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Commentary

Controlling muscle mitochondrial content

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Summary

Mitochondrial content, a chief determinant of aerobic capacity, varies widely among muscle types and species. Mitochondrial enzyme levels in vertebrate skeletal muscles vary more than 100-fold, from fish white muscle to bird flight muscles. Recent studies have shed light on the transcriptional regulators that control mitochondrial gene expression in muscle fiber differentiation and development, and in the context of pathological conditions such as neuromuscular disease and obesity. While the transcriptional co-activator PGC-1α (peroxisome proliferator-activated receptor gamma co-activator 1) has emerged as a *master controller* of mitochondrial gene expression, it is important to consider other mechanisms by which coordinated changes in mitochondrial content could arise. These studies, largely using biomedical models, provide important information for comparative biologists interested in the mechanistic basis of inter-species variation in muscle aerobic capacity.

Key words: skeletal muscle, oxidative phosphorylation, energy metabolism, peroxisome proliferator-activated receptor (PPAR), PPAR gamma coactivator 1 (PGC-1).

Introduction

Aerobic metabolism depends on the capacity of mitochondria to generate ATP at rates sufficient to meet the energetic demands of tissues. Metabolic regulation of enzymes allows cells to use the existing pool of mitochondria to match energy production to energy demand, even under rapidly changing conditions. Cells can regulate energetics by altering the structural architecture of mitochondria, such as the dynamics of the mitochondrial reticulum (Bach et al., 2003). The efficiency of the mitochondrial metabolism can also be altered, without inducing global changes in mitochondrial structure or enzymology (Harper and Himms-Hagen, 2002). For example, changes in the magnitude of the proton leak can influence the amount of energy used by mitochondria to maintain the proton motive force, an important contribution to resting metabolic rate (Cadenas et al., 2001). When changes in energy demands persist for long periods, most cells can respond by modifying the rates of mitochondrial biogenesis to induce compensatory changes in mitochondrial capacity. Modulation of muscle mitochondrial content begins early in embryonic development, when muscle precursors differentiate and diverge to form distinct fiber types. Muscle mitochondrial content remains plastic throughout adulthood, increasing in response to hypermetabolic conditions, or decreasing with periods of reduced activity. Mitochondrial content of homologous muscles varies widely between species in relation to body size and activity levels. Taking into consideration the entire scope of variation between tissues and species, mitochondrial content varies by at least two orders of magnitude among vertebrate muscles (Fig. 1). The scope of this variation can be attributed to muscle specializations, allometric scaling, and inter-species differences in activity levels. Recent studies in control of gene expression have provided insight into how mitochondria are built and how their levels are altered during development and in response to environmental cues (reviewed in Moyes and Hood, 2003). Comparative biochemists, physiologists and cell biologists can use this information to identify targets that might contribute to evolutionary variation.

The process of building, maintaining and modifying muscle mitochondria is very complex. Global changes in mitochondrial content require exquisite coordination of hundreds of different genes located in the nucleus, in parallel with the genes encoded by mitochondrial DNA (mtDNA). Synthesis of the appropriate amounts of mitochondrial proteins also requires coordination of protein synthesis in both the cytoplasm and mitochondria. Furthermore, most enzymes within mitochondria change, more or less, in parallel to maintain enzyme ratios or stoichiometries. For example, mitochondrial content differs almost tenfold between red and white muscles of fish, yet the ratios of mitochondrial enzymes are nearly identical (Leary et al., 2003). How are complex pathways altered, while retaining intrinsic stoichiometries in enzyme levels?

