Promoter activity did not follow the expected trend with body size. Basal PGC-1α promoter activity did not reflect differences in mitochondrial content relative to body size. Instead a positive slope of 0.13 was observed.

![Graph](image)

**Figure 9: PGC-1α promoter activity**

A) PGC-1α relative mRNA expression in rodent cell lines through myogenesis B) human PGC-1α reporter construct promoter strength relative to day 0 in sol8 cells.
Figure 10: Rodent PGC-1α promoter activity in Sol8 cells.

Promoters were cloned into firefly luciferase pGL3 constructs and dual transfected with Renilla vector, collected on day 3 of differentiation. All data were corrected for transfection efficiency with the use of the promoter-driven Renilla luciferase (pRL)-thymidine kinase (TK).
Chapter 4: Discussion

Allometric scaling of metabolic rate is a well known phenomenon, but less studied is the impact of body size on metabolic enzymes. The main goals of my analysis were (i) to establish whether similar scaling relationships exist in multiple muscle types and (ii) explore the underlying mechanisms that control the size-dependent mitochondrial enzyme activities. Few studies have compared muscle enzyme levels in relation to size in an appropriate phylogenetic context. For example, the earliest studies on scaling of metabolic enzymes in mammals (Kunckel et al., 1956; George and Talesera, 1961; Emmett and Hochachka, 1981) drew from numerous placental mammals without regard for phylogenetic relatedness. In assessing the hierarchical control of enzyme levels through transcriptional regulation, these studies represent an important contribution to the understanding of a question that has been unresolved for more than 50 years.

Metabolic enzyme activities

Prior to these studies, there has been a general consensus on scaling of muscle enzymes. The scaling coefficients for aerobic enzymes are less than those for aerobic metabolism. Larger animals are less oxidative and more glycolytic than smaller animals, based upon muscle enzyme patterns (Emmett and Hochachka, 1981) and fish (Somero and Childress 1980). When I examined lower hindlimb muscle enzyme activities in rodents of different size, several surprising trends emerged.
Fiber type differences in muscle enzymes

I chose three muscles for my study that I expected to differ in mitochondrial content because of their muscle fiber type composition. Though muscle fiber types are defined on the basis of the myosin heavy chain (MHC) isoform (I, IIa, IIb, IIx/d), there are correlates with both fiber dimensions and mitochondrial content (Eng et al., 2008). For example, type I fibers have the smallest diameter, greatest staining for mitochondria and lowest glycolytic enzyme activities, whereas type IIb fibers have the largest diameter, lowest staining for mitochondria and greatest glycolytic enzyme activities. Each muscle is a mosaic of different fiber types. Soleus is a postural muscle and requires long-term steady state force generation. It is typically composed of type I or IIa fibers. For example, 80% of the fibres in rat soleus are type I MHC (Eng et al. 2008). Muscle that contribute to leg movement have greater requirements for short-term, high intensity activity and thus have a higher proportion of type larger IIx/d and the largest IIb fibers. In rats, the dorsiflexor TA has relatively more IIx and less IIb fibers than does the plantarflexor gastrocnemius, though both are primarily fast-twitch (Eng et al., 2008). Because mammalian muscles are not composed of a single fibre type, the proportion of each type for each individual muscle may be driving some of the differences between species.

Tetrapods muscles are mosaics of different fiber types, but the relative abundance of each type also differs regionally within a muscle. Slow twitch fibres usually lie deep in limb muscles, though the degree is dependent on the species (Tasic et al., 2003). Gastrocnemius muscle typically has a considerable mix in fiber types in different regions of the muscle; the medial gastrocnemius is typically richer in type I fibers than the lateral gastrocnemius. In my study, I powdered the entire muscle to avoid the effects of depth-dependent heterogeneity,
thus values for tissues are influenced by fiber type composition but not anatomical region of sampling.

Prior to this study, I expected that soleus muscles would have a higher mitochondrial enzyme activity than TA or gastrocnemius. I was surprised to find that only 2 of 12 species showed this pattern. In most cases, TA and gastrocnemius had similar COX activities. The differences between COX activities for a given species diminished with size, to the point where the largest species had similar activities in all 3 muscle fiber types.

