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PGC-1 Coactivators Regulate MITF and the Tanning Response

Jonathan Shoag[1,4], **Rizwan Haq**[5,2], **Mingfeng Zhang**[3], **Laura Liu**[1], **Glenn C. Rowe**[1], **Aihua Jiang**[1], **Nicole Koulisis**[1], **Caitlin Farrel**[1], **Christopher I. Amos**[7], **Qingyi Wei**[7], **Jeffrey E. Lee**[8], **Jiangwen Zhang**[6], **Thomas S. Kupper**[3], **Abrar A. Qureshi**[3], **Rutao Cui**[9], **Jiali Han**[3,10,12], **David E. Fisher**[5,2,11,12], and **Zoltan Arany**[1,11,12,*]

[1]Cardiovascular Institute, Beth Israel Deaconess Medical Center Harvard Medical School, Boston, MA 02115, USA

[2]Department of Dermatology, Massachusetts General Hospital Harvard Medical School, Boston, MA 02115, USA

[3]Departments of Medicine and Dermatology, Brigham and Women's Hospital Harvard Medical School, Boston, MA 02115, USA

[4]Division of Health Sciences and Technology, MIT and Harvard Medical School, Cambridge, MA 02139, USA

[5]Massachusetts General Hospital Cancer Center, Boston, MA 02114, USA

[6]Research Computing, Faculty of Arts and Sciences, Harvard University, Cambridge, MA 02138, USA

[7]Department of Epidemiology MD Anderson Cancer Center, The University of Texas, Houston, TX 77030, USA

[8]Department of Surgical Oncology MD Anderson Cancer Center, The University of Texas, Houston, TX 77030, USA

[9]Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA

[10]Department of Epidemiology, Harvard School of Public Health, Boston, MA 02115, USA

[11]Harvard Stem Cell Institute, Cambridge, MA 02138, USA

SUMMARY

The production of pigment by melanocytes tans the skin and protects against skin cancers. UV-exposed keratinocytes secrete α-MSH, which then activates melanin formation in melanocytes by inducing the microphthalmia-associated transcription factor (MITF). We show that PPAR-γ coactivator (PGC)-1α and PGC-1β are critical components of this melanogenic system in melanocytes. α-MSH signaling strongly induces PGC-1α expression and stabilizes both PGC-1α and PGC-1β proteins. The PGC-1s in turn activate the MITF promoter, and their expression correlates strongly with that of MITF in human melanoma cell lines and biopsy specimens. Inhibition of PGC-1α and PGC-1β blocks the α-MSH-mediated induction of MITF and melanogenic genes. Conversely, overexpression of PGC-1α induces pigment formation in cell culture and transgenic animals. Finally, polymorphism studies reveal expression quantitative trait

*Correspondence: zarany@bidmc.harvard.eduhttp://dx.doi.org/10.1016/j.molcel.2012.10.027.
[12]These authors contributed equally to this work

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loci in the PGC-1β gene that correlate with tanning ability and protection from melanoma in humans. These data identify PGC-1 coactivators as regulators of human tanning.

INTRODUCTION

Skin is the most common site of cancer in humans, and skin color and ease of tanning are the best predictors of skin cancer (Lin and Fisher, 2007). Melanin, produced by melanocytes, absorbs UV radiation and protects skin cells from UV-induced damage. Understanding how pigmentation occurs is therefore critical. Melanins derive from tyrosine and are primarily produced by melanocytes. Tyrosinase is the rate-limiting enzyme in this process, and humans with defective tyrosinase are albino. Melanin is packaged in the melanosomes, secreted, and internalized by adjacent keratinocytes, where it is positioned on the sun-exposed side of the nucleus, thereby affording it protection from damaging UV radiation.

The molecular mechanisms by which melanin formation is induced in response to UV radiation are beginning to be understood. UV sensing occurs in the keratinocyte, where DNA damage caused by UV is sensed in a P53-dependent fashion, leading to the secretion of the peptide α-MSH (Cui et al., 2007). α-MSH then engages the Gs-coupled melanocortin 1 receptor (MC1R) on melanocytes, activating cyclic AMP (cAMP) cascades and inducing the lineage-restricted melanocyte regulator MITF, a key transcriptional regulator of multiple enzymes involved in melanogenesis (Levy et al., 2006). The regulation of *MITF* is complex and incompletely understood. cAMP pathways impinge on the cAMP responsive element binding protein (CREB), which strongly activates *MITF* expression. The melanocyte-specific transcription factor SOX10 is required in conjunction with CREB for optimal *MITF* expression and provides lineage specificity (Huber et al., 2003). A number of other pathways, including the RAF/MAPK systems, also impinge on *MITF* expression (Saha et al., 2006). The importance of fully understanding the regulation of *MITF* is additionally underscored by its key role in melanocyte differentiation and survival, as well as in melanoma carcinogenesis (Garraway et al., 2005; Levy et al., 2006).

PGC-1 proteins are transcriptional coactivators that potently regulate mitochondrial biogenesis and other metabolic programs in numerous tissues (Handschin and Spiegelman, 2006; Rowe et al., 2010). They form a small family, including PGC-1α, PGC-1β, and the more distant cousin, PRC. PGC-1α and PGC-1β coactivate a large number of transcription factors, including most of the nuclear receptors, and thereby play important roles in numerous metabolic processes in different tissues. PGC-1α, for example, activates gluconeogenic genes in the liver in response to fasting (Yoon et al., 2001), angiogenesis in skeletal muscle in response to exercise (Chinsomboon et al., 2009), and thermoregulation in brown adipose tissue in response to cold (Puigserver et al., 1998). The function of PGC-1β significantly overlaps with that of PGC-1α, but PGC-1β also has unique functions that include the regulation of lipid handling in the liver (Lin et al., 2005; Wolfrum and Stoffel, 2006) and immune modulation in macrophages (Vats et al., 2006). The PGC-1 coactivators are thus well-established regulators of broad genetic programs in numerous tissues. No role for the PGC-1 s has yet been described in the skin.

We show here that α-MSH signaling impinges on the PGC-1s, which activate expression of the *MITF* gene, which in turn mediates the induction of *TYROSINASE* and melanogenesis in melanocytes. Human studies reveal a correlation between tanning ability and expression quantitative trait loci (eQTLs) in the *PGC-1β* gene. These studies thus provide insight into the complex molecular regulation of human tanning.