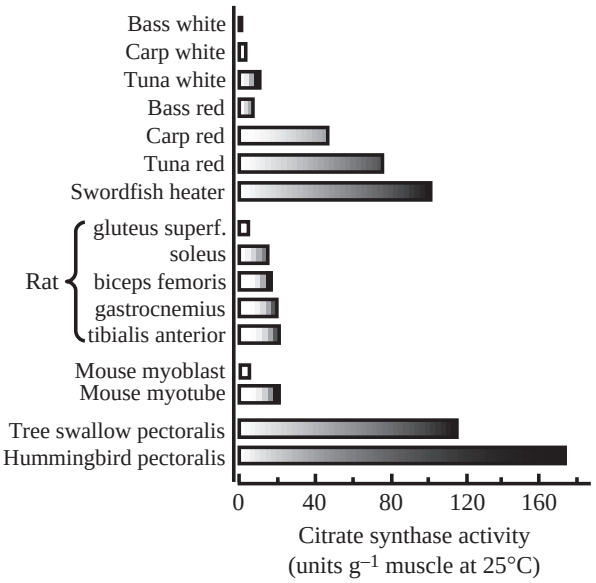

Fig. 1. A survey of the levels of the mitochondrial enzyme citrate synthase found in skeletal muscles of vertebrates. Values were obtained from the literature for tree swallows *Tachycineta bicolour* (Burness et al., 2001) rufous hummingbird *Selasphorus rufus* (Suarez et al., 1991), carp *Cyprinus carpio* (Moyes et al., 1992), skipjack tuna *Katsuwanis pelamis* (Moyes et al., 1992), swordfish (Tullis et al., 1991) and cultured mouse myoblasts (Moyes et al., 1997). Values for rat muscles, largemouth bass *Micropterous salmoides* are unpublished. All values are standardized to 25°C using a Q_{10} of 1.9 for citrate synthase (Moyes et al., 1992).

PGC-1 as a master regulator of mitochondrial gene expression

The genetic control of the glycolytic pathway illustrates one way in which cells maintain enzyme stoichiometries in complex pathways. When oxygen becomes limiting, hypoxia-inducible factor 1α (HIF1α) is stabilized, leading to coordinated activation of genes for glycolytic enzymes, glucose transporters and even enzymes involved in synthesis of oxygen transport proteins (Semenza, 1998). Other transcriptional regulators also contribute to the control of glycolytic genes, but HIF-dependent regulation is essential for mounting coordinated responses. In the world of mitochondrial gene expression, there is growing evidence to suggest that the transcriptional regulator PGC-1α (peroxisomal proliferator-activated receptor gamma coactivator-1α) may be the *master controller* of mitochondrial biogenesis. In conjunction with a network of transcription factors, PGC-1α helps to coordinate the expression of genes involved in aerobic energy metabolism. Many nuclear genes encoding mitochondrial enzymes are directly responsive to PGC-1α and the transcription factors with which it interacts. The PGC-1α axis also stimulates expression of other transcription factors involved in coordinating mitochondrial genes, such as nuclear respiratory factor 1 (NRF-1) and NRF-2, which control the expression of regulators of mtDNA transcription and replication (see Scarpulla, 2002; Puigserver and Spiegelman, 2003). While most recent studies extend the role of PGC-1α (reviewed by Knutti and Kralli, 2001; Berger and Moller, 2002; Puigserver and Spiegelman, 2003), particularly in the context of metabolic disorders (e.g. Mootha et al., 2003), it is important to recognize that other transcriptional and translational regulators can play important roles in governing mitochondrial content.

PGC-1α stimulates transcription by acting as a transcriptional coactivator. It does not bind DNA directly, but attaches to transcription factors already bound to DNA at specific recognition sequences, or elements (Fig. 2). PGC-1α binds heterodimers of transcription factors from the nuclear hormone receptor (NHR) family, including the retinoic acid receptor (RxR), the thyroid receptor (TR), and peroxisome-proliferator activated receptor (PPAR) (see reviews by Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003). PGC-1α possesses an N-terminal leucine-rich region that binds to the NHR. Both RxR and TR must bind their ligands (retinoic acid and thyroid hormone, respectively) before they can bind PGC-1α. However, PGC-1α can bind PPARγ even if no ligand is present. PGC-1α binds the heterodimer then undergoes a conformational change that allows it to act as a docking site for other proteins. These proteins (e.g. SRC-1, CBP/p300) remodel chromatin and help assemble the general transcriptional machinery (Puigserver et al., 1999). Although first identified as a coactivator of PPAR responsive genes, PGC-1α has since been shown to interact with other transcription factors, such as myocyte enhancer factor 2c (MEF2c) (Micheal et al., 2001) and NRF-1 (Wu et al., 1999). These interactions involve regions of PGC-1α that are distinct from the domains involved in binding NHR. In addition to its role as a transcriptional coactivator, PGC-1α also has a post-transcriptional role, participating in mRNA processing and export (see Knutti and Kralli, 2001). These transcriptional and post-transcriptional responsibilities place PGC-1α at the center of regulatory pathways controlling genes for proteins of aerobic metabolism.