**Muscles differ in scaling relationships**

Most of the studies that have explored scaling of muscle enzymes have focused on a single muscle. For example, the earliest mammalian study by Emmett and Hochachka (1981) focused on gastrocnemius muscle, in which they found a slope of -0.106. I found that the degree of scaling differed among muscles. Soleus muscle COX activities were largely independent of body size ($b = -0.004$) whereas more significant allometric scaling was observed in both gastrocnemius ($b = -0.07$) and TA ($b = -0.18$). In contrast, the greatest scaling coefficients for glycolytic muscle were seen in soleus ($b = 0.16$), followed by TA ($b = 0.12$) and gastrocnemius ($b = 0.08$). As animals increased in size, the magnitude of the divergence between oxidative and glycolytic enzymes was greatest for TA (0.30), then gastrocnemius (0.16) and soleus, (0.14).

In terms of the oxidative enzyme profile, the muscles that showed the greatest allometric scaling were TA and gastrocnemius. In my study, the scaling coefficient for gastrocnemius was lower than that reported by Emmett and Hochachka (1981) (-0.07 versus -0.106). As mentioned, this difference might be due to species selection. In their study, the curve was highly influenced by the smallest species, the shrew. TA showed a greater scaling
coefficient, but still less than one would expect for a scaling coefficient for metabolic rate. The most intriguing pattern appears in the rodent soleus. This muscle shows isometric scaling of COX activities. This suggests that the aerobic function of postural muscles may be less affected by body size.

Overall, these studies show that scaling of metabolic enzymes show a reciprocal relationship in all muscles, but that only some muscles show allometric scaling of oxidative enzymes. One possible explanation for the differences between tissues and species in mitochondrial content might be myonuclear domain.

*Myonuclear domain does not account variation in mitochondrial enzyme activity in relation to body size*

Focusing on the mitochondrial side of the metabolic phenotype, I explored the underlying basis of the patterns by assessing the potential roles for each level of gene expression. One factor that plays a role in determining the muscle metabolic phenotype is the number of myonuclei in a fibre. Whether considering it as myonuclear domain (fibre volume per nucleus) or nuclear content (DNA/g tissue), the abundance of nuclei can in principle have a dramatic effect on constitutive gene expression. Each nucleus produces mRNA to provide the precursors in support of the surrounding cytoplasm (Ralston and Hall, 1992). It follows that more nuclei per g tissue would lead to more precursors per g tissue (Edgerton and Roy, 1991; Tseng et al., 1994). If there are changes in gene expression it should be reflected when corrected for total DNA content per gram. Thus, higher levels of nuclear gene transcripts (per g tissue) can arise even if there are no differences in gene expression *per se*. In muscle, the fiber diameter varies inversely with both nuclear content and mitochondrial content. Because
nuclear and mitochondrial content covary, muscle mitochondrial content may not be actively regulated through differential gene expression, but rather, it would depend primarily on muscle nuclear content. A recent study by Liu et al. (2009) showed a strong relationship myonuclear domain size and body mass in mammalian muscle fibres. Likewise, in previous studies with fish, consideration of DNA content largely negated differences in mitochondrial enzyme specific activity in relation to size (Davies and Moyes, 2007) and between fibres (Dalziel et al. 2005). In this study, many of the differences in mitochondrial content in relation to fibre type and body size are diminished when taking into account differences in myonuclear content.

In this study, accounting for DNA content greatly reduced COX activity differences between muscles in each of the species. Thus, it appears that many of the differences between fiber types of a given species can be largely attributed to myonuclear domain. However, accounting for DNA content did not negate the allometric patterns seen in TA and gastrocnemius. Developing scaling coefficients on the basis of COX activities per mg DNA had little effect on gastrocnemius ($b$ decreases from -0.066 to -0.054) or TA ($b$ decreases from -0.18 to -0.14). In these tissues, DNA content showed allometric scaling with scaling coefficients of -0.01 (gastrocnemius) and -0.04 (TA). In these tissues, it appears that there are few differences in myonuclear content in relation to size in relation body size and thus other mechanisms must explain the differences in mitochondrial content in relation to body size.

The soleus, in contrast, was the only muscle to show a pronounced effect of size on nuclear content (Figure 2). Muscle nuclear content increased with size ($b$=0.13) and as a result, the isometric relationship seen with COX per g transforms to an allometric relationship with COX per mg DNA ($b$=-0.14).
As a result of these transformations, each of the muscles showed a similar allometric relationship when COX activities are expressed per mg DNA. Thus, for each muscle there remains an unanswered question of how myonuclei are regulated to generate the allometric differences in COX enzyme activity. Before assessing the influence of transcriptional regulation on the metabolic phenotype, there were a number of other interspecies differences that emerged from my study.