RESULTS

PGC-1α Induces Melanogenesis

While studying the role of PGC-1α in tumor metabolism, we noticed that over-expression of PGC-1α in B16-F10 melanoma cells rendered the cells darker. Cells with stable integration of retrovirus expressing PGC-1α were visibly hyperpigmented, both under light microscopy (Figure 1A, right panels) and when pelleted (left panels). Conversely, cells infected with lentivirus expressing short hairpins against PGC-1α became markedly hypopigmented (Figure 1D). Melanoma cells originate from melanocytes, which produce the dark melanin pigment in the skin. Tyrosinase converts tyrosine to DOPA and dopaquinone and is the rate-limiting enzyme of melanin synthesis (Lin and Fisher, 2007). Cells overexpressing PGC-1α markedly increased both Tyrosinase enzymatic activity (Figure 1B) and expression of the *Tyrosinase* gene (Figure 1C). Conversely, *Tyrosinase* gene expression and enzymatic activity were markedly diminished in cells in which PGC-1α was knocked down (Figures 1E and 1F). PGC-1α thus regulates *Tyrosinase* expression and activity in these cells.

PGC-1 Coactivators Induce *MITF* Expression

Initial experiments failed to show that PGC-1α regulates the *Tyrosinase* promoter directly (data not shown). The expression of *Tyrosinase* and other melanogenic enzymes is controlled by MITF, a transcription factor thought to be the “master regulator” of both melanocyte differentiation and pigment production (Lin and Fisher, 2007). Overexpression of PGC-1α induced the expression of the melanocyte-specific form of *Mitf* (*Mitf-M*) in B16-F10 cells (Figure 2A), as well as the expression of the well-defined Mitf target gene *Trpm1* (Miller et al., 2004). Conversely, PGC-1α knockdown by short hairpin RNA (shRNA) significantly reduced *Mitf-M* expression (Figure 2B). Repression of PGC-1α similarly led to marked reductions in *MITF-M* expression and MITF protein in a number of human melanoma cells (Figure 2C; Figures S1A–S1D available online) and in human primary melanocytes isolated from discarded foreskins (Figure 2D).

The *MITF-M* promoter is regulated by a number of transcription factors, including SOX10 and CREB (Lin and Fisher, 2007). Cotransfection into UACC-257 cells of an *MITF-M* promoter-luciferase construct with a plasmid expressing SOX10 led to activation of the *MITF-M* promoter (Figure 2E), as has been shown elsewhere (Huber et al., 2003). The addition of a plasmid expressing *PGC-1*α further induced activity of the *MITF-M* promoter 5-fold (Figure 2E). The activity of the *MITF-M* promoter can also be further activated by PAX3, MAPK, or β-catenin (Lin and Fisher, 2007). In all of these cases, the addition of PGC-1α induced *MITF-M* promoter activity approximately 5-fold (Figures S1E–S1G). Dominant-negative inhibition of CREB, which is known to interact with and be critical for SOX10 activity (Huber et al., 2003), abolished all increased activity (Figure 2F). PGC-1α is known to bind to and coactivate the SOX10 family member SOX9 in chondrocytes (Kawakami et al., 2005). Together, these data suggested that PGC-1α coactivates SOX10 on the MITF-M promoter. Consistent with this, PGC-1α and SOX10 could be immunoprecipitated in a complex (Figure S1H). Furthermore, chromatin immunoprecipitation (ChIP) studies revealed that PGC-1α binds the *MITF-M* promoter near the well-established SOX10-binding site, near the CRE site, and near the transcriptional start site (which may reflect looping of the transcription factor complexes with the initiating transcription machinery) (Figure 2G). PGC-1α thus directly induces the activity of the MITF-M promoter, probably in large part by coactivation of SOX10.

PGC-1β shares homology with PGC-1α and has numerous overlapping functions (Handschin and Spiegelman, 2006; Rowe et al., 2010). Stable transfection of B16 cells with either PGC-1α or PGC-1β (Figure S1I) led to a 2-fold increase in MITF-M expression

(Figure 2H), demonstrating that activation of MITF-M is a shared activity of PGC-1α and PGC-1β Conversely, inhibition of PGC-1β also strongly decreased *MITF-M* expression in melanoma cells (Figure 2I) and primary melanocytes (Figure S1J), and knockdown of either PGC-1α or PGC-1β diminished the amount of MITF protein in human melanomas (Figure S1A).

Like PGC-1α, PGC-1β also induced activity of the *MITF-M* promoter, in a manner dependent on the presence of SOX10 (Figure 2J). Interestingly, multiple shRNAs against PGC-1β inhibited both *PGC-1β* and *PGC-1α* expression, despite the absence of significant sequence homology with the PGC-1α transcript (Figure S1K), suggesting that PGC-1β may also regulate *PGC-1α* expression. In support of this, PGC-1β induced activity of the *PGC-1α* promoter in luciferase-based assays (Figure S1 L). SHPGC-1α constructs did not inhibit PGC-1β expression (Figure S1M). However, PGC-1β induced the expression of *MITF-M* 3.5-fold whether in the presence of shPGC-1α or scrambled control (Figure S1N), indicating that PGC-1β probably acts mostly independently of PGC-1α. Thus, PGC-1β probably regulates *MITF-M* directly, and perhaps indirectly via PGC-1α. Together, these data demonstrate that both PGC-1 coactivators regulate MITF-M expression in melanocytes.

Consistent with the finding that PGC-1α regulates *MITF*, their expressions correlate in melanoma cell lines and biopsy specimens. Like that of *MITF, PGC-1α* expression is higher in melanoma cells lines than in other cancer cell lines in the NCI-60 collection (Figure 3A, $p < 0.05$). Expression of *MITF* and *PGC-1α* is remarkably correlated in melanoma cell lines (Figures 3B and 3C, $p < 0.0001$), melanoma biopsy specimens (Figures 3D, $p < 0.0001$), and melanoma cell cultures (Figure S2A, $p < 0.0001$). *PGC-1α* expression is also correlated with that of *TYROSINASE* in melanoma cell lines and cultures, although less closely than with *MITF* (Figures S2B–S2D). Similar microarray studies showed strong correlation between the expression of *PGC-1β* and *MITF* in melanoma cell lines (Figure 3E, $p < 0.0001$), as did quantitative PCR (qPCR) analyses with a set of 42 complementary DNAs (cDNAs) prepared from melanoma biopsies (Figure S2E, $p = 0.03$). *PGC-1β* expression also correlated well with that of *TYROSINASE* (Figure S2F). The expression of both coactivators thus correlates well with the expression of *MITF*, consistent with both PGC-1 coactivators controlling *MITF* expression.