Each element of the PGC-1α axis (ligands, NHRs, PGC-1α) can, under the right conditions, stimulate many aspects of mitochondrial biogenesis. PGC-1α levels correlate with muscle mitochondrial content in both fiber type comparisons (Lin et al., 2002a) and adaptive responses to exercise training (Pilegaard et al., 2003). Increasing PGC-1α levels directly, using transgenic mice or transfected cells, increases mitochondrial content (Lehman et al., 2000; Wu et al., 1999). The levels of the NHR and its ligands can also influence mitochondrial gene expression. During fasting, increases in both PPARα, and its putative ligand (fatty acids) trigger an increase in enzymes of mitochondrial β-oxidation (Leone et al., 1999; Kersten et al., 1999). This could also be the mechanism by which high fat diets stimulate muscle mitochondrial biogenesis, although the physiological ligands for PPARs remain uncertain (Michalik et al., 2003; Puigserver and Spiegelman, 2003). Since the ability to stimulate transcription by these NHR requires heterodimerization and DNA-binding, it can be regulated by competition for binding partners and

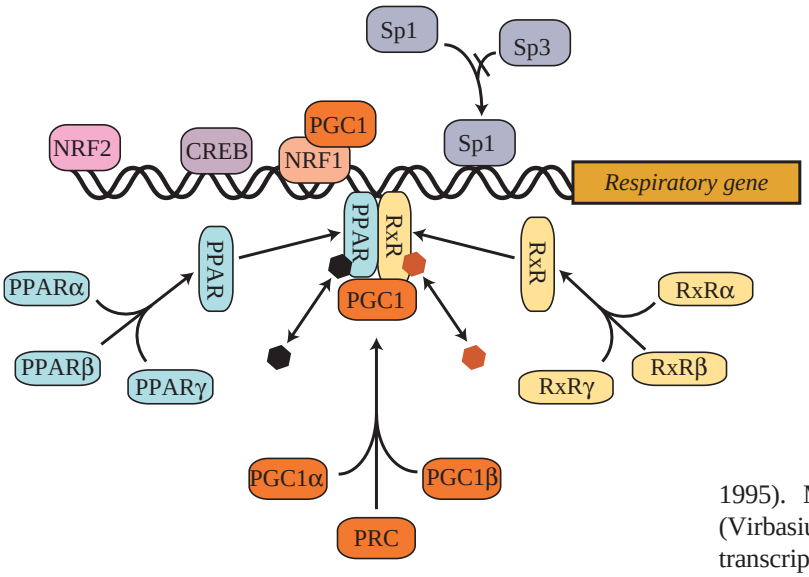

Fig. 2. Transcriptional regulators of a typical nuclear-encoded respiratory gene. Mitochondrial genes typically lack TATA boxes and constitutive expression is usually mediated by the specificity protein 1 (Sp1) family of transcription factors. The promoters for mitochondrial genes often possess binding sites for CREB, PPAR, NRF-1 and NRF-2. The members of the mammalian PPAR and RxR families are shown but the extent of the family in other vertebrates is not yet established. Not every tissue expresses each of the RxR and PPAR family members. Hexagons represent the specific agonists for the nuclear hormone receptors.