**Phylogenetic trends in muscle enzyme activities**

Many factors can influence muscle mitochondrial content, so it is not surprising that I saw differences in animals of similar size. Squirrels possessed COX activities that were higher than would be expected based on the regression of all rodents. The interspecies differences were most obvious when comparing chipmunk and red squirrel to gerbil and hamster, which are comparable in size. There are a several possible explanations for this pattern. First, the squirrels were wild-caught whereas gerbils and hamsters were colony animals. Activity level can play a major role in metabolic rates and thus the higher than expected COX activity may constitute something akin to a training effect. However, it was interesting to note that lab mice and wild deer mice had similar COX activities. Even if we had used wild-caught hamsters and gerbils, it is likely that they would have had lower activities than squirrels because of their more sedentary lifestyle. Athletic species can increase oxygen consumption by about 30 fold with activity, whereas sedentary animals of similar size have a scope of only about 12-fold (Hoppeler et al., 1987). Thus, the higher COX activity may be a reflection of requirements for maximal activity rather than BMR. The incorporation of other factors, such as diet and habitat, is known to change the scaling exponent because of the influence on BMR (McNab, 2008).
still the major factor in interspecies differences in metabolic rate, accounting for up to 96% of variation with size (McNab et al., 2008). Phylogenetic relatedness may also lead to problems when comparing any trait to body mass. The problem with comparative data such as that seen in this study is that it is not necessarily independent due to descent from common ancestors (Garland et al., 2005). Species may share common traits due to shared ancestry rather than simply due to a factor such as body size. This means that a mouse may be more like a rat than a squirrel in terms of metabolic rate not because of size but because of degree of relatedness. Correcting for phylogenetic independence using an accurate phylogeny may also decrease observed trends both due to body size and activity level. However, correcting for phylogenetic relatedness generally requires that the true phylogeny is known and not all the species used have sequence information. Though PGC-1α promoter information was obtained for all species, it does not provide an accurate phylogeny.

**Transcriptional regulation**

What is driving these changes in enzyme activity may be difficult to determine. Myonuclear domain does not account for the differences in enzyme activity seen between species, so there must be a role for active regulation of muscle mitochondrial content. If differences in protein levels or enzyme activity levels correlate with differences in mRNA, then the most parsimonious explanation is that the patterns are due to transcriptional regulation. Mitochondrial content has been shown to be under transcriptional control in response to energy challenges such as exercise and dietary stress (reviewed in Finck and Kelly, 2002). The question becomes whether interspecies differences are driven by the same factors. Therefore I asked if the ‘master controller’ of mitochondrial biogenesis was regulated in a way that would
account for scaling patterns in mitochondrial enzymes. Though the proximal promoter was
generally well conserved across mammals, certain patterns of gain and loss did appear. As well,
the proximal promoter may be central to gene regulation, but it is difficult to assess the
potential role of distal regulatory elements.

**PGC-1α promoter analysis reveals possible size dependent trends**

I analyzed about 800bp of the PGC-1α promoter from the transcription start site to an
upstream conserved element for IRS. Though I assessed the location, number and arrangement
of transcription factor binding elements, I also used the sequence to construct neighbour-
joining trees. I was not expecting such a short sequence to provide a faithful rendering of
mammalian phylogeny but I was surprised at how much the resulting tree differed from
accepted phylogeny. Each gene has functional constraints that can cause them to evolve in
ways that misrepresent phylogeny (Abril et al., 2005). Noncoding regions, such as promoters
and introns, can experience many point mutations, substitutions, insertions and deletions with
little selective penalty. However, promoters, as well as introns, possess sequences that are
conserved because they are specific binding sites (Mattick, 2001, Dermitzakis et al., 2002). The
main focus of my analysis was the evaluation of the number and arrangement of the putative
regulatory elements within the PGC-1α promoter.