α-MSH Induces PGC-1α Expression

Tanning occurs when UV light triggers keratinocytes to upregulate secretion of α-MSH, which engages the MC1R on melanocytes and activates cAMP signaling and *MITF* expression. To investigate the role of PGC-1α in this pathway, we measured the response of PGC-1α to α-MSH signaling. As shown in Figure 4A, α-MSH and cAMP signaling dramatically induce *PGC-1α* expression in B16-F10 cells (left panels) and primary human melanocytes (right panels). Most strikingly, expression of transcripts initiated at an alternative *PGC-1α* promoter located 14 kb further upstream of the proximal PGC-1α promoter (Chinsomboon et al., 2009; Miura et al., 2008) is induced more than 400-fold by α-MSH in B16-F10 cells and 200-fold by the cAMP activators forskolin and IBMX in primary melanocytes (Figure 4A, top panels). PGC-1α protein encoded from this alternative transcript differs by a few amino acids in the N terminus (Chinsomboon et al., 2009; Miura et al., 2008) and is as active in induction of the MITF-M promoter as canonical PGC-1α (Figure S3A). Both of the *PGC-1α* promoters contain canonical cAMP regulatory sequences (Figure 4B, bottom). Transfection of *PGC-1α* promoter-luciferase constructs into B16-F10 cells, followed by treatment with α-MSH, induces the activity of both of the *PGC-1α* promoters (Figure 4B). Consistent with these findings, PGC-1α protein is markedly induced by α-MSH (Figure 4C). Furthermore, topical treatment of mice genetically engineered to contain epidermal melanocytes (which are normally limited to deeper hair follicles) with forskolin and the phosphodiesterase inhibitor Rolipram (Khaled et al., 2010) led to 2-fold

induction of MITF expression and >30-fold induction of alternative *PGC-1α* expression in the skin (Figure 4D). *PGC-1β* messenger RNA (mRNA) expression was not induced by α-MSH (Figure 5A). These data show that α-MSH potently induces *PGC-1α* expression in melanocytes.

α-MSH secretion by keratinocytes occurs in response to UV radiation and is part of a complex signaling crosstalk between keratinocytes and melanocytes. To directly test the effect of UV-induced factors secreted from keratinocytes, PAM212 mouse keratinocytes were irradiated with 10 mJ/cm^2 of UVB light, and media from these cells were incubated with B16-F10 cells (Figure 4E, left). Such conditioned media induced the expression of *Mitf-M* 2-fold, *PGC-1α* 2-fold, and alternative *PGC-1α* 5-fold in B16 melanocytes (Figure 4E, right). Direct UVB irradiation of melanocytes inhibited *PGC-1α*, *PGC-1β*, and *MITF-M* >5-fold (Figure S3B), demonstrating that UV does not induce *PGC-1α* directly in melanocytes but rather does so via keratinocytes. Irradiation of discarded human foreskins similarly led to 5-fold induction of alternative *PGC-1α* transcripts (Figure S3C). UV thus induces the expression of *PGC-1α* both in cell culture and in intact human skin.

The PGC-1s Mediate Induction of MITF in Response to α-MSH

The findings above suggested that the PGC-1s are required for the induction of MITF and the melanogenic program by α-MSH and cAMP signaling. Three approaches were taken for testing this notion. In the first approach, UACC-257 cells were made to express short hairpins against either PGC-1α or green fluorescent protein (GFP) as a control. Treatment of the control cells with the cAMP activators forskolin and IBMX induced the expression of MITF and its target gene TRPM1 (Figure 4F). In contrast, the induction of MITF and TRPM1 was abrogated when PGC-1α was knocked down (Figure 4F). In a second approach, B16 melanocytes were transfected with small interfering RNAs (siRNAs) against the PGC-1s and treated with forskolin + IBMX. *MITF-M* was induced >20-fold in control cells but <5-fold in cells with PGC-1α and PGC-1β knockdown (Figure S3D). Finally, in a third approach, primary melanocytes were derived from mice homozygous for floxed alleles of PGC-1α and PGC-1β. The cells were then infected with adenovirus expressing CRE recombinase for deletion of PGC-1α and PGC-1β. Cells infected with adenovirus expressing GFP served as a control. Activation of cAMP signaling in control cells strongly upregulated *Mitf-M* and *Tyrosinase*, and this was completely abolished in the absence of PGC-1α and PGC-1β (Figure 4G). Conversely, expression of either PGC-1α of PGC-1β strongly potentiated the induction of *MITF-M* in response to α-MSH in B16-F10 cells (Figure S3E). Together, these data show that PGC-1 coactivators are required for induction of MITF and melanogenic genes by cAMP signaling.

α-MSH Stabilizes PGC-1 Proteins

We noted in the above studies that in primary human melanocytes, the induction of PGC-1α mRNA by α-MSH was delayed compared with that of MITF (Figure 4A, right panels). Moreover, the induction of PGC-1α protein after cAMP activation preceded that of *PGC-1α* message in B16-F10 cells (Figure 5A). This prompted us to test whether α-MSH could impact PGC-1α protein earlier than it affects *PGC-1α* mRNA expression. PGC-1α is known to have a short half-life, and posttranslational modification by the p38 MAPK can significantly stabilize PGC-1α protein (Puigserver et al., 2001). As shown in Figure 5B, adding α-MSH to cells that were transfected with a plasmid constitutively expressing PGC-1α led to marked stabilization of PGC-1α protein. Surprisingly, inhibition of p38 MAPK (with SB203580) did not block the stabilization of PGC-1α (Figure S4A). In contrast, inhibition of protein kinase A (PKA), a known target of α-MSH and cAMP, did prevent the stabilization of PGC-1α (Figure 5C). Conversely, induction of cAMP by forskolin was sufficient for stabilizing PGC-1α (Figure 5D). Levels of GFP protein,

expressed from the same plasmid as PGC-1α, were not increased by forskolin (Figure 5D). Stimulation of cAMP by the combined action of forskolin and IBMX increased the halflife of PGC-1α from less than 15 min to at least 2 hr (Figure 5E). Murine PGC-1α contains 12 predicted PKA phosphorylation sites that are conserved with human PGC-1α (Figure 5F). Mutation to alanine of the seven serines with the highest predicted scores almost completely abolished the ability of cAMP activation to stabilize PGC-1α (Figures 5F and 5G). In contrast, mutation of the three serines phosphorylated by p38 MAPK (Puigserver et al., 2001) had no impact on the ability of cAMP activation to stabilize PGC-1α (Figure S4B). PGC-1β was similarly stabilized by either α-MSH or cAMP activation (Figure 5H). Thus α-MSH signaling via cAMP both induces PGC-1α mRNA expression (Figure 4) and stabilizes PGC-1α and PGC-1β proteins (Figure 5).