DNA elements. Interestingly, each of these ligand-receptor combinations (PPAR agonists/PPAR, retinoic acid/RxR, thyroid hormone/TR) can induce transcriptional changes in mitochondrial proteins (e.g. Barbe et al., 2001). However, the interactions between these receptors are very complex. For example, PPAR selectively inhibits the effects of TR by competing for RxR (Juge-Aubry et al., 1995) and TR can, in turn, inhibit PPAR effects on transcriptional activation (Miyamoto et al., 1997). The complexity of this PGC-1α axis allows animals to create distinct tissue-specific metabolic properties and mount responses to metabolic challenges. It also presents a challenge to comparative biologists exploring the genetic basis of inter-species variation in aerobic metabolism.

How might evolutionary variation in the PGC-1 axis account for differences in mitochondrial content between animals?

While the PGC-1α axis may be central to changing mitochondrial content in individuals, it is not clear if it can account for differences between species. Inter-specific variation is evident in relation to activity levels (e.g. athletic *vs* sedentary species) or body size (i.e. allometric scaling) (e.g. Weibel et al., 1992). In principle, an active species could achieve an elevated muscle mitochondrial content by targeting the same effectors that mediate changes within an individual. However, evolution also allows for the possibility of genetic variation in the regulatory elements of the respiratory gene promoters and structural changes in the transcriptional regulators themselves. Evolutionary variation in transcriptional activity is usually attributed to variations in the *cis*-binding elements (i.e. the promoters) rather than the structure of the transcription factors themselves (see Hsia and McGinnis, 2003). Promoter variation can include changes in the sequence of transcription factor binding sites, their number and their organization within the promoter. For example, the PPAR responsive element is a highly conserved direct repeat (AGGTCANAGGTCA), but the elements surrounding this site influence the ability of the transcription factor to bind the element (Wahli et al., 1995). Multiple copies of elements, such as NRF-2 sites (Virbasius and Scarpulla, 1994), can increase the potency of the transcription factor. In general though, the case for promoter variation as a route of evolutionary variation in muscle mitochondrial content is not very compelling.

PGC-1 variation

PGC-1α has multiple distinct binding sites for unrelated transcriptional regulators. The N-terminal leucine-rich NHR-binding motif of PGC-1α resembles that of other proteins which bind NHR (Glass and Rosenfeld, 2000). It possesses unique structural features that bind other transcription factors, such as NRF-1 and MEF2c. Its C-terminal RNA-binding motif resembles other proteins that process mRNA. The structural motifs within PGC-1α suggest an intriguing evolutionary history that probably involved fusion of functional domains of different genes. In fact, very little is known of the evolutionary history of PGC-1α. Since it was first reported (Puigserver et al., 1998), two other coactivators have been identified that share structural features with PGC-1α: PGC-1 related coactivator (PRC) (Andersson and Scarpulla, 2001) and PGC-1β (Lin et al., 2002b). Only PGC-1α has been studied in detail, but the other members of the family appear to have similar capacities, although distinct developmental profiles and inter-tissue distributions (see Knutti and Kralli, 2001). For example, both PGC-1α and PGC-1β induce mitochondrial proliferation but there are subtle differences in the kinetics of proton leak (St Pierre et al., 2003). The evolutionary origins of the gene family are also not yet established. Zebrafish and *Xenopus* have expressed sequence tags (ESTs) or genes that show superficial similarities to PGC-1α in either the N-terminal or C-terminal regions (see Puigserver and Speigelman, 2003). Complete phylogenetic analyses have not yet been reported but it will be interesting to see the relationship between the origins of the ancestral PGC-1 gene, the evolutionary diversification the PGC-1 gene family, and the diversification of muscle fiber types in tetrapod evolution.