Many short strings of nucleotides that appear to be sites may appear but in reality these
are far in excess of true binding sites. The genetic function of these sites, if any, depends on
conditions or factors that cannot be inferred from the sequence itself but are often context
dependent. This includes things such as the positioning and presence of additional promoter
and enhancer elements, the existence of associated coding regions and sufficient availability of
the activated protein to bind (Balmer and Blomhoff, 2009). Elements may act as binding sites in vitro or in engineered settings but be transcriptionally irrelevant in vivo (Balmer and Blomhoff, 2009).

Further experimental analysis is necessary to determine if the putative sites are truly binding the transcription factors responsive to different conditions. For the purposes of this thesis, I focused on sites that have been found to bind factors in other studies.

Certain regulatory elements in the promoter of PGC-1α are found in the promoters of many nuclear-encoded mitochondrial genes. These include cAMP responsive elements (CRE), insulin responsive sequence (IRS), and sites for Sp1, estrogen-related regulators, and PPARs (Franco et al., 2008). For most of these elements, there were instances when select species showed gain or loss but in ways that reflected no particular phylogenetic or size pattern. For example, the presence and number of IRS sites is variable across mammals as well as within certain families. However, there were intriguing size-dependent patterns that implicate 2 elements in scaling of metabolic enzymes: CRE and Sp1 site.

CRE is a common regulatory element that seems to follow a size-related pattern. In Rodentia, only larger rodents appear to have a CRE element while the smaller members of the family are lacking this site. This appears to be based more on size than on relatedness as rat has this element but other murids didn’t. The regulatory element CRE binds CREB and activating transcription factor 1 (ATF-1). These transcription factors are activated by phosphorylation in response to stimuli such as elevation of intracellular cAMP, increasing Ca\(^{2+}\) or stimulation with growth factors (Handschin et al., 2003). In addition to the complex control through phosphorylation, there are also CREB splice variants that differ in their ability to activate
promoters (Franco et al., 2008). What is interesting about this trend is that the presence of a CRE site may increase activity, yet it is found in larger members of the family. Promoter activity would be expected to be lower in these species if PGC-1α gene expression follows a scaling trend. However, rodents that lack this site appear to have an NFAT site, another transcription factor responsive to calcium through CnA and CAMK activities.

Gain or loss of a site can have unpredictable consequences. Sp1 regulates many constitutive genes, particularly those with TATA-less promoters (Segal et al., 1999). The loss of a Sp1 site can increase or decrease promoter activity. Sp1 works through forming multimers that allow Sp1 sites to act synergistically. Sp1 initially forms a tetramer and subsequently assembles multiple tetramers due to a DNA loop forming (Mastrangelo et al., 1991). Therefore additional Sp1 sites can result in increased activity. For example, deletion of one of two tandem Sp1 sites caused a 50% reduction in the citrate synthase promoter activity (Kraft et al., 2005). However, the Sp1 family includes inhibitory factors, such as Sp3. The inhibitory region of Sp3 is positioned at the N-terminus of the protein whereas in Sp1 this is located in front of the zinc-finger domain. This positioning is thought to be the reason for the distinct function of Sp1 and Sp3. However, these proteins bind to the same GC-rich promoter sites (identified in this thesis as Sp1 sites) using Cys2His2 zinc fingers (reviewed in Lin et al., 2004). Thus, an Sp1 site can be inhibitory or stimulatory based on which variants are expressed by the cell. In addition, if Sp3 binds to the promoter, it prevents Sp1 multimers from binding multiple sites, disrupting their synergistic effect. If this is the case, a loss of a Sp1 site could actually result in increased activity. It is striking that there are several examples where a clade generally has 1 Sp1 site, but the largest members of the family possess 2 sites: all rodents except capybara and beaver, all
primates except gorilla, and all carnivores except ursids. Of course, since simple sequence analysis does not reveal whether these sites are active, or how stimulatory Sp1 and inhibitory Sp3 interact \textit{in vivo}, but it is an intriguing pattern that this should be tested experimentally.

\textbf{PGC-1α promoter activity does not follow mitochondrial enzyme scaling patterns}

To assess the proximal promoter strength, I made reporter constructs of 12 rodents with 2 other placental mammals for outgroup comparison using a mouse myoblast line as a common background. Unlike the muscle analysis, I was able to use experimental material from a wider range of rodents, from the largest extant species (capybara) to one of the smallest. Based on the mean of the scaling coefficients I found in COX activity per mg DNA, I would predict a scaling coefficient of -0.15. This means the construct from mouse should have promoter activity that was 3-fold more than capybara. What I found was that there was no significant effect of body size on PGC-1α promoter activity. This suggests that, the PGC-1α proximal promoter has not evolved in a way that is dominated by size effects.