PGC-1α Can Induce Pigmentation in Rodents

Mice lack epidermal melanocytes and thus do not tan. Instead, murine melanocytes reside adjacent to hair follicles and provide melanin for the dark pigmentation of fur. In most mice, activation of melanogenesis in hair follicles is constitutive. A^y/a mice, however, aberrantly express the agouti protein, which inhibits MC1R signaling, yielding a yellow (“blond”) coat color (Bultman et al., 1992). A^y/a mice thus provide the opportunity to test for the activation of pathways downstream of MC1R. A^y/a mice were crossed with mice transgenically expressing PGC-1α exclusively in melanocytes (see Experimental Procedures for details). Expression of the transgene was easily detectable in the skin (Figure S4C) and led to a 2-fold induction of *PGC-1α* over endogenous levels in the entire skin, indicating robust induction of PGC-1α in melanocytes, which comprise only a small subset of cells in the skin (Figure S4D). As shown in Figure 6A, melanocytic expression of PGC-1α noticeably darkened the coat color of A^y/a mice. Consistent with this, the expressions of *Mitf-M, Dct*, and *Tyrosinase* all trended to a 2- to 3-fold increase in PGC-1α-overexpressing mice (Figure S4D). Melanocytic expression of PGC-1α in A^y/a mice significantly induced the presence of melanin in hair follicles, as detected by Fontana Masson staining (Figure 6A). PGC-1α can thus induce pigmentation in vivo, albeit in a fairly artificial system. The coat color did not become completely black, probably because the agouti protein also inhibits melanogenesis via other poorly understood and cAMP-independent inhibitory pathways in these mice (Hida et al., 2009), which are probably not rescued by activation of PGC-1α. Interestingly, deletion of PGC-1α and PGC-1β in melanocytes did not alter the baseline coat color in C57/Bl6 mice (Figure S4E), suggesting that the PGC-1s play an important role in the epidermal response to UV, but not in basal hair follicular pigmentation, two processes that probably evolved disparately (Jablonski and Chaplin, 2010).

PGC-1β Polymorphisms Are Associated with Tanning Ability and Melanoma Susceptibility in Humans

To look for more direct evidence that PGC-1 coactivators regulate pigmentation in humans, we turned to human polymorphism studies. A genome-wide association study was recently performed in a cohort of 2,166 subjects for identifying polymorphisms associated with tanning ability (Nan et al., 2009). Three of the top 20 SNPs identified in this study were in the *PGC-1β* gene (*PPARGC1B*), including the SNP rs32579, located in intron 4 of *PGC-1β* ($p < 1 \times 10^{-06}$). To determine whether these polymorphisms were indeed associated with tanning ability, we focused on rs32579, which had the highest association with tanning ability in the original data set, and significantly enlarged the sample size by recruiting an additional 4,341 subjects. The variant allele of rs32579 (A allele) was significantly associated with an increased tanning ability in this enlarged set (Table 1, n = 5,140, p = 0.01), reaching a p value of 9.86×10^{-07} when the discovery and replication sets were pooled (Table 1). The associations between tanning ability and SNPs in the 100 kb region surrounding the *PPARGC1B* gene were substantially attenuated after adjusting for the single

SNP rs32579 (Figure 6B), suggesting that the SNPs are in linkage disequilibrium (LD) and that a single SNP is responsible for the tanning association. The presence of the variant A allele of rs32579 in *PPARGC1B* thus correlates strongly with increased tanning ability.

How variant alleles affect phenotype and whether the mechanism involves the gene in which the SNP resides is often unclear. One way to test whether a given SNP affects a given gene is to evaluate for correlations between the SNP and the mRNA expression of the gene. We thus correlated the presence of the variant A allele at rs32579 with *PPARGC1B* mRNA expression, using transcript-expression profiling data of 87 HapMap CEU (Utah residents with ancestry from northern and western Europe from the CEPH collection) cell lines (National Center for Biotechnology Information Gene Expression Omnibus database). This eQTL analysis revealed that the expression of PGC-1β increased in proportion to the number of A alleles (Figure 6C). The effect showed a strong dose response and is highly statistically significant for all comparisons ($p = 2.5 \times 10^{-7}$ for GG versus AA, 8.1×10^{-6} for GA versus AA, and 5.8×10^{-3} for GG versus GA). Presence of the variant A allele at rs32579 thus increases both *PGC-1β* expression and the propensity for tanning. The variant allele did not correlate with PGC-1α expression (data not shown), indicating that PGC-1α-independent effects of PGC-1β dominate in this context. This observation strongly suggests that the correlation of the rs32579 SNP with tanning ability results from its effect on *PGC-1β* rather than some distant gene. The observation also strongly suggests that, mechanistically, the causal SNP modifies tanning ability by affecting the expression of *PGC-1β*.

Finally, we wished to test whether the protanning alleles of rs32579 correlated with protection from skin cancer. Melanoma is the most lethal skin cancer, and its incidence continues to rise worldwide (Levy et al., 2006). The ability to tan is strongly associated with melanoma risk in humans (Baccarelli and Landi, 2002; Elwood et al., 1985; Landi et al., 2001). Two data sets encompassing a total of 8,699 subjects were queried for the relationship between rs32579 and the incidence of melanoma. Homozygosity of the protanning A allele at rs32579 correlated with a 20% reduction in the odds ratio for the incidence of melanoma (Table 2), a considerable reduction given the multifactorial nature of melanoma. The variant *PGC-1β* rs32579 polymorphism thus correlates in a number of independent studies with (1) tanning ability, (2) increased *PGC-1β* expression, and (3) protection from melanoma.

DISCUSSION

The ability to tan plays important roles in primary protection from basal and squamous cell carcinomas, melanoma carcinogenesis, vitamin D homeostasis, and cosmesis (Fisher and James, 2010; Sturm, 2002). We show here, using a combination of molecular studies, cell culture, mouse data, and human polymorphism studies, that the PGC-1 coactivators are regulators of melanin production and the tanning response via regulation of MITF (see Figure 6D). These studies thus identify components of the complex mechanism by which UV irradiation leads to the induction of MITF and tanning.