Transcription factor levels and properties

Evolutionary variation in transcription factor structure is a bit of a paradox. Since each individual transcription factor may

regulate hundreds of genes, even subtle variations could have broad ramifications for gene expression. For this reason, it has been argued that mutations in transcription factors would have generally deleterious effects on integration of gene expression, and would not be advantageous in the context of natural selection (see Hsia and McGinnis, 2003). The very existence of transcription factor subfamilies, however, argues that such subtle structural variations can be fixed within populations, although gene duplications may be a necessary precondition. Rather than disrupting genetic and metabolic integration, the subtly different transcription factors within a family can provide the organisms with a degree of developmental and physiological flexibility.

The diverse members of the NHR family probably arose from a single gene early in metazoan evolution. Each lineage evolved distinct transcription factors, although each NHR retains sufficient structural homology to be recognized as a member of the family that arose more than 400 million years ago. For example, the vertebrate RxR most closely resembles the invertebrate *Ultraspiracle* gene product, which binds juvenile hormone III (Jones and Sharp, 1997). Both share a role in developmental regulation, but respond to completely different regulatory ligands. While there is clearly great structural similarity in these transcription factors, they mediate very different processes at the cellular level. Even the multiple members of individual receptors, such as RxR and PPAR subfamilies, possess distinct cellular roles despite very high degrees of homology. Consequently, it is not beyond the realm of possibility to imagine that a transcription factor variant might contribute to interspecies differences in mitochondrial content in specific tissues, such as skeletal muscle. On a micro-evolutionary scale, population-level polymorphisms provide clear examples of how even single amino acid variations can alter transcription factor function. For example, PPARγ exists as two allelic variants that differ in position 12. People with the Ala_{12} allele have a lower body mass index and lower risk of type 2 diabetes. When recombinant constructs of the two PPAR alleles were compared, the ALA_{12} variant had lower DNA binding activity (Beamer et al., 1998). Another PPAR polymorphism ($PRO_{115}GLN$) is adjacent to a regulatory serine residue (SER_{114}) (Hu et al., 1996), and correlates with obesity (Ristow et al., 1998). Finally, an increase in a severe type of type 2 diabetes was associated with two other polymorphisms ($PRO_{467}LEU$, $VAL_{290}MET$). The effects of these polymorphisms were dominant, exerting negative effects on people who where heterozygous. Collectively, the studies on allelic variation demonstrate how subtle changes in structure could alter metabolic properties, manifesting a phenotype that could be subject to natural selection.

Mitochondrial content can also be controlled by post-transcriptional processes

While I have focused on the potential role of transcriptional regulators, it is important to recognize the difficulties in using transcriptional strategies as a route of achieving evolutionary variation in muscle mitochondrial content. Evolutionary changes in PGC-1α efficacy, for example, could affect every tissue in which PGC-1α is expressed. Genetic control of mitochondrial content faces major challenges in terms of coordinating hundreds of genes in parallel. Consequently, there is an important potential role for a mechanism that can lead to a coordinated alteration in the levels of functionally related proteins. This would obviate a change in the constitutive expression, and preserve the inherent stoichiometries. Post-transcriptional mechanisms in particular may more readily account for differences in mitochondrial content in physiological and evolutionary contexts.

Translational effectors

One mechanism to increase mitochondrial enzyme synthesis is to target the translational processes. Translational control is important in many different cell types, influencing both global translation and mRNA-specific translation (Gray and Wickens, 1998; Wilkie et al., 2003). Many nutrients exert their effects on protein synthesis through the mTOR (mammalian target of rapamycin) signalling pathway that targets the translational machinery, including initiation factors, elongation factors, their respective binding proteins and the small ribosomal subunit (S6) (see Proud, 2002). For example, iron-dependent gene expression exerts both specific and global controls on expression in many tissues, particularly hemopoietic tissues such as erythroblasts (Torti and Torti, 2002; Templeton and Liu, 2003). Iron-dependent repression of translation is probably responsible for the coordinated reduction in mitochondria during erythrocyte aging (Moyes et al., 2002). Mechanisms acting through global translational control have the potential to alter the levels of proteins, without any change in transcriptional regulation or the inherent stoichiometric relationships. For many individual mRNA species, information in the 3′ and 5′ untranslated regions controls the efficiency of translation (see MacDonald, 2001; Wilkie et al., 2003). The best example of this type of regulation is cytoplasmic polyadenylation. A longer $poly(A)^+$ tail is better able to recruit the $poly(A)^+$ binding protein, which is necessary to initiate translation.