Though I am confident that my results are accurate in this system, they may not be representative of the actual promoter activity \textit{in vivo}, so some caution must be used in interpreting the data. Consider for example the difference in luciferase activity between two mice species. The sequence information reveals that the promoters are essentially the same, but I found the luciferase activity for the reporter constructs differs. There are several possible explanations for experimental variation in my results. Transfection efficiency varies dramatically between cell types and depends on subtle differences in purity of plasmid preparations and the state of the cells used. The efficiency is addressed using a second plasmid to account for transfection efficiency. My transfections were highly repeatable, but the
replicates were performed on the same plasmid preparation. If plasmid purity or heterogeneity was an issue, I could resolve this by using independent plasmid preparations; this is a low copy number plasmid so 100 ml cultures and midipreps were needed to generate enough plasmid at sufficient concentration to avoid a precipitation step. This variation in plasmid preparation quality may be what is driving some of the patterns, as species with promoters that are essentially identical appear to have different activity.

A great deal of effort went into selecting the appropriate cell line to test the PGC-1α promoter reporter constructs. I wanted to use a muscle model, because of my previous focus on muscle. PGC-1α plays a role in the development of oxidative capacity during myogenesis. PGC-1α knockout animals display a 20-30% reduction in muscle mitochondrial content (Leick et al., 2008) and overexpression of PGC-1α in muscle creates an increase in oxidative metabolism (Lin et al., 2002). I also wanted to employ a cell line that was amenable to culture and has demonstrated a mitochondrial biogenesis in response to PGC-1α. Muscle differentiation is accompanied by mitochondrial proliferation, as the energy demands of the cells can increase by at least fivefold (Franco et al., 2008). In the murine muscle cell line C2C12, activities of mitochondrial marker enzymes increase several fold with myogenesis (Moyes et al., 1997; Remels et al., 2009). This mitochondrial proliferation during differentiation appears to be driven by PGC-1α (Chang et al., 2006, Remels et al., 2009). The many myoblasts cell lines display distinct phenotypes. For example, Sol8 and C2C12 are both murine muscle cell lines. Sol8 myoblasts are purported to show a slow-twitch phenotype since they are derived from soleus muscle (Daubas et al., 1988) whereas C2C12 myoblasts show a mixed phenotype (Zebedin et al., 2004). However, the differences between the two lines in terms of oxidative capacity may
be minimal (Lyons et al., 2004). In both lines the expression level of PGC-1α is low in proliferating cells but increases as cells differentiate (Figure 9), similar to changes in COX (Lyons et al., 2004). This means that the appropriate transcriptional factors for PGC-1α expression are available at this time, allowing for increased promoter activity. However, the absolute level of PGC-1α is different between cell lines. Lower constitutive levels in the background mean that a greater change may be seen as cells differentiate, but the constitutive level should be high enough to know that PGC-1α is active in the cells. Therefore it made more sense to use Sol8 cells, the line with higher levels of PGC-1α.

Choosing any particular point during differentiation could also have consequences on the expression patterns shown. For example, the Sp1 to Sp3 ratio changes through myogenesis (Fandos et al., 1999). Since some species have two Sp1 binding sites, this may influence how my reporters respond to myogenesis. By comparing constructs with one or two sites during different stages of differentiation the relative role of background levels of Sp1 on promoter activity could be inferred.

Work with immortalized mammalian myoblasts is restricted to rodent sources, either rat or mouse in origin. Using only one common background could lead to some issues as some rodent constructs would be more closely related to the mouse background than others. The structure of transcription factors tends to be conserved across broad taxonomic comparisons (Hsia and McGinnis, 2003) but there are examples of evolution variation in wider taxa (e.g., Sp1 (Kolell and Crawford, 2002). Likewise transcription factor elements sites appear to be conserved across mammalian species. However, there is a remote possibility of significant effect of evolutionary variation in transcription factors. A greater potential concerns is a
peculiarity in the cell line itself that affects promoter activities (e.g., deficiency or dysfunction in a transcription factor). For these reasons, it would have been useful to employ another cell line (e.g., rat L6 cells) to confirm the results.