The identification of a role for PGC-1α and PGC-1β in melanin production provides an interesting link between metabolism and pigment formation. Melanin production and transport is an energy-demanding process. Melanin production is also an oxidative process that is thought to generate high levels of reactive oxygen species (ROS) (Wittgen and van Kempen, 2007). The PGC-1 s are known to regulate broad protective programs against ROS (St-Pierre et al., 2006; St-Pierre et al., 2003), and MITF has been implicated in controlling ROS (Liu et al., 2009). Coregulation of melanogenesis and an anti-ROS program by PGC-1s may thus be critical for protecting activated melanocytes from ROS damage.

We highlight here a link between the PGC-1 coactivators and the regulation of MITF in response to α-MSH. The regulation of MITF is highly complex and integrates multiple signaling cascades, including α-MSH/cAMP and RAF/MAPK signaling. PGC-1α protein is also regulated by both MAPK (Knutti et al., 2001; Puigserver et al., 2001) and cAMP cascades (this study), and thus probably contributes to the integration of information from these second-messenger cascades. cAMP regulation of PGC-1α also occurs via glucagon in the liver and via adrenergic signaling in the muscle, strongly supporting the plausibility of the PGC-1s mediating the tanning response in the skin and suggesting that the PGC-1s may also contribute to other cAMP-mediated processes in other tissues. The powerful activation by α-MSH of an alternative PGC-1α promoter is also similar to that found in skeletal muscle in response to adrenergic signaling (Chinsomboon et al., 2009; Miura et al., 2008). It is possible that the alternative PGC-1α protein generated by the use of this alternative promoter and splicing of exon 1 has unique properties that may favor a role in melanogenesis. The functional differences between PGC-1 splice variants remain an area of active research (Chang et al., 2010; Miura et al., 2008; Trausch-Azar et al., 2010). PGC-1β had not previously been thought to be regulated by cAMP. The observation here that PGC-1β protein is stabilized by cAMP signaling suggests that its role in cAMP-mediated signaling in the liver, muscle, and elsewhere should also be investigated.

It is interesting that PGC1-α and PGC-1β regulate MITF and melanogenesis in response to UV and α-MSH but may not regulate the baseline expression of pigment, at least in fur. This observation is in line with the similar observation that deletion of p53, which is required for pro-opiomelanocortin (POMC) induction (i.e., α-MSH), also does not cause changes in coat color (Cui et al., 2007). In fact, POMC deficiency itself causes only a subtle coat-color deficiency (Challis et al., 2004; Yaswen et al., 1999), underscoring the complexity of this system. The observation is also in agreement with the finding that the identified *cis*-eQTL in the human PGC-1β gene correlates highly with tanning ability, but not with hair color. This role for the PGC-1s is also consistent with the roles that these coactivators play in multiple other contexts, wherein they have been demonstrated to control the physiologic adaptation to external stimuli, but often not the basal phenotype. This is the case, for example, with PGC-1 control of vascular density in skeletal muscle (normal vessel density in knockout animals, but absence of angiogenic response to exercise [Chinsomboon et al., 2009]) and thermogenesis in brown fat (normal temperature at baseline, but lack of resistance to cold [Lin et al., 2004]). Finally, the observation is also consistent with the finding that, with the exception of redheads, hair color and tanning ability in humans are only loosely associated (Flanagan et al., 2001; Healy et al., 2000; Wong et al., 2010). Human skin pigmentation probably evolved in response to the loss of body hair to ease thermoregulation during prolonged exercise (Jablonski and Chaplin, 2000, 2010), and tanning and baseline pigmentation probably evolved distinctly, with the ability to tan evolving secondarily (Jablonski and Chaplin, 2010).

MITF also plays a critical but complex and incompletely understood role in melanoma biology that may be separate from its role in protection from sun exposure (Garraway et al., 2005; Lin and Fisher, 2007; McGill et al., 2002). PGC-1α expression is enriched in melanoma cells and correlates highly with that of MITF (Figure 3), suggesting that PGC-1α contributes to this role. The role of MITF in melanoma progression is highly complex and incompletely understood (Levy et al., 2006). On the one hand, MITF is amplified in a subset of melanomas, where it is thought to serve as a “lineage addiction” oncogene associated with poor prognosis and resistance to chemotherapy (Garraway et al., 2005). In this context, PGC-1α may thus support oncogenesis, given that expression of PGC-1α and MITF are so tightly coexpressed. On the other hand, high levels of MITF are antiproliferative (Carreira et al., 2005; Selzer et al., 2002), and MITF is downregulated in advanced melanomas, wherein low levels of MITF promote metastasis (Cheli et al., 2011; Cheli et al., 2012; Salti et al.,

2000). In this context, activation of the PGC-1s in melanoma may thus be beneficial. A rheostat model has been proposed to account for these differing MITF functions in melanoma, in which high levels of MITF are associated with differentiation, lower levels promote proliferation, and a stem cell-like phenotype promotes invasiveness, whereas the absence of MITF causes senescence (Carreira et al., 2006; Goding, 2011; Hoek and Goding, 2010; Strub et al., 2011). The PGC-1s are well-established regulators of oxidative metabolism in numerous tissues and have recently been shown to regulate angiogenesis (Arany et al., 2008a). Cellular metabolism and vascular homeostasis are increasingly appreciated as playing critical roles in cancer. Furthermore, as noted above, the PGC-1s are known to activate anti-ROS programs, and ROS have also been implicated in both the development of melanoma and as a potential therapeutic target (Tuma, 2008). Elucidating the probable complex roles of PGC-1s in melanoma progression and the way in which their regulation of metabolism, vasculature, and ROS interacts with the MITF rheostat model will thus be of great interest.