While translational control has been shown to be important in many contexts, its potential to explain inter-species differences in mitochondrial content remains largely unexplored. The inherent differences in cytochrome oxidase activities in Antarctic and polar eelpout, for example, appear to be due to differences in translation, rather than transcription (Hardewig et al., 1999).

Organelle stabilization

Mitochondria and mitochondrial macromolecules possess discrete half-lives within cells. Proteins, lipid and DNA are readily damaged by oxidative stress in particular. Superoxide production at electron transport system (ETS) Complexes I and III can initiate a cascade of cytotoxic reactive oxygen species (ROS) production, enhanced by metals that accelerate Haber–Weiss reactions. Mitochondria can mitigate oxidative

damage using anti-oxidant defence pathways including ROS scavengers (e.g. thioredoxin, cytochrome *c*) and anti-oxidant enzymes (e.g. glutathione peroxidase, glutathione-S-transferase, Mn^{2+} superoxide dismutase). Some damaged macromolecules can be repaired directly within mitochondria. For example, mitochondria possess molecular chaperones, such as Hsp60 and Grp75, that catalyze the folding of mitochondrial proteins. Cells can mount a mitochondria-specific heat shock response (Zhao et al., 2002) and cells with enhanced mtHsp60 levels are more tolerant of oxidative damage (Cabiscol et al., 2002). Once proteins become irreversibly damaged, they are degraded by proteases located in each mitochondrial compartment. The Krebs cycle enzyme aconitase, with its four iron–sulfur centers, is one of the most sensitive enzymes in mitochondria. The mitochondrial Lon protease is very effective at detecting and degrading mildly damaged aconitase (Bota and Davies, 2002). Despite the anti-oxidant protection, repair chaperones and macromolecular degradation pathways, mitochondria inevitably accrue damage. Once individual mitochondria or regions of mitochondria accumulate enough damage, they are targeted for degradation. The mitochondria fragment, then depolarize to trigger autophagy (Elmore et al., 2001). These multifaceted quality control pathways ensure optimal mitochondrial function, but they can also be used during periods of active remodeling of cellular energetics. Acute reductions in energy demand can cause relatively rapid reductions in mitochondrial content. For example, anti-hypertensive treatment of spontaneously hypertensive rats can lead to a 30% reduction in mitochondrial content of the left ventricle within 10 days (Leary et al., 2002). In principle, animals could increase the mitochondrial content of a tissue by enhancing the half-life of mitochondrial macromolecules and the organelle itself. Comparisons of muscles from different tissues (Leary et al., 2003) and species (see Beckman and Ames, 1998) show that anti-oxidant enzyme levels tend to covary with mitochondrial capacity. However, the relative levels of the other aspects of quality control pathways have not been explored.

Models for studying evolutionary variation in energetics

The increasing amount of genetic and genomic information has presented comparative biologists with many opportunities to explore long-standing questions at a new level of sophistication. It is important to recognize that the considerations that go into the choice of experimental model have changed. Animals such as *Drosophila* may not be spectacular athletes but they are useful models for studying mitochondrial biogenesis because of the spectrum of genetic data, ease of genetic manipulation, sequenced genome, commercial microarrays, and databases that integrate mitochondrial bioinformatics (e.g. Mitodrome; Sardiello et al., 2003). While comparative physiologists are increasingly reliant on 'phylogenetically correct' comparisons, the field of evolutionary and developmental biology (Evo-Devo) relies extensively on broad inter-species comparisons (e.g. yeast, flies, worms, *Xenopus*, zebrafish, mice). These broad comparisons are useful for assessing evolutionary relationships because of the relative conservation of genetic mechanisms, such as transcription factor structure–function relationships. The study of the evolution of energy metabolism can effectively draw upon both the traditional closely related comparative physiological models and traditional developmental models. However, many of the models that have been used to great advantage in comparative physiological studies of energy metabolism may prove to be too intractable to explain inter-species variation in energetics at the genetic level. Comparative analyses of distantly related species that differ in activity level or body size (e.g. Weibel et al., 1992) are prohibitively complicated by genetic and genomic variation. Even studies comparing closely related species must be cognisant of the complexity that can arise after even a few thousand years of natural selection. Consider, for example, the many lineages of fish and frogs that have experienced recent genome duplications, resulting in total or partial polyploidy.