**Transcriptional regulators of PGC-1α could play a role in interspecies differences in COX activity**

My data suggest that the promoters do not differ in ways that can account for the differences in COX activity in muscle in relation to body size. This does not preclude a role in regulation of the PGC-1α promoter, as it is likely to be an important master regulator in all mammals. Differences in mitochondrial gene expression may arise from through the transcriptional regulators of PGC-1α. The expression of mitochondrial genes is dependent on both extrinsic control and intrinsic control, most of which feeds through PGC-1α in some manner.

Extrinsic control of mitochondrial gene expression includes external regulatory factors that control the fibre-type contractile phenotypes such as hormones and neurotransmitters, which can also exert effects on metabolic enzymes through crosstalk with signalling pathways. This may include insulin-like growth factors (IGF) and thyroid hormone, both of which are known exert effects on PGC-1α expression (Ircher et al., 2008). Thyroid hormone in particular has been regarded as a major endocrine controller of energy expenditure; its turnover levels and whole body oxygen consumption are allometrically related (Harper and Seifert, 2008). Thyroid hormone is directly involved in control of PGC-1α, but its effects on mitochondrial gene expression are independent of PGC-1α activation (Wulf et al., 2007).
Intrinsic control refers to the contractile phenotype inducing changes within the muscle that alter patterns of gene expression, such as exercise. This can include changing signals such as innervations patterns, IGF and thyroid hormone leading to changes in metabolic phenotype. Either way, changes in the cell environment may be the source of differences in enzyme levels. If this level of regulation is responsible for determining the metabolic phenotype across species, then regardless of the species from which the promoter is derived, the activity will reflect “mouse” levels due solely to the background of mouse myoblasts.

Though these sites may play important roles in guiding the PGC-1α transcriptional response to external stimuli, constitutive expression and presumably interspecies differences appear to be guided by factors located closer to transcription start site (TSS). In large scale analyses of human promoters, a bias towards transcription start site in functional binding is observed (Tabach et al., 2007). In terms of mitochondrial genes encoded by the nuclear genome, promoters have many common features all located relatively close to TSS (Franco et al., 2008). The probability is that binding sites further from start site are less important in terms of constitutive expression patterns. Nonetheless, transcription factors binding to sites located further from TSS may contribute to size-dependent patterns (Tabach et al., 2007). A thyroid response element (TRE) has been identified in the promoter of mice PGC-1α approximate 4 kb upstream from TSS (Wulf et al., 2008). Thyroid hormone is required for the normal function of nearly all tissues and can have major effects on oxygen consumption and metabolic rate. Thyroid hormone triggers effects similar to those of PGC-1α, such as mitochondrial biogenesis, adaptive thermogenesis, and hepatic gluconeogenesis (Yen, 2001). It has been shown to play a direct role in regulation of PGC-1α (Irrcher et al., 2003), while PGC-1α coactivates active thyroid
hormone receptor to create an autoregulatory feed-forward loop of PGC-1α activation (Wulf et al., 2008). The question becomes whether thyroid hormone is guiding basal expression of not only PGC-1α but also mitochondrial content. Thyroid-mediated gene expression patterns are also not completely dependent on PGC-1α activation (Wulf et al, 2007). If this plays a role in interspecies differences it may be able to act on mitochondrial genes independently of PGC-1α. Another example of more distally located site is MEF2 (myocyte enhancer factor). MEF2 proteins are a small family of transcription factors that play pivotal roles in striated muscle differentiation, development, and metabolism. Two sites has been identified approximately 1800 and 2500 bp from the TSS. MEF2c is the MEF2 family member that is most important in skeletal muscle (Handschin et al., 2003); it may also be playing a role in developmental differences between rodents in terms of fibre type. Divergence in the distal promoter made it difficult to make longer constructs in our breadth of species.