The identification of a *cis*-eQTL in the human PGC-1β gene that correlates highly with tanning ability provides strong support for the notion that PGC-1β regulates tanning in humans. It is important to note that the effect size of a SNP on a phenotype does not necessarily correlate with the importance of the identified pathway in the phenotype. For example, SNPs in the HMG-CoA reductase gene are associated with only mild effects on low-density lipoprotein (LDL) levels (~3 mg/dl), despite the undeniably key role this enzyme plays in LDL biology (Kathiresan and Srivastava, 2012; Teslovich et al., 2010). It is thus difficult to conclude the extent to which PGC-1s contribute to tanning in humans based on the effect size of the polymorphism alone. In this context, however, the observations that PGC-1β polymorphisms confer a 20% protective effect from melanoma and that PGC-1s have a large effect experimentally on pigmentation pathways suggest that the role may be large. The fact that the expression of PGC-1β correlates with the variant SNPs also provides at least one possible mechanism by which the causal SNP affects tanning ability and also strongly supports the notion that the SNP affects tanning by affecting PGC-1β. We have focused on the rs32579 SNP in these studies because it has the highest correlation with tanning, but our studies cannot identify precisely which SNP(s) in the LD block is/are in fact causal. Identifying the causal SNP(s) will be interesting, though probably challenging. Finally, it will also be interesting to see whether the eQTLs identified here also impact any of the numerous metabolic diseases thought to be affected by the PGC-1s.

In summary, we identify here a molecular mechanism by which the PGC-1 coactivators regulate tanning. Efforts to identify small molecules that modulate the PGC-1s (Arany et al., 2008b; Wu and Boss, 2007) may thus have applications in human pigmentation and melanoma.

EXPERIMENTAL PROCEDURES

Cells and Reagents

Cells were grown in Dulbecco's modified Eagle's medium 10% fetal bovine serum unless otherwise indicated in the Supplemental Experimental Procedures. Forskolin and IBMX were used at 10 μM. α-MSH was used at 1 or 2 μM. UV irradiations were performed with a Stratalinker equipped with UVB bulbs with a precalibrated dose of 10 mJ/cm^2. Adenoviruses expressing Cre recombinase and GFP were from the Harvard Gene Therapy Initiative. PGC-1α antibodies were from Calbiochem. Lentiviruses expressing PGC-1α and PGC-1β shRNA were acquired from the shRNA library of the Broad Institute and the Dana Farber Cancer Institute. SiRNA constructs were obtained from Sigma-Aldrich. Renilla luciferase activity (Promega) was measured per the manufacturer's protocol.

Mice

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Mice expressing PGC-1α specifically in melanocytes were generated by crossing transgenic Tyr::rtTA mice (kindly provided by Dr. Lynda Chin) with transgenic TetO::PGC-1α mice (kindly provided by Dr. Dan Kelly). PGC-1α floxed and PGC-1β floxed mice were gifts from Dr. Bruce Spiegelman and Dr. Dan Kelly, respectively.

Gene Expression

RNA was isolated using QIAGEN TurboCapture and reverse transcribed using the Applied Biosystems RT-PCR kit. qPCR was performed using SYBR green. For microarray data, the data sets used are indicated in the figure legends. Cancer Cell Line Encyclopedia (CCLE) data sets were obtained from the Broad Institute CCLE website (http://www.broadinstitute.org/ccle/home). For PGC-1β qPCR expression data, the Melanoma TissueScan cDNA Array (OriGene) was used.

Statistics

Student's two-tailed t test was used throughout. For microarray correlations, Stata statistical software (College Station, TX, USA) was used to calculate the Pearson correlation coefficient (r) and the Spearman's rho (log rank test), which was used for generating p values.

Human Polymorphism Studies

All studies were approved by and conducted in accordance with the institutional review board at the relevant institution. The individuals in the tanning-propensity study consisted of participants in the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS). The individuals in the melanoma-propensity studies consisted of participants from a hospital-based melanoma case-control study of the MD Anderson Cancer Center, and individuals in the melanoma case-control study were from the NHS and HPFS. See Supplemental Information for additional details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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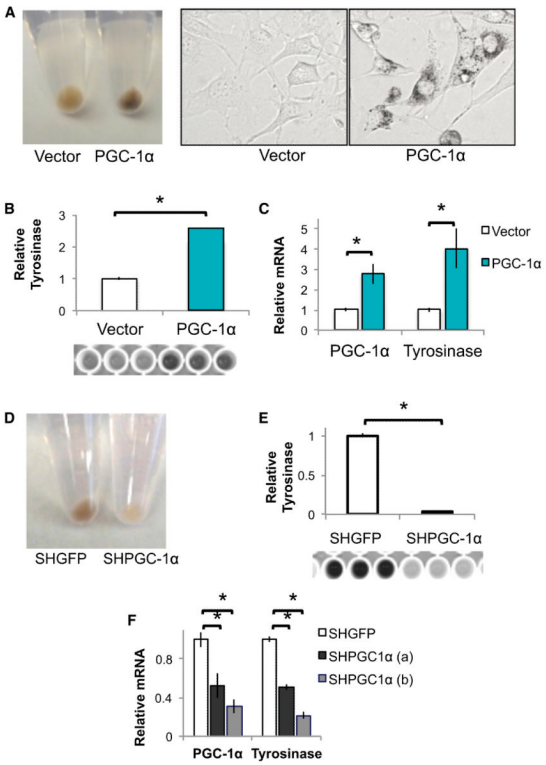

Figure 1. PGC-1α Induces Melanogenesis

(A) B16-F10 cells were infected with retrovirus overexpressing PGC-1α or vector control. Cell pellets (left panel), and light microscopy (right panel) are shown.

(B) Tyrosinase activity in extracts from cells, as in(A).

(C) Relative mRNA expression of the indicated genes in cells, as in (A).

(D) Cell pellets of B16-F10 cells infected with lentivirus expressing shPGC-1α or shGFP.

(E) Tyrosinase activity in extracts from cells, as in(D).

(F) Relative mRNA expression of the indicated genes in cells, as in (D).

*$p < .05$. $n \geq 3$ for all groups. Error bars represent mean ± SD.

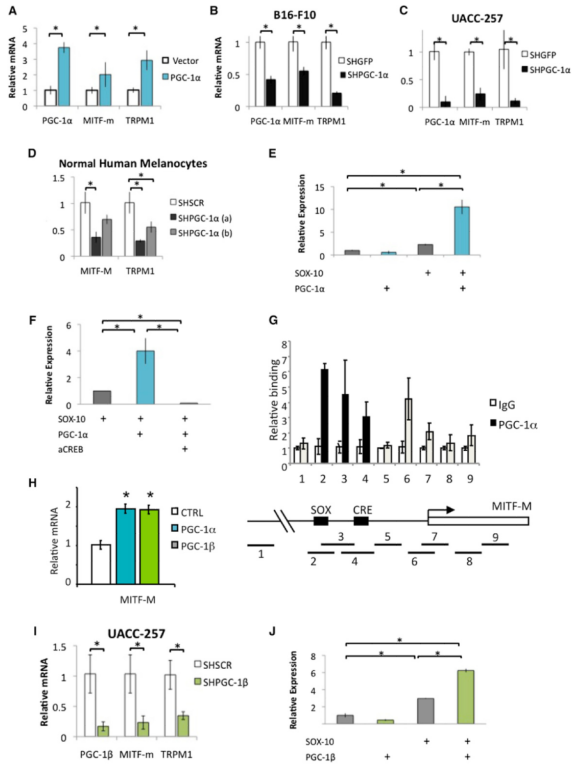

Figure 2. PGC-1 Coactivators Induce *MITF-M* Expression

(A–D) mRNA expression of the indicated genes in B16-F10 cells stably overexpressing PGC-1α versus vector control (A) and lentiviral knockdown of PGC-1α in B16-F10 (B), UACC-257 (C), and normal human melanocytes (D).