With the growing awareness of the importance of considering phylogenetic relationships, many researchers focus on intra-species variations that have arisen since relatively recent geographic isolation events. Since mtDNA sequence variation is often used to distinguish closely related populations and species, it is possible to assess the impact of the variants on mitochondrial function (reviewed in Moyes and Hood, 2003). There are relatively few model systems where clear differences in mitochondrial content arise among populations of a given species, without the complications associated with physiological and phenotypic plasticity. For example, wing polymorphic insects can display profound population-level variation in flight muscle bioenergetics but these differences in energetics are likely to be secondary to developmental differences in life history strategies (Winchell et al., 2000). Garland has used artificial selection to establish lines of mice that exhibit profoundly different levels of voluntary exercise; active lines exhibit about 20% more mitochondrial enzymes than do sedentary lines, but both active and sedentary lines elevate mitochondrial content to the same extent with exercise training (Houle-Leroy et al., 2000). Thus, even population-level variation in the constitutive expression of respiratory genes does not preclude the ability to induce further increases with training. The genetic basis of population-level variation in mitochondrial content in this powerful model remains unclear.

Much of the previous discussion has focused on the role of developmental studies, inter-tissue comparisons, and pathophysiology in highlighting potential steps at which natural selection could act. Advances in biomedical sciences can have tremendous implications for comparative physiologists studying essentially evolutionary variation. Because of the role of mitochondria in numerous pathological conditions, our understanding of mitochondrial genetics is greatly aided by studies involving targeted mutations in

transgenic mice. Transgenic mice can also be used to form reasonable, testable hypotheses addressing the genetic basis of evolutionary variants. Of the many lines of transgenic mice that exhibit elevated mitochondrial enzyme levels, the effects of most transgenes can be attributed to PGC-1α directly, or indirectly through its regulators such as CamK (Wu et al., 2002). Other transgenic mice that exhibit increased mitochondrial enzymes are not so clearly linked to PGC-1α. Myogenin overexpressing mice (Hughes et al., 1999) have elevated slow muscle mitochondrial content. Knockout mice lacking muscle-specific adenine nucleotide translocase demonstrate proliferation of mitochondria (Graham et al., 1997). Mice overexpressing muscle-specific lipoprotein lipase also show increased mitochondrial content, with enhanced capacity for fatty acid oxidation (Hoefler et al., 1997). In many of these transgenic studies, it is important to note that the mice often exhibited mild or severe myopathies and mitochondrial abnormalities. This, of course, has important ramifications for studying the genetic basis of evolutionary variation in mitochondrial biogenesis. Any genetic change must lead to a coordinated increase in mitochondrial enzymes that is functionally beneficial and limited to specific tissues. The answer may lie in evolutionary variation in specific transcriptional regulators, but it is important to keep in mind the potential role of other process, such as post-transcriptional regulation.

Abbreviations used

EST, expressed sequence tag
ETS, electron transport system
HIF, hypoxia inducible factor
MEF, myocyte enhancer factor
mtDNA, mitochondrial DNA
mTOR, mammalian target of rapamycin
NHR, nuclear hormone receptor
NRF, nuclear respiratory factor
PPAR, peroxisome proliferator activated receptor
PGC, PPAR gamma coactivator
PPRE, PPAR response element
RxR, retinoic acid receptor
ROS, reactive oxygen species
Sp1, specificity protein 1
TR, thyroid hormone receptor.

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