**PGC-1α expression does not follow mitochondrial enzyme scaling patterns**

I hypothesized that PGC-1α expression patterns would follow oxidative enzyme activity. Since PGC-1 is under some level of transcriptional control (Cao et al., 2004; Daitoku et al., 2003; Handschin et al., 2003), its expression may be expected to reflect the scaling patterns seen in the genes for mitochondrial enzymes it controls. Since the promoter strength did not vary in relation to body size (with caveats), the next most likely explanation is that the promoter is regulated in ways that leads to differences in expression and steady state mRNA levels. However, the transcript levels of PGC-1α also did not correlate with mitochondrial enzyme activity level across rodent samples. Thus, there is little evidence to this point that the master controller of mitochondrial biogenesis in a species explains the variation in mitochondrial
content between species in relation to body size. The regulators of expression that drive scaling relationships may include the DNA-binding transcription factors that regulate mitochondrial biogenesis in conjunction with PGC-1α, namely NRF-1 and NRF-2 (Wu et al., 1999). It would be useful to extend our studies to include these transcription factors.

Although PGC-1α mRNA levels were not consistent with the overall scaling pattern, they were correlated with differences in mitochondrial content between select taxa. For example, members of the Sciruidae family had higher COX activity and greater PGC-1α transcript levels.

Since neither PGC-1α transcript levels nor promoter activity reflect the differences in mitochondrial content, it would appear that PGC-1 may not be the source of variation, at least on the transcriptional level. It still may be playing a role in regulating interspecies differences.

**Post-translational modifications of PGC-1α**

To this point, I have shown that in the rodent TA and gastrocnemius COX scales when expressed per g tissue and just a bit less so when taking into account differences in myonuclear content. Though transcriptional regulation would appear to be a sensible mechanism by which to achieve the differences between species, neither PGC-1α mRNA or promoter activity can explain the interspecies patterns. PGC-1α may still be responsible for interspecies differences in mitochondrial content even if its expression does not seem to follow scaling trends. Disconnects between mRNA levels and protein levels are certainly possible. Unfortunately, in our lab, PGC-1α levels were not reliably measured via immunoblot of muscle homogenates to an extent where we could compare between species. At this time, we did not feel this approach would yield useful comparative data. Perhaps future studies could include analysis of nuclear extracts of muscles where PGC-1α immunoquantification is more reliable.
Like most coregulators, PGC-1α activity is also strongly post-translationally regulated. Activity may be regulated by phosphorylation by MAPK, p38, AKT and AMPK, deacetylation by SIRT1, arginine methylation by PRMT1, ubiquitination by SCF and interactions with repressors (reviewed in Hock and Kralli, 2009). These modifications influence PGC-1α localization, stability and how it interacts with other proteins, affecting the overall effect on gene expression. There are examples of both positive and negative regulation of PGC-1α post-translationally. For example, several transcription factors including Src-1, SIRT1, LRP130 and PRDM16 have been identified as positive regulators (Cooper et al., 2006; Lagouge et al., 2006) while others such as GCN5 and p160MBP suppress activity (Fan et al., 2004; Lerin et al., 2006). Because some of these factors, such as AMPK and SIRT1, are also involved in sensing metabolic changes it is not surprising that activity may be changed at both a transcriptional as well as posttranslational level. Covalent changes to proteins can be fast-acting responses but may also be reflective of constitutive levels playing a role in maintaining cellular energy balance. Since the important role of PGC-1α appears to be changes in coactivation of its targets in response to different stimuli, PGC-1α expression may not necessarily be changing itself.

Interspecies trends may be regulated by something else in the transcriptional machinery. Modifications in the PGC-1α protein, or in transcription factors it interacts with also affect function. This includes coordination with transcription factors such as YY1. YY1 binds directly to mitochondrial gene promoters and that PGC-1α functions as a transcriptional coactivator for YY1 in an mTOR-dependent manner (Cunningham et al., 2007). Since mTOR (mammalian target of rapamycin) is a kinase that regulates cell growth, size and survival, its physical interaction with PGC-1α-YY1 complex balances energy metabolism through
transcriptional control of mitochondrial oxidative function. It is possible that conditions allowing for interactions between two proteins are guiding interspecies trends rather than simply the expression pattern of PGC-1α. Unfortunately this study does not account for variations in the level of PGC-1α protein. The levels are too low to be measured in our muscle extracts, and we could not generate nuclear extracts from our material.

An important factor to keep in mind is that there are multiple layers of regulation of mitochondrial biogenesis. This includes many transcription factors responsive to many elements. Other transcription factors that work with PGC-1α may correlate more closely with mitochondrial enzyme activity, such as NRF-1. However, none of these transcription factors or coactivators alone appears to be sufficient to regulate the entire set of genes encoding mitochondrial proteins during biogenesis (Franko et al., 2008). When PGC-1α is knocked out, other factors will ensure that mitochondrial biogenesis will still occur in response to stimuli, though efficiency may be compromised (Handschin et al., 2007).