(E) Relative activity of luciferase expressed from the MITF-M promoter, cotransfected into UACC-257 cells with plasmids expressing the indicated proteins.

(F) Relative luciferase activity, as in (E).

(G) Occupancy by PGC-1α on the MITF-M promoter at the indicated sites. Black bars correspond to binding near the SOX and CRE sites. IgG, immunoglobulin G.

(H) Relative MITF-M mRNA in B16-F10 cells stably overexpressing PGC-1α or PGC-1β. CTRL, control.

(I) mRNA of indicated genes in UACC-257 cells following lentiviral knockdown of PGC-1β.

(J) MITF-M promoter activity in UACC-257 cells expressing the indicated proteins.

*$p < 0.05$. Error bars represent mean ± SD.

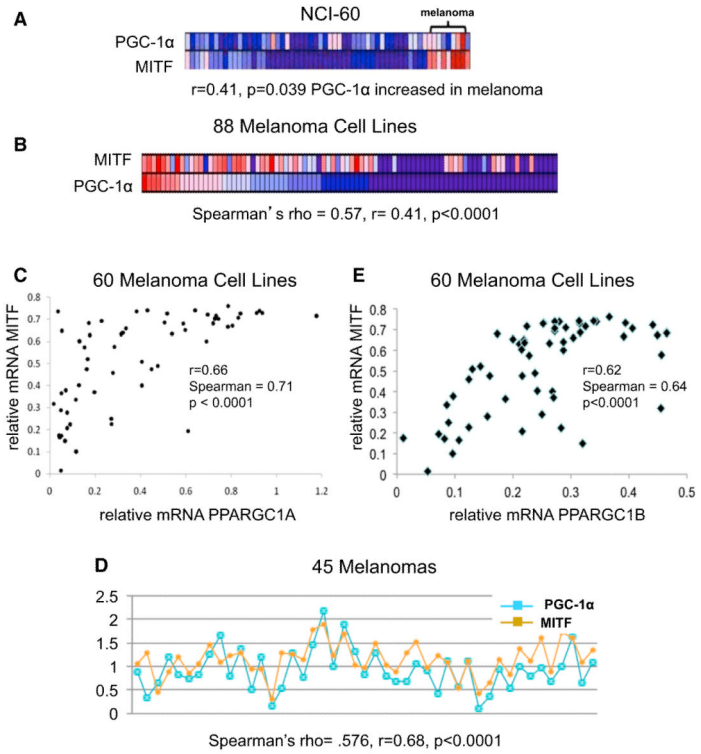

Figure 3. PGC-1 and MITF Expression Correlates in Multiple Melanoma Cell Lines and Biopsies

(A–D) Relative expression of PGC-1α and MITF in (A) the NCI-60 cell lines (GSE5846), (B) 88 melanoma cell lines (Lin et al., 2008; Lee et al., 2007), (C) 60 melanoma cell lines (Broad Institute CCLE), and (D) 45 melanoma biopsies (GSE3189) (Talantov et al., 2005). (E) Relative expression of PGC-1β and MITF in 60 melanoma cell lines (Broad Institute CCLE).

p values for Spearman's rho are as indicated.

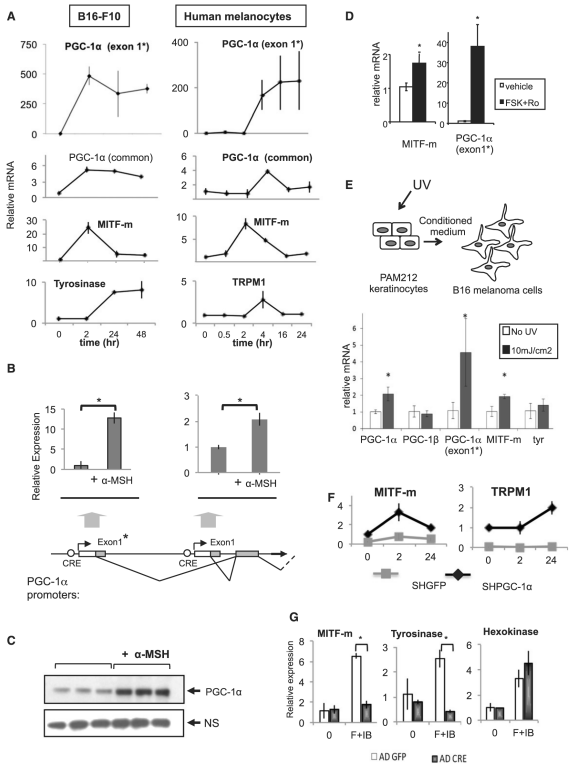

Figure 4. Regulation of PGC-1α and MITF by α-MSH

(A) mRNA expression of the indicated genes in B16-F10 cells treated with α-MSH (left panels) and normal human melanocytes treated with forskolin + IBMX (right panels) for the indicated times.

(B) Relative activity of the two PGC-1α promoters in B16-F10 cells treated with α-MSH, as assessed by luciferase activity. Schematic of PGC-1α promoters and the alternatively spliced exon 1* is shown below.

(C) PGC-1α protein in B16-F10 cells treated with α-MSH for 16 hr. NS, nonspecific band.

(D) MITF-M and PGC-1α induction in C57Bl6$^{e/e}$ K14 SCF mice topically treated with vehicle or forskolin + Rolipram (FSK + Ro) for 6 hr.

(E) Expression of the indicated genes in B16-F10 cells after 5 hr incubation in medium that had been conditioned for 16 hr by PAM212s exposed to UV or mock treatment. The experimental outline is schematized on the left.