Future directions

Based on these results several other things should be examined to further develop this thesis. This study primarily focuses on the rodent family except in terms of sequence analysis. Despite the fact that the expected scaling pattern was not reflected in promoter activity within the rodent family, this does not discount the possibility that a broader taxa trend may be seen. This was not prioritized for this study as a representative family possessed many of the sequence differences seen across placental mammals. However, expanding the approach to include representatives of multiple families may help to solidify this interspecies trend.
The size-related trends that are seen within both mitochondrial and glycolytic enzymes in rodentia do reflect what has been shown in previous studies across mammals (Emmett and Hochachka, 1982). However, the impact of phylogeny on scaling of enzyme activity has not been firmly established. Determining the importance of relatedness in size specific activity may be another contributing factor outside of myonuclear domain in the patterns seen. In order for this to be done with more conclusiveness an appropriate marker gene must be chosen and sequenced for all of the rodents used for enzyme analysis, as well as an outgroup. Having a non-rodent species of similar size also involved in analysis would also help to solidify trends.

Another problem related to certain species being closer in phylogeny than others involves the primers used for mRNA analysis. The impact of consensus primers being more efficient with certain species than others was considered through dilution series, but not completely determined. Introducing other consensus primer sets would establish whether the trends are due to differences in mRNA level, or interspecies differences in primer efficiency. The housekeeping gene should also be confirmed as being a true marker of constitutive protein expression, again possibly by choosing a second one to confirm the results seen here. This analysis can also be more developed in terms of establishing a mitochondrial marker, such as a gene for one of the subunits of COX that is regulated transcriptionally, as well as establishing a measure of the mRNA levels of the transcription factors itself. Gaining more of an understanding of the transcript levels beyond simply PGC-1α may provide more of a basis for the amount of transcriptional regulation that is going on.

A simple problem to clarify involves the use of only one common background. By using another cell line such as the rat L6, questions about whether certain promoters work better
based on relatedness to the background may be addressed. Further questions about tissue
specific activity may also be addressed by using fibroblasts, to determine if promoter activity is
tissue specific. Because it may be that the background is controlling the activity of the
promoter, using a cell line that has a different set of demands may yield a very different
answer. Certain factors such as MyoD will play a crucial role in myoblast differentiation, but
may not be as predominant in fibroblasts, resulting in different constitutive patterns.

Constitutive expression is likely regulated by the suite of possible binding sites located
close to TSS as discussed about. However, looking into the possibilities further from this point
may again help us understand interspecies differences in transcriptional regulation. This may be
as simple as sequence analysis of the more distal promoter, or possibly similar promoter
analysis with the rodent family. Another unknown in this study is whether the identified sites
are actually playing a role in transcriptional regulation in all the species examined. Identifying
which of these putative sites are actually binding the expected transcription factors could both
clarify which are true sites and which are important for constitutive activity. The specific role of
each transcription factor in guiding interspecies differences can also be examined through site-
specific mutagenesis. This could essentially make a guinea pig look more like a capybara in
terms of number of putative Sp1 sites, seeing if variance in promoter activity can be explained
as simply as this. Though from this study it seems unlikely that changes in PGC-1α promoter
activity are guiding size related differences in mitochondrial content, it does not discount the
importance of understanding whether different mammals utilize the same suite of transcription
factors for both constitutive and inducible expression.
Conclusions

This thesis examined whether transcriptional regulation of PGC-1α was a factor in interspecies differences of mitochondrial content. Mitochondrial enzyme activity scaled negatively with body mass in lower limb muscles of rodents while glycolytic activity followed a positive trend. Myonuclear domain accounts for the variation in the degree of scaling between rodent muscle enzyme activity, but does not completely explain interspecies scaling trends. As master controller of mitochondrial biogenesis in mammals, PGC-1α appeared to be a candidate in guiding interspecies trends. However, PGC-1α mRNA expression and promoter activity did not follow the scaling pattern, indicating that interspecies differences in mitochondrial content may not be dependent on this level of transcriptional regulation. Though PGC-1a is an important regulator of mitochondrial content within a species, it may not be guiding interspecies trends.
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