(F) MITF-M and TRPM1 mRNA in UACC-257 cells expressing shPGC-1α and treated with forskolin + IBMX.

(G) MITF-M and Tyrosinase induction in response to forskolin + IBMX (F+IB) in primary mouse melanocytes lacking PGC-1α and PGC-1β. AD, adenovirus.

*p < .05. Error bars represent mean ± SD.

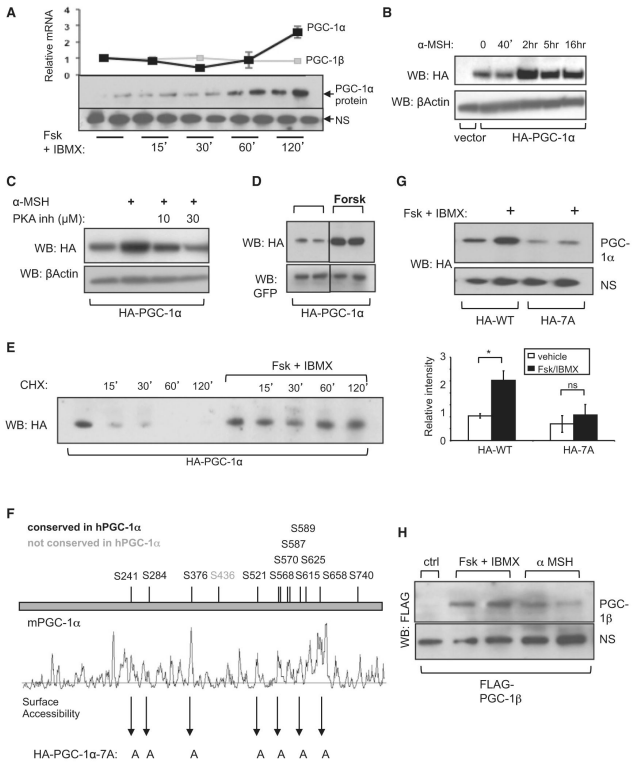

Figure 5. Stabilization of PGC-1α Protein by α-MSH

(A) Time course of induction of PGC-1α and PGC-1β mRNA (top) and PGC-1α protein (bottom) in B16-F10 cells treated with forskolin and IBMX (Fsk + IBMX) for the indicated times. (B) Stabilization of PGC-1α by α-MSH. WB, western blot.

(C) The PKA inhibitor (H89) blocks stabilization of PGC-1α protein.

(D) PGC-1α protein stabilization in cells treated with the adenylate cyclase activator (forskolin). GFP (lower panel) is expressed by the same promoter (CMV).

(E) Half-life of PGC-1α in B16-F10 cells treated with forskolin + IBMX. CHX, cycloheximide.

(F) Schematic of murine PGC-1α and predicted PKA target sites. Sites mutated to alanine in the 7A mutant are indicated below.

(G) Absence of stabilization of PGC-1α mutated at PKA target sites. The quantification of five experiments is shown below. WT, wild-type.

(H) Stabilization of PGC-1β in B16-F10 cells treated with forskolin + IBMX or α-MSH. NS, nonspecific band. *$p < 0.05$. Error bars represent mean ± SD.

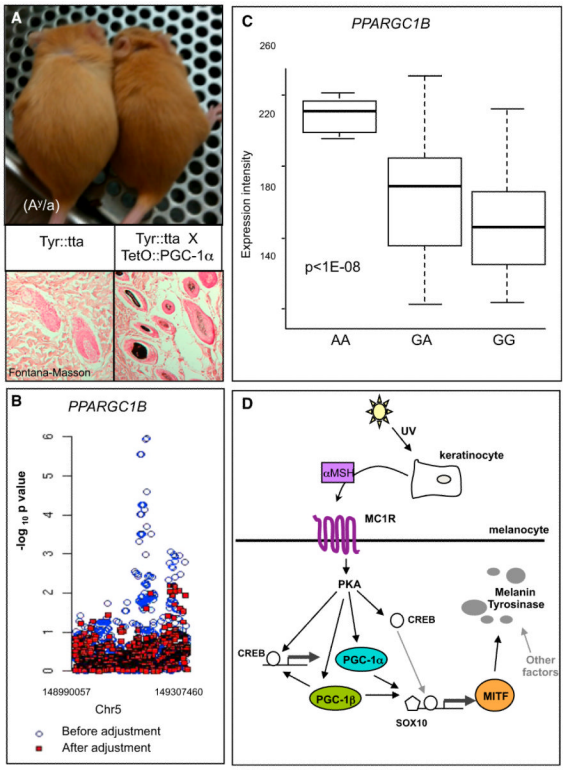

Figure 6. Evidence in Humans and Rodents for Regulation of Pigmentation by PGC-1 Coactivators

(A) Melanocytic overexpression of PGC-1α in mice darkens the fur of A^y/a mice (top panel) and increases melanin in hair follicles (bottom panels). Representative of n > 4 for each group.

(B) Manhattan plot of the 100 kb region surrounding the human PGC-1β gene (*PPARGC1B*), and the association of SNPs within this region with tanning ability.

(C) Relative expression of PGC-1β in 87 HapMap human cell lines according to their genotype at the rs32579 SNP. Interquartile ranges (boxes) and mean values (lines in boxes) are shown.

(D) The PGC-1 coactivators regulate MITF and melanin production.

Table 1

The Variant Allele of rs32579 in the Human *PPARGC1B* Gene Correlates with Tanning Ability

	WT/VT	Sample Size	Beta	SE	p Value
Discovery set	G/A	2,166	0.32	0.06	4.80×10^{-7}
Replication set	G/A	5,140	0.1	0.04	0.01
Pooled set	G/A	7,306	0.17	0.03	8.90×10^{-7}

A, adenine; G, guanine; VT, variant type; WT, wild-type.

Table 2

The Variant Allele of rs32579 in the Human *PPARGC1B* Gene Correlates with Protection from Melanoma in Individuals of European Ancestry

WT/VT (G/A)	Cases (%)	Controls (%)	OR (95% CI)	p Value
WT (GG)	1,222 (51.3)	3,173 (50.2)	1.00 (reference)	AA versus
Het (GA)	982 (41.3)	2,610 (41.3)	1.01 (0.90–1.14)	GG = 0.047
VT (AA)	176 (7.4)	536 (8.5)	0.80 (0.64–0.997)	

A, adenine; CI, confidence interval; G, guanine; Het, heterozygote; OR, odds ratio; VT, variant type; WT, wild-